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PROGNOSTIC VALUE OF TOLL-LIKE RECEPTORS IN COLORECTAL CANCER

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And if at first you don't succeed,
Then dust yourself off and try again.
Aaliyah

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Beilmann-Lehtonen I, Böckelman C, Mustonen H, Koskensalo S, Hagström J, Haglund C. The prognostic role of tissue TLR2 and TLR4 in colorectal cancer. *Virchows Arch*. 2020;477:705–715.

II Beilmann-Lehtonen I, Hagström J, Mustonen H, Koskensalo S, Haglund C**, Böckelman C**. High tissue TLR5 expression predicts better survival in colorectal cancer patients. *Oncology*. 2021;99:589–600.

III Beilmann-Lehtonen I, Hagström J, Kaprio T, Stenman U-H, Strigård K, Palmqvist R, Gunnarsson U, Böckelman C**, Haglund C**. The relationship between the tissue expression of TLR2, TLR4, TLR5, and TLR7 and systemic inflammatory responses in colorectal cancer patients *Oncology*. 2021;99:790–801.

IV Beilmann-Lehtonen I*, Kasurinen J*, Hagström J, Kaprio T, Haglund C**, Böckelman C**. High tissue expression of TLRs combined with a high density of tumor-infiltrating lymphocytes predicts better prognosis in colorectal cancer patients. Under review.

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ABSTRACT

Background and aims

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide with 1.9 million new cases occuring globally in 2020. The prognosis of CRC patients has improved, although 30–50% of all local colon cancer patients treated develop recurrence. Biomarkers serve as diagnostic, prognostic, and predictive tools that help to detect disease without clinical symptoms, to identify early recurrence at follow-up, and to determine which patients might need more aggressive treatment.

Subtyping cancers using biomarkers can enable targeted and personalized therapy. Chronic inflammation may promote cancer development and dissemination. Notably, cancer may cause both systemic and local inflammation. CRC patients with a strong systemic inflammatory response (SIR) indicated by an elevated C-reactive protein (CRP) value exhibit a worse prognosis, whereas CRC patients with high local immune cell infiltration have a better prognosis. Furthermore, CD3-positive and CD8-positive immune cells are critical in local adaptive immune responses. Toll-like receptors (TLRs) are transmembranous proteins crucial in initiating innate and adaptive immune responses after recognizing pathogen-associated or host-originating patterns. In malignancies, TLRs play pro- and antitumorigenic roles. Thus, this dissertation project aimed to evaluate the prognostic role of different TLRs in CRC. Moreover, this project aimed to assess the possible relationship between TLRs and CRP, and between local innate (TLRs) and adaptive (CD3-positive and CD8-positive cells) immune responses in CRC.

Methods

Expressions of TLR2, TLR4, TLR5, and TLR7 were assessed in tumor cells through the immunohistochemistry of tissue microarray (TMA) slides from 1308 CRC patients who underwent surgery in the Department of Surgery at Helsinki University Hospital, Finland, between 1982 and 2005. The associations between the immunoexpressions of TLRs in tumor cells, clinicopathological characteristics, and survival were evaluated. Among a subgroup of 549 patients surgically treated between 1998 and 2005, the relationship between tumor cell expressions of TLR2, TLR4, TLR5, and TLR7 and plasma CRP was analyzed. Blood samples were taken preoperatively, and plasma CRP levels were measured using a high-sensitivity method. Finally, the relationship between the tumor cell expressions of TLRs and the immunoexpression of CD3-positive and CD8-positive T-cell densities in the tumor and stroma in the same samples was analyzed. A CD3–CD8 tumor–stroma index was established based on the density levels of CD3-positive and CD8-positive T cells in the tumor and stroma, resembling the well-known *Immunoscore®*.

Results

Patients with a high TLR2 expression, a high TLR5 expression, and a positive TLR7 expression exhibited a better disease-specific survival (DSS) in the cohort of 1308 CRC patients. Furthermore, stage III subgroup patients with a high TLR2 immunoexpression exhibited a better outcome. A high TLR5 value served as a positive prognostic factor among younger patients, patients with a higher pT stage, patients with lymph node–negative disease, and patients with a lower WHO grade. A positive TLR7 immunoexpression emerged as a positive prognostic factor among higher pT stage and lower WHO grade patients. In a cohort of 549 CRC patients, individuals with a high preoperative CRP level exhibited a worse DSS. Among patients with a high CRP level, those with a low TLR4 immunoexpression exhibited a better prognosis. In the low CRP subgroup, patients with a high TLR2, a high TLR5, and a positive TLR7 immunoexpression exhibited a better survival. Interestingly, TLR4 immunoexpression carried no prognostic value in the entire cohort. Furthermore, none of the TLRs emerged as an independent prognostic factor in the multivariate analyses. High expressions of tumoral and stromal CD3-positive and CD8-positive T cells associated with high expressions of TLR2, TLR4, and TLR5. Among all TLR subgroups except the negative TLR7 subgroup, patients with a low CD3–CD8 tumor–stroma index exhibited a worse prognosis.

Conclusions

Survival was better among patients with a high expression of TLR2, TLR5, and TLR7 in the tumor cells. By contrast, survival was worse among patients with a high CRP level. Furthermore, survival was better among patients with a low CRP level and with high tumor cell expressions of TLR2, TLR5, and TLR7, as well as among patients with a high CRP level and a low TLR4 expression. High expressions of immune cell densities in the tumor and stroma associated with high expressions of TLRs in the tumor cells. Thus, TLRs may play a prognostic role either alone or in combination with CRP or immune cell densities. However, further studies are needed to more fully understand the prognostic value of TLRs in CRC.

TIIVISTELMÄ (FINNISH ABSTRACT)

Tausta ja tavoitteet

Paksu- ja peräsuolisyöpä (kolorektaalisyöpä) on kolmanneksi tavallisin syöpätauti maailmassa ja vuonna 2020 todettiin 1,9 miljoonaa uutta tapausta. Kolorektaalisyöpää sairastavien potilaiden ennuste on parantunut, mutta silti 30–50 % hoidetuista paikallisista syövistä uusiutuu. Biomerkkiaineet ovat diagnostisia ja ennusteellisia molekyylejä, joiden avulla voi löytää aggressiivisemmasta hoidosta ja seurannasta hyötyvät potilaat. Syövän alatyypitys biomerkkiaineiden avulla voi auttaa löytämään apuvälineitä millä voi kohdentaa hoidot paremmin. Krooninen tulehdus voi edesauttaa syövän kehittymistä ja leviämistä. Osalle kolorektaalisyöpäpotilaista kehittyy syövän yhteydessä vahva systeeminen tulehdusvaste ja näillä potilailla on huonompi ennuste. C-reaktiivinen proteiini (CRP) on hyvin tunnettu systeemisen tulehdusvasteen merkkiaine. Korkea paikallinen T-imusolujen tiheys kasvainalueella taas liittyy hyvään ennusteeseen. CD3- ja CD8-positiiviset T-imusolut ovat keskeinen osa paikallista adaptiivisen immuunivastetta. Tollin kaltaiset reseptorit (TLR:t) ovat transmembraaniproteiineja, jotka tunnistavat mikrobiaalisista taudinaiheuttajista ja isännästä lähtöisin olevia tekijöitä ja ovat erittäin tärkeitä luontaisen ja adaptiivisen immuunijärjestelmän aktivoinnissa. Tollin kaltaisilla reseptoreilla on sekä syövän kehitystä edistäviä että syövän kehittymiseltä suojaavia vaikutuksia. Tämän tutkimuksen tavoitteena oli arvioida TLR:ien ennusteellista roolia kolorektaalisyövässä. Lisäksi oli tarkoitus arvioida TLR:ien ja systeemisen tulehdusvasteen yhteyttä sekä paikallisen luonnollisen (TLR:t) ja adaptiivisen (CD3- ja CD-8 positiiviset solut) immuunivasteen yhteyttä kolorektaalisyövässä.

Potilaat ja menetelmät

Tutkimuksessa mitattiin TLR2, TLR4, TLR5 ja TLR7 ilmentymätasoa kasvainsoluissa immunohistokemiallisen värjäyksen avulla 1308 kolorektaalisyöpäpotilaan kudosmikrosirublokeista (tissue microarray). Potilaat oli leikattu kolorektaalisyövän vuoksi Helsingin yliopistollisessa sairaalassa vuosina 1982–2005. Arvioitiin TLR:ien kudosilmentymän yhteyttä kliinispatologisiin tekijöihin ja elossaoloon. Pienemmässä 549 potilaan ryhmässä, jonka potilaat oli hoidettu vuosina 1998–2005, tukittiin TLR2, TLR4, TLR5 ja TLR7 sekä plasman CRP-tason yhteyttä. Potilailta oli otettu verinäytteitä ennen leikkausta ja plasman CRP määriteltiin herkällä mittausmenetelmällä (high-sensitivity method). Lopuksi arvioitiin samasta potilasryhmästä TLR:ien syöpäsoluilmentymän ja CD3-positiivisten ja CD8-positiivisten T-immuunisolujen tiheyden yhteyttä. Kasvainsolukon ja tukikudoksen (strooman) immuunisolujen tiheystasot laskettiin erikseen ja niiden perusteella muodostettiin CD3-CD8 kasvain-strooma indeksi (tumor-stroma index) vastaavalla tavalla kuin yleisesti tunnetussa *Immunoscoressa*®.

Tulokset

Havaitsimme 1308 kolorektaalisypää sairastavan potilaan ainaistossa, että korkeaan TLR2-, korkeaan TLR5- ja positiiviseen TLR7-ilmentymään liittyi pidempi tautispesifinen elossaolo. Levinneisyysasteen III (stage III) alaryhmän potilailla, joilla oli korkea TLR2-ilmentymä, oli parempi ennuste. Korkea TLR5 oli positiivinen ennusteellinen merkki seuraavissa alaryhmissä: nuoremmat potilaat, korkeamman kasvusyvyystason (pT) kasvain, imusolmukenegatiivinen tauti (pN0) ja hyvän erilaistumistason (gradus 1–2) syöpä. Positiivinen TLR7-ilmentymä oli positiivinen ennusteellinen merkki korkeassa pT-ryhmässä ja potilailla, joilla oli hyvän erilaistumistason syöpä. Pienemässä 549 potilaan aineistossa oli lyhyempi tautispesifinen elossaolo potilailla, joilla oli korkea leikkausta edeltävä CRP-taso,. Korkean CRP:n alaryhmässä oli huonompi ennuste niillä, joilla oli matala TLR4-ilmentymä kasvainsoluissa. Lisäksi matalan CRP:n alaryhmässä oli parempi ennuste potilailla, joilla oli korkea TLR2-, TLR5- ja TLR7-ilmentymä kasvainsoluissa. Yksikään tutkituista merkkiaineista ei noussut itsenäiseksi ennusteelliseksi tekijäksi monimuuttujaanalyysissä. Korkea CD3- ja CD8-positiivisten T-solujen ilmentymä kasvainkudoksessa ja vastaava stroomassa oli yhteydessä korkeaan TLR2-, TLR4- ja TLR5-ilmentymään kasvainsoluissa. Kaikissa TLR- alaryhmissä paitsi negatiivisessa TLR7-ryhmässä oli huonompi ennuste potilailla, joilla oli matala CD3-CD8 kasvain-strooma indeksi.

Yhteenveto

Tulosten perustella voi päätellä, että ennuste on parempi potilailla, joilla on korkea TLR2-, TLR5- ja TLR7-ilmentymä kasvainsoluissa. Korkea CRP on huonoon ennusteen merkki. Ennuste on parempi potilailla, joilla on matala CRP ja korkea TLR2-, TLR5- ja TLR7-ilmentymä kasvainsoluissa. Matala TLR4-ilmentymä kasvainsoluissa oli hyvän ennusteen merkki potilailla, joilla on korkea CRP-taso. T-immuunisolujen korkea tiheys kasvainkudoksessa liittyi tutkittujen TLR:ien korkeaan ilmentymään kasvainsoluissa. TLR:t voisivat toimia ennusteellisinä tekijöinä yksin tai yhdistettynä muihin merkkiaineisiin, mutta tarvitaan kuitenkin lisää tutkimuksia TLR:ien ennuisteellisen roolin selventämiseksi.

ABBREVIATIONS

AJCC	American Joint Committee on Cancer
AP-1	Activator protein 1
APC	Adenomatous polyposis coli
BCG	Baccilus Calmette–Guerin
CA125	Carbohydrate antigen 125
CA19-9	Carbohydrate antigen 19-9
CA242	Carbohydrate antigen 242
CD3	CD3- positive T lymphocyte
CD8	CD8- positive T lymphocyte
CEA	Carcinoembryonic antigen
CI	Confidence interval
CIMP	Cytosine–guanine dinucleotide group island methylator phenotype
CIN	Chromosomal instability
CME	Complete mesocolic excision
CMS	Consensus molecular subtype
CpG	Cytosine-Guanine dinucleotide group
CRC	Colorectal cancer
CRM	Circumferencial resection margin
CRP	C-reactive protein
CT	Computed tomography
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-lymphocyte–associated antigen 4
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
DFS	Disease-free survival
DSS	Disease-specific survival
EGF	Epidermal growth factor
EGRF	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
FAP	Familial adenomatous polyposis
FDG–PET	Fluorodeoxyglucose–positron emission tomography
FGF	Fibroblast growth factor
FIT	Fecal immunochemical blood test
FOBT	Fecal occult blood test
GPS	Glasgow prognostic score
gFOBT	Guaiac-based fecal occult blood test
HIPEC	Hyperthermic intraperitoneal chemotherapy

HNPCC	Hereditary nonpolyposis colorectal cancer
HR	Hazard ratio
IBD	Inflammatory bowel disease
IFN	Interferon
IL	Interleukin
IQR	Interquartile range
IRF3	Interferon-regulatory factor 3
KRAS	Kirsten ras homolog
MAPK	Mitogen-activated protein kinase
MC	Mast cell
mGPS	Modified Glasgow prognostic score
MHC	Major histocompatibility complex
MMP8	Matrix metalloproteinase 8
MMR	Mismatch repair
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
MSS	Microsatellite stability
MyD88	Myeloid differentiation primary response gene 88
NF-κB	Nuclear factor kappa B
OS	Overall survival
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PD1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PDGF	Platelet-derived growth factor
PET	Positron emission tomography
ROC	Receiver operating characteristic
SCNA	Somatic copy number alteration
SIR	Systemic inflammatory response
TATI	Tumor-associated trypsin inhibitor
TAT2	Tumor-associated trypsin 2
TaTME	Transanal total mesorectoral excision
TGF-β	Transforming growth factor beta
Th1	T-helper type 1 cell
Th2	T-helper type 2 cell
Th17	T-helper type 17 cell
TIL	Tumor-infiltrating lymphocyte
TIMP1	Tissue inhibitor of metalloproteinase 1
TIR	Toll-interleukin-1 receptor
TLR	Toll-like receptor
TMA	Tissue microarray

TME	Total mesorectal excision
TNF	Tumor necrosis factor
TNM	Tumor Node Metastasis Classification of Malignant Tumors
TP53	Tumor protein 53
Treg	Regulatory T cells
TR-IFMA	Time-resolved immunofluorometric assay
TRIF	TIR-related adaptor protein-inducing interferon
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1 INTRODUCTION

Cancer is among the three leading causes of death before the age of 70 years in nearly all countries globally (Sung et al., 2021). In most developed countries, cancer is the leading cause of death, followed by cardiovascular diseases. In 2019, over 35 000 people were diagnosed with cancer in Finland and more than 13 000 cancer-related deaths were reported (Pitkäniemi et al. 2019, Finnish Cancer Registry).

Colorectal cancer (CRC) is the world's second most deadly cancer surpassed only by lung cancer (Sung et al., 2021). In Finland, CRC is the second most diagnosed cancer among men and women after prostate cancer and breast cancer. Interestingly, CRC mortality has decreased since the 1990s in Finland, but similar to most other countries the incidence rate continues to rise due to the aging population and the rapid adoption of lifestyle behaviors that increase cancer risk, including dietary habits, obesity, alcohol consumption, and a lack of physical exercise (Pitkäniemi et al. 2019, Finnish Cancer Registry).

The radical surgical removal of cancer tissue represents the cornerstone of curative treatment for CRC patients fit for surgery. Metastasized disease may be curatively operable, comprising surgical removal of both the primary tumor and metastases, combined with oncological treatment administered before and after surgery (Cutsem et al., 2016; Hyöty et al., 2019). As many as one-third of CRC patients present as emergency cases with obstruction or perforation as the first symptom of disease. Prognosis among such patients is worse compared to those who undergo planned surgery (Biondo et al., 2019). Treatment protocols relay on tumor staging classifications, which consists of the tumor location, size, invasiveness, lymph node involvement, and metastases. Adjuvant treatment is recommended for patients with lymph node involvement or distant metastases, although several risk factor have emerged that favor adjuvant treatment at an earlier stage as well (Argiles et al., 2020; Hyöty et al., 2019). Neoadjuvant treatment is commonly administered to rectal cancer patients enabling resection margins free of cancer cells and in order to improve outcomes (Hyöty et al., 2019).

The stage at diagnosis is still the most important prognostic factor among CRC patients. For instance, patients diagnosed with metastasized disease experience the worst prognosis, while patients who present with local disease experience better survival (Howlader et al., 2021). Achieving diagnosis at an early stage remains challenging since patients are often asymptomatic even when presenting with advanced disease. As such, screening programs have been developed and initiated in many countries including in Finland, enabling early detection and improving prognosis among patients (Heinävaara et al., 2019).

CRC develops from normal epithelial cells that transform into precancerous lesions and further into malignant cells after escaping mechanisms that control proliferation and achieving uncontrollable growth and the capability of metastasizing. This transformation occurs due to the accumulation of genetic mutations and epigenetic changes.

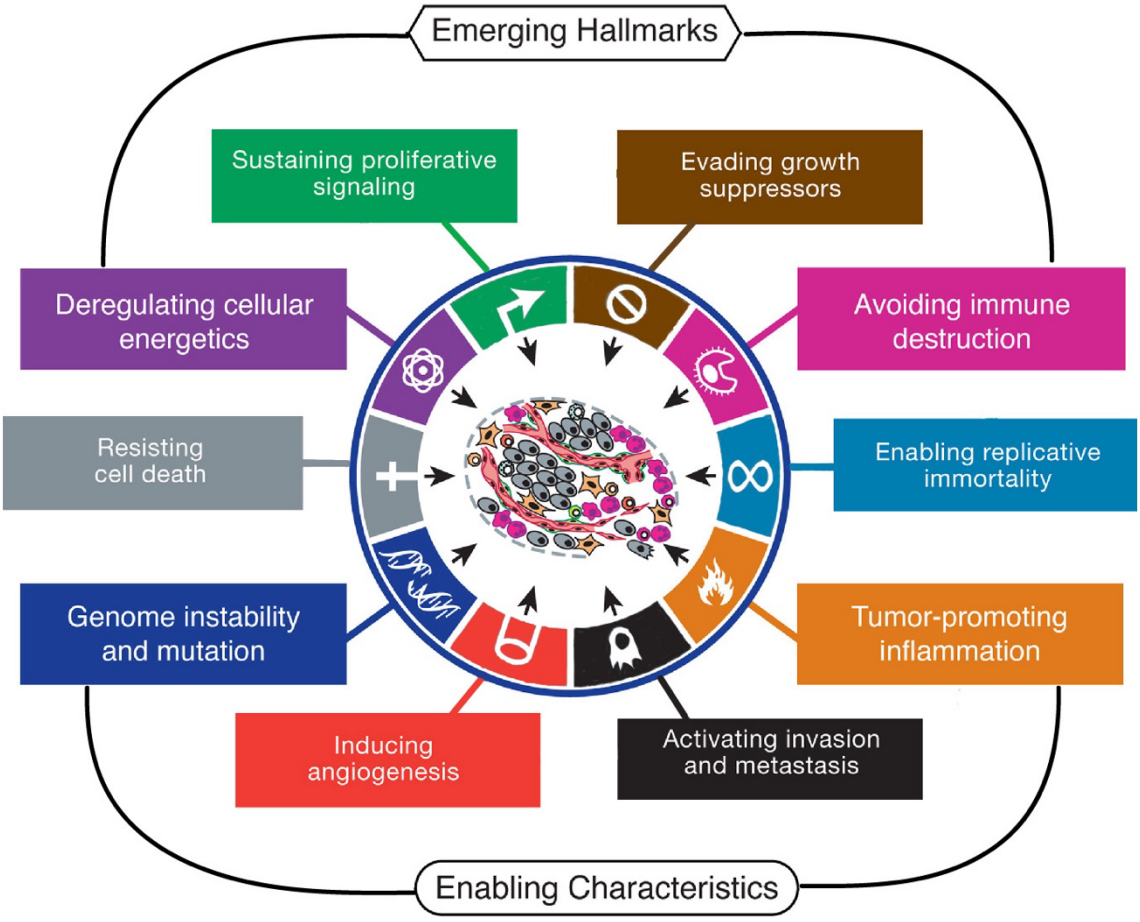

Figure 1. *Hallmarks of cancer. Image modified from Hanahan and Weinberg, 2011.*

Mutations occur in oncogenes, tumor suppressor genes, and genes linked to DNA repair. Hereditary syndromes cause 5% of CRC cases, although as many as one-third of sporadic cases have an underlying family history (Rawla et al., 2019).

Our understanding of cancer biology is continuously expanding. In 2000, Hanahan and Weinberg (2011) reported six distinctive and complementary hallmarks characterizing the capabilities cells acquire during the multistep development towards malignancy — tumoral cells sustain proliferative signalling, become insensitive to antigrowth signals, develop a limitless replicative potential, induce angiogenesis, resist apoptosis, and become capable of invading and metastasizing (Figure 1). Two emerging hallmarks were later identified — tumors' capabilities of modifying cellular energetics to work in favor of tumor proliferation and tumors' capabilities of avoiding hosts' immunological responses and destruction (Hanahan and Weinberg, 2011). In addition, genome instability and mutation along with tumor-promoting inflammation were introduced as two underlying enabling hallmarks.

The connection between tumorigenesis and inflammation has been widely studied. Strong immune cell infiltration and an elevated expression of proinflammatory cytokines are observed in colorectal tumors, even in cancers that do not arise from inflammatory bowel disease (Terzić et al., 2010). Acute inflammation is necessary for the elimination

of pathogens, although chronic inflammation contributes to cancer development. In the tumor microenvironment the cytokine profile shifts to becoming protumorigenic and contributes to tumor cell proliferation, angiogenesis, invasion, and metastasis. As tumors become dependent upon the proinflammatory microenvironment, the components become promising targets for cancer prevention and therapy (Terzić et al., 2010).

Cancer biomarkers as measurable biochemicals associated with a malignancy play an important role in cancer detection, treatment, and follow-up. These biomarkers may be produced by tumor cells or by the host as the response to a tumor. Biomarkers can help identify individuals at a higher risk of developing a malignancy. During screening biomarkers help identify cancer at an early stage. Moreover, tumor markers are helpful tools for cancer diagnosis and for identifying recurrence during follow-up (Duffy et al., 2011). In addition, predictive markers guide therapeutic choices since they identify whether patients might benefit from a specific treatment intervention, and prognostic biomarkers predict the natural course of disease and thus distinguish between patients with a good or poor prognosis. An ideal tumor biomarker should be rather cheap, easily measurable, and feature a high sensitivity leading to early detection. Furthermore, a biomarker should have a high specificity with an early and high expression level in individuals with disease and remain undetectable among healthy people. Ideally, the value should correlate with the severity of disease and rapidly respond to treatment (Carlomagno et al., 2017; Madu and Lu, 2010).

Heterogenous tumors with different molecular alterations have different natural histories, responses to chemotherapy, and outcomes. Molecular profiling is an irreplaceable tool to determine the genetic features of each tumor and finding the best suitable treatment for a patient (El-Deiry et al., 2019). Based on gene expressions, a new and universal molecular classification of four consensus molecular subtypes (CMSes) has been established in CRC (Guinney et al., 2015). There is, however, an urgent need for new biomarkers to provide earlier cancer detection and improved prognostics. Furthermore, biomarkers may help to distinguish patients likely to benefit from adjuvant treatment from patients who might be spared intense and expensive adjuvant therapy (Benson et al., 2018; Böckelman et al., 2014).

In this thesis, the significance of potentially prognostic inflammatory biomarkers was analyzed in patients undergoing surgery for primary CRC, focusing on the tissue expression of four distinct toll-like receptors (TLRs) — TLR2, TLR4, TLR5, and TLR7. In addition, plasma C-reactive protein (CRP) and the tissue expression of CD3-positive and CD8-positive lymphocytes were examined. Finally, the association between biomarkers and clinicopathological characteristics was analyzed and survival data were used to identify those patients at a higher risk of a poorer prognosis.

2 REVIEW OF THE LITERATURE

2.1 EPIDEMIOLOGY

Colorectal cancer (CRC) is the third most common malignancy worldwide with 1.9 million new cases occuring annually. With an estimated 935 000 cancer deaths in 2020, CRC is the second leading malignancy in terms of cancer mortality (Sung et al., 2021). In 2020, a total of 1 414 259 new colon cancer cases were diagnosed with 600 896 cases among men and 547 619 among women. For rectal cancer, incidence was 443 358 among men and 288 852 among women (Sung et al., 2021).

Incidence rates differ between geographic regions, remaining higher in many developed and some transitioning countries due to aging populations, obesity, and dietary patterns (Arnold et al., 2017; Sung et al., 2021). In Finland, incidence continues to rise, with 3628 new cases diagnosed in 2019. With the CRC incidence rate reaching 73.6 per 100 000 person-years among men and 50.9 among women, it stands as the third most common cancer following prostate and breast cancer in Finland (Figure 2) (Pitkäniemi et al. 2019, Finnish Cancer Registry).

Mortality is falling in developed countries given better screening, earlier detection, and improved treatment availability and methods, all in contrast to transitioning countries

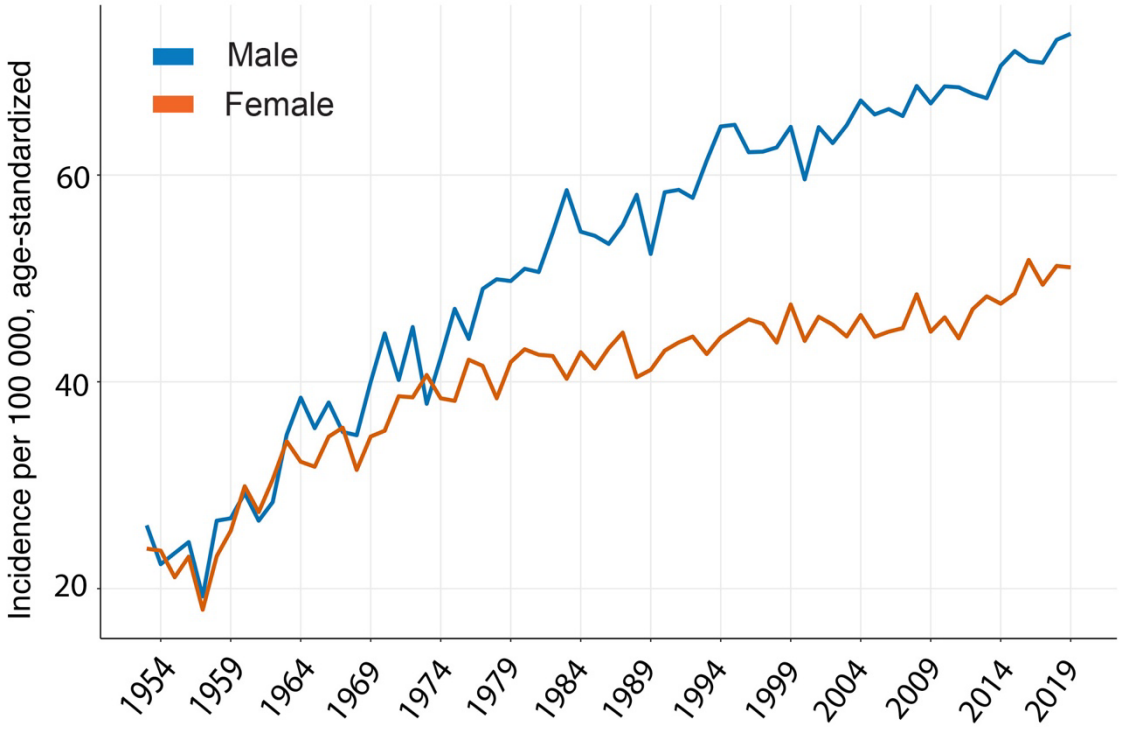

Figure 2. *Age-adjusted CRC incidence in Finland among men and women. Modified from the Finnish Cancer Registry, 2019.*

where mortality rates continue to rise (Arnold et al., 2017; Sung et al., 2021). In Finland, mortality rates are declining (Pitkäniemi et al., 2019). The prognosis for CRC patients in Finland has improved in recent decades, with a 5-year survival rate of 67.1% for colon cancer and 69.8% for rectal cancer patients (Pitkäniemi et al. 2019, Finnish Cancer Registry).

2.2 ETIOLOGY AND RISK FACTORS

Several genetic, environmental, and lifestyle factors impact the development of CRC. Among sporadic CRC cases, up to one-third have a family history of CRC. The risk of CRC is more than two times higher among individuals who have one first-degree relative with a history of disease, with the risk increasing if the relative was diagnosed at a younger age or if more relatives, also beyond first-degree, were affected (Rawla et al., 2019; Sawicki et al., 2021).

The so-called Western lifestyle — characterized by a history of smoking, heavy alcohol consumption, a high intake of red, processed meat and animal-derived fat, and low physical activity leading to obesity — is a major risk factor in the increasing burden of CRC (Rawla et al., 2019). Changing these modifiable lifestyle factors allows for a reduction in the risk of CRC. A varied diet with a high fruit, vegetable, and whole grain intake, in addition to the adequate consumption of calcium and vitamin D supplements, and the prolonged use of nonsteroid anti-inflammatory drugs may reduce CRC risk (Rawla et al., 2019; Sawicki et al., 2021).

The risk of developing CRC increases with age, whereby the majority of individuals diagnosed with CRC are over 50 years old (Siegel et al., 2020), although an increasing incidence is observed among people aged 20–49 (Rawla et al., 2019). The CRC incidence rate is 31% higher among men, although the gender difference is exceedingly small in cases occurring before the age of 45 (Siegel et al., 2020). Moreover, incidence and mortality rates are significantly higher among African Americans and Native Americans in the United States. Ethnic differences have significantly increased since the 1980s, and are primarily associated with other risk factors, such as obesity, smoking habits, a low socioeconomic status, and a worse uptake of screening programs (Rawla et al., 2019; Siegel et al., 2020).

2.3 HEREDITARY COLORECTAL CANCER SYNDROMES

Around 5% of CRC cases are caused by known hereditary nonpolyposis or polyposis syndromes, of which the most common, responsible for 3% of CRC cases, is Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer (HNPCC) (Ma et al., 2018). Inherited in an autosomal-dominant manner, HNPCC is caused by a germline mutation in DNA mismatch repair (MMR) genes (MLH1, MSH2, MSH6, or PMS2)

leading to a microsatellite instability (MSI) (Ma et al., 2018; Seppälä et al., 2020). Lynch syndrome can also be caused by a germline deletion in the epithelial cell adhesion molecule (EpCAM) leading to MSH2 deactivation (Sinicrope, 2018).

Individuals with Lynch syndrome have a 50% lifetime risk of developing CRC and an increased risk for other malignancies, with endometrium and ovary cancer representing the most common extracolonic manifestations (Sinicrope, 2018). All new colorectal and endometrial cancer cases are recommended for testing for an MMR deficiency. Patients without cancer who fulfil the Amsterdam II criteria are recommended for genetic screening (Seppälä et al., 2020). Colonoscopy screening every 2–5 years beginning at the age of 25–35, depending on the mutation type, is also recommended (Seppälä et al., 2020).

Familial adenomatous polyposis (FAP) is the second most common hereditary syndrome, causing up to 1% of all CRC cases (Ma et al., 2018). FAP is inherited in an autosomal-dominant manner and caused by a mutation in the adenomatous polyposis coli (APC) gene (Ma et al., 2018). By early adolescence, all FAP patients develop hundreds or thousands of colorectal adenomatous polyps, and if untreated all patients develop CRC by the age of 40–50 (Ma et al., 2018). Polyps may occur and develop into cancer elsewhere in the gastrointestinal tract as well, especially in the stomach and duodenum (Aihara et al., 2014). Individuals with first-degree relatives diagnosed with FAP should undergo genetic screening. If an APC mutation is found, an annual colonoscopy is recommended until a prophylactic proctocolectomy or colectomy is performed (Aihara et al., 2014).

Other less common syndromes such as Peutz–Jeghers syndrome and juvenile polyposis are inherited in an autosomal dominant manner, and MUTYH-associated polyposis is inherited in a recessive manner (Ma et al., 2018).

2.4 PATHOGENESIS

A stepwise accumulation of diverse genetic alterations and epigenetic changes leads to the dysregulation of epithelial cell homeostasis and a transition to premalignant lesions and ultimately CRC. Faeron and Vogelstein introduced a multistep model of CRC development known as the adenoma–carcinoma sequence (Figure 3) (Fearon and Vogelstein, 1990). This model, initially based on carcinogenesis among FAP patients, applies to the most common chromosomal instability (CIN) pathway (Palma et al., 2019). The natural history of carcinomas is, however, variable and morphomolecular heterogeneity can be seen between tumors as well as in the same tumor. Currently, two other genetic instability pathways — namely, a microsatellite instability (MSI) and the cytosine–guanine dinucleotide group (CpG) island methylator phenotype (CIMP) pathway — of sporadic CRC are recognized. In addition, CRC does not develop only according to one pathway, whereby overlapping features may occur (Grady and Markowitz, 2015).

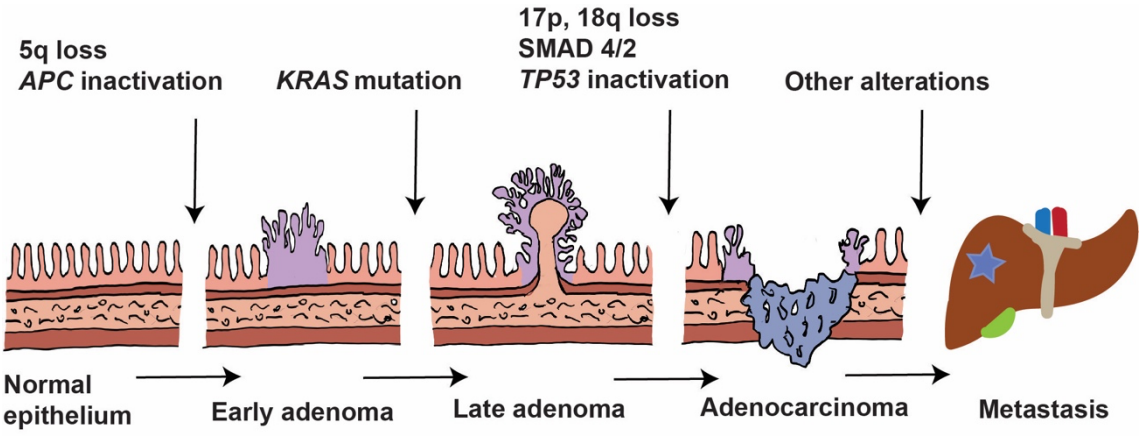

Figure 3. *Adenoma–carcinoma sequence in colorectal cancer (Armaghany et al.,2012; Fearon and Vogelstein, 1990).*

The development from a normal epithelial cell to a carcinoma takes years or decades depending on the specific pathway. Yet, in certain hereditary syndromes the mutations accumulate faster, and a carcinoma can develop in just a few years (Nguyen et al., 2020; Palma et al., 2019).

Adenomatous polyps are precursors to the majority of CRC cases, but 15–30% of CRC cases arise through an alternative serrated pathway from serrated benign lesions (Simon, 2016). Serrated lesions usually carry *BRAF* mutations and less frequently *KRAS* mutations, but do not have *APC* mutations. The serrated pathway is associated with the MSI and CIMP pathways (Palma et al., 2019).

2.4.1 Chromosomal instability (CIN) pathway

The chromosomal instability (CIN pathway) results in 70–85% of CRC cases. These nonhypermutated tumors are characterized by a high rate of gains or losses of whole chromosomes or chromosomal regions leading to aneuploidy, subchromosomal genomic amplifications, a high frequency of heterozygosity loss, and chromosome imbalances (Bali et al., 2021; Nguyen et al., 2020). Mutations in several tumor-suppressor genes and oncogenes are typical for CIN, such as the inactivation or deletion of the APC tumor-suppressor gene, the activation of the Kirsten ras homolog (KRAS) oncogene, the loss of chromosome 18q, the deletion of chromosome 17p, and the inactivation of tumor protein 53 (TP53) suppressor gene, although all CIN tumors do not present all of these mutations (Bali et al., 2021; Nguyen et al., 2020).

An APC mutation is an early event during carcinogenesis, occurring in 85% of tumors. The main pathway involved in CIN tumors is the Wnt cellular pathway, activated in almost all CIN tumors (Nguyen et al., 2020). APC suppression leads to the upregulation of Wnt signalling, resulting in the uncontrolled nuclear accumulation of β-catenin and the proliferation of undifferentiated cells.

A KRAS mutation, another early event, occurs in 45–60% of cases. KRAS is a downstream effector of growth factor pathways, such as the epidermal growth factor receptor (EGFR) pathway. The mitogen-activated protein kinase (MAPK) pathway is upregulated if mutations in KRAS and/or BRAF genes occur, stimulating RAS and RAF proteins and cell proliferation (Armaghany et al., 2012).

TP53 inactivation is a late event in carcinogenesis, present in 60% of CIN tumors (Nguyen 2020). The p53 protein normally regulates the cell cycle, providing time for DNA repair and inducing apoptosis if DNA repair is unsuccessful (Armaghany et al., 2012).

2.4.2 Microsatellite instability (MSI) pathway

The MSI pathway does not affect chromosomal integrity, but is characterized by a generalized instability in short, repeated DNA sequences called microsatellites. Normally nucleotide mismatches in DNA replication are corrected by MMR enzymes, but mutations in MMR genes lead to a deficient MMR function with an inability to repair DNA replication errors (Nguyen et al., 2020). In sporadic MSI tumors, methylation of the CpG-rich MLH1 promoter leading to MLH1 expression silencing occurs (Bali et al., 2021; Nguyen et al., 2020). MSI is responsible for 15–20% of sporadic CRC cases. MSI mutations lead to the disruption of several genes involved in the regulation of cell proliferation, apoptosis, and DNA repair. For example, a TGFB receptor-2 gene (*TGFBR2*) mutation is found in more than 90% of MSI tumors, leading to the activation of cell proliferation (Nguyen et al., 2020). In sporadic MSI tumors, the morphological precursor lesion of CRC is a sessile serrated adenoma, frequently with a *BRAF* mutation, whereas MSI tumors in Lynch syndrome patients arise from adenomatous polyps, tumors which lack *BRAF* mutations (Nguyen et al., 2020; Søreide et al., 2009).

Tumors are classified as MSI high, MSI low, and microsatellite stable (MSS) depending on the number of positive microsatellite markers in a testing panel (Nguyen et al., 2020). Patients with MSI-high tumors exhibit a better prognosis (Søreide et al., 2009).

2.4.3 CpG island methylator phenotype (CIMP) pathway

The hypermethylation of numerous promotor CpG island loci and subsequent inactivation of tumor suppressor genes, such as *APC* and *MLH1*, is an epigenetic alteration common to 15–20% of CRC cases, called CpG island methylator phenotype (CIMP) tumors (Bali et al., 2021). These tumors typically have a *BRAFV600E* mutation. Sessile serrated adenomas are precursors to CIMP tumors. A majority of sporadic MSI tumors have methylation of the *MLH1* gene (80%), although CIMP tumors may occur without an MLH1 methylation or MSI (Nguyen et al., 2020; Søreide et al., 2009).

Tumors are classified as CIMP high or CIMP low depending on the amount of simultaneous hypermethylated islands. CIMP-high tumors are associated with a proximal

location, an advanced stage, an older age, a higher rate of tumor-infiltrating lymphocytes, and being female (Bali et al., 2021). CIMP-high tumors have high *BRAF* mutation rates and low *TP53* mutation rates, while CIMP-low tumors more often have *KRAS* mutations (Bali et al., 2021; Palma et al., 2019).

2.5 CRC ASSOCIATED WITH INFLAMMATORY BOWEL DISEASE

CRC risk among patients with inflammatory bowel diseases (IBD) is well known (Kim and Chang, 2014). A defective mucosal barrier and failed homeostatic balance between intestinal microbiota and immune responses contributes to tumor development in IBD patients (Lasry et al., 2016). *APC* loss is less frequent and occurs later in IBD-associated cancer, while a *TP53* mutation is seen early in a majority of colitis-associated cancers and in nondysplastic mucosa (Kim and Chang, 2014).

CRC risk is up to twofold higher among IBD patients compared with the general population although a much higher risk has been reported in some previous studies (Annese et al., 2015). A long disease duration, wide extent of disease, and diagnosis at a younger age are CRC risk factors among IBD patients. Additionally, simultaneous primary sclerosing cholangitis increases the risk of CRC remarkably (Kim and Chang, 2020; Lutgens et al., 2013). Among a Finnish cohort of 1915 IBD patients, 21 CRC cases were diagnosed and the risk of CRC was only moderately higher compared with the general population (Manninen et al., 2013).

According to recent studies, IBD–CRC patients exhibit a survival similar to that among sporadic CRC patients (Leowardi et al., 2016; Lin et al., 2022). In the Finnish cohort mentioned above, no increase in CRC mortality was observed among IBD pateints compared with the general population (Manninen et al., 2012).

2.6 SCREENING

A fecal occult blood test (FOBT) is the most common screening method for CRC worldwide. Patients with a positive FOBT carry a higher risk of dying from CRC (Libby et al., 2018). Two types of FOBTs are available: guaiac-based FOBT (gFOBT) testing and a fecal immunochemical blood test (FIT) (Robertson et al., 2017). Previously, gFOBT was widely used, but over time FIT has proven superior to gFOBT, both in detecting cancer and dysplastic lesions and in the participation rate (Hassan et al., 2012; Robertson et al., 2017). With one-time testing, the sensitivity of FIT for detecting cancer is around 80% and 2–30% for detecting advanced dysplastic lesions (Robertson et al., 2017).

In Finland, around 180 000 patients were screened between 2004 and 2012 with no impact on CRC mortality, possibly because the follow-up time was too short to determine the mortality effect (Pitkäniemi et al., 2015). Screening was stopped for a few years, but

new data of later health outcomes detected a decrease in CRC mortality also among the control group, which did not undergo gFOBT. Thus, the decrease in CRC mortality with screening was 5% (Miettinen et al., 2017). Nationwide CRC screening with FIT began again in 2019 among people aged 60–66 (Heinävaara et al., 2019). According to the CRC screening decree amended by the Finnish Government in August 2021, screening will be extended stepwise to people aged 56–74 by 2031. FIT testing is conducted every second year, and in the case of a positive FIT, a high-quality colonoscopy is recommended (Heinävaara et al., 2019).

Flexible sigmoidoscopy or colonoscopy screening resulted in at least a 40% decrease in cancer incidence and 60% mortality (Brenner et al., 2014). The advantage of endoscopy over fecal screening lies in the opportunity to prevent CRC by removing premalignant lesions, although its high cost and possible complications diminish its use as a screening method.

To achieve the optimal cost-effectiveness of a screening program, the appropriate cut-off point must be chosen for the test. For men, FIT has a higher sensitivity and lower specificity than for women, and thus gender-specific cut-offs should be applied (Arana-Arri et al., 2017). To detect one advanced neoplasia, 59 colonoscopies must be performed on men and 92 on women at a FIT cut-off of 20-µg Hb/g feces. If the Hb cut-off is increased to 60-µg Hb/g feces, the number of colonoscopies needed increases to 230 in order to detect one advanced neoplasia. Since screening increases the demand for colonoscopies, increasing the cut-offs could rule out as many as 50% of colonoscopies, but result in detecting 43% fewer adenomas and 22% fewer CRC cases. As many as 70% of lost CRC cases present with early-stage tumors (Arana-Arri et al., 2017).

In ongoing European screening programs, participation rates vary from between 48% to 79%, with the highest rate found in Finland (Sarkeala et al., 2021). The positive test rate was 2.4% for the first screening year in Finland, while in other countries it falls between 6.0% and 9.6%. The CRC detection rate is 1.7 per 1000 participants in Finland and 3.1–5.3 in other countries. The positive predictive value of a colonoscopy for CRC is 6.2–8.9%, and 22.4–41.1% for advanced adenoma (Sarkeala et al., 2021).

2.7 SYMPTOMS AND DIAGNOSIS

CRC symptoms may be variable and vague, or patients may be asymptomatic. Due to CRC growing to the lumen and the bowel wall, symptoms accompany relatively large tumors. Changes in bowel habits, rectal bleeding, unintended weight loss, fatigue, iron-deficiency anemia, and discomfort or pain in the abdominal region are common symptoms among CRC patients (Hamilton et al., 2005). Weight loss and fever are more common in the presence of metastasized disease, although even the spread of disease can be asymptomatic. Up to 30% of patients present as emergency cases, which may be their first indication of CRC (Pisano et al., 2018). Obstruction, most commonly located in the

sigmoid colon, causes 80% of emergency cases, while perforation, typically at the tumor site, causes the remaining 20% (Pisano et al., 2018).

Histological, immunohistochemical, and genetic analyses of biopsies taken from a tumor during colonoscopy serve as the basis of CRC diagnosis (Argiles et al., 2020). Synchronic CRC is present in 3.6% of patients and visualizing the complete colon is important either preoperatively or within 3–6 months postoperatively (Argiles et al., 2020). Digital examination is also important in the diagnosis of rectal tumors. Computed tomographic colonography may be performed if colonoscopy is impossible (Argiles et al., 2020; Spada et al., 2021). Contrast-enhanced computer tomography (CT) of the chest, abdomen, and pelvis is the primary radiologic examination used to determine the locoregional extent of the tumor and the distant spread of disease (Argiles et al., 2020). Finding locally advanced tumors is highly important since such patients may benefit from preoperative treatment (Seligmann and Group, 2020). The sensitivity of CT in finding such tumors is around 90% with a specificity of around 70%. Often CT cannot distinguish neoplastic pericolic fat infiltration from a desmoplastic reaction, and may lead to overstaging. Furthermore, the determination of the nodal status using CT imaging is limited with a 78% sensitivity and a 68% specificity (Nerad et al., 2016). Although the sensitivity for detecting distant metastases is 85 % and the specificity is 98%, very small liver metastases may not be visible in a CT (Velde et al., 2014).

Among rectal cancer patients, magnetic resonance imaging (MRI) of the pelvic region is conducted for preoperative staging, making it possible to assess tumor location, size, invasion, relationship to the mesorectal fascia, local lymph node involvement, vascular invasion, and sphincter involvement, all crucial in deciding whether the patient can undergo upfront surgery or requires neoadjuvant treatment before surgery (Velde et al., 2014). Fluorodeoxyglucose–positron emission tomography (FDG–PET) scan (or PET with CT) is sometimes used to determine the extent of metastatic disease (Argiles et al., 2020; Cutsem et al., 2016).

The serum level of carcinoembryonic antigen (CEA) is measured for patients diagnosed with CRC (Glynne-Jones et al., 2017; Locker et al., 2006; Velde et al., 2014). Unfortunately, the specificity and sensitivity of CEA are too low for single use in follow-up and diagnosis (Liemburg et al., 2021; Shinkins et al., 2017). The sensitivity is around 59% with a specificity 89%. Using CEA at a threshold of 5 μg/L would lead to missing half of all cases (Liemburg et al., 2021). CEA measurement is used for staging and planning treatment, to monitor the efficiency of treatment, and to detect possible recurrence. An increased CEA level indicates a more advanced stage (Polat et al., 2014). An elevated preoperative CEA is independently associated with survival (Tarantino et al., 2012), but also an early postoperative CEA indicates a worse outcome (Auclin et al., 2019; Lin et al., 2011). The 5-year disease-free survival (DFS) is 62% for stage II patients with an elevated postoperative CEA level compared with 87% for patients with a low postoperative CEA level (Lin et al., 2011). Furthermore, high-risk stage II patients with

an elevated postoperative CEA level appear to benefit from adding oxaliplatin to treatment (Auclin et al., 2019).

2.8 TUMOR LOCATION

Based on the anatomical location, CRCs are classified as colon and rectum tumors. Around 70% of CRCs are diagnosed in the colon and 30% in the rectum (Paschke et al., 2018). The rectum is 15 cm long involving the large bowel from the sacral promotorium to the anocutaneous line and is divided into the low, middle, and high rectum. Although the rectum belongs embryologically to the left colon, debate surrounds whether tumors in either location should be classified as different diseases (Paschke et al., 2018; Stintzing et al., 2017).

Proximal and distal CRCs are seen as different diseases biologically, immunologically, and genetically. The division is usually made at the splenic flexure based on an embryological origin (Figure 4) (Stintzing et al., 2017). The blood supply to the distal colon and rectum stems from the inferior mesenteric artery, while the proximal colon is supplied from the superior mesentery artery (Sakorafas et al., 2006). Some argue that a continuum model and multisegmental approach would be more appropriate than a dichotomous division of the colorectum since the frequency of MSI high, CIMP high, and *BRAF* mutations increase linearly from the rectum to the ascending colon. The caecum, however, is unique with a high *KRAS* mutation rate (Yamauchi et al., 2012).

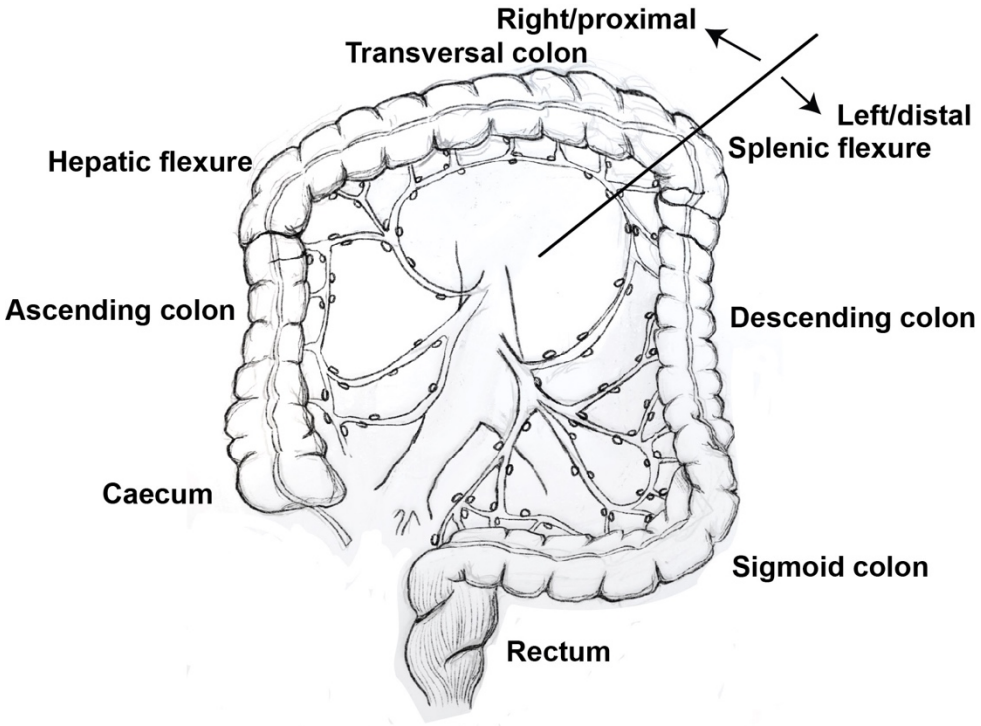

Figure 4. *Anatomical parts of the colorectum region and division into right/proximal and left/distal at the splenic flexure. Image drawn by Aletta Beilmann.*

Left-sided tumors more often arise via the CIN pathway and have *APC* and *TP53* mutations. *KRAS* and *BRAF* mutations and MSI high tumors are more common among patients with right-sided disease (Stintzing et al., 2017). Among hereditary syndromes, tumors associated with Lynch syndrome arise in the colon, in particular the proximal colon, whereas FAP patients develop cancer more often in the left colon and rectum (Paschke et al., 2018).

A proximal tumor location is considered a negative prognostic factor (Brungs et al., 2017; Petrelli et al., 2017; Qiu et al., 2018), although a stage-dependent effect related to the tumor location occurs. Within stage I–II, patients with proximal disease exhibit a better prognosis than those with distal disease. By contrast, among stage III patients, those with right-sided cancer exhibit a worse prognosis (Brungs et al., 2017; Turner et al., 2019; Warschkow et al., 2016). It remains unclear why such differences among stages occur between right- and left-sided disease. Perhaps MSI serves as one explanation for MSI high patients experience a better survival. Additionally, right-sided tumors are more often found at a more advanced stage and more often in elderly individuals (Brungs et al., 2017).

2.9 STAGING

The current and eighth edition of the Tumor Node Metastases Classification of Malignant Tumors (TNM) is used for tumor staging. This classification is based on the anatomical extent of the tumor, where T describes tumor infiltration (Figure 5), N describes the

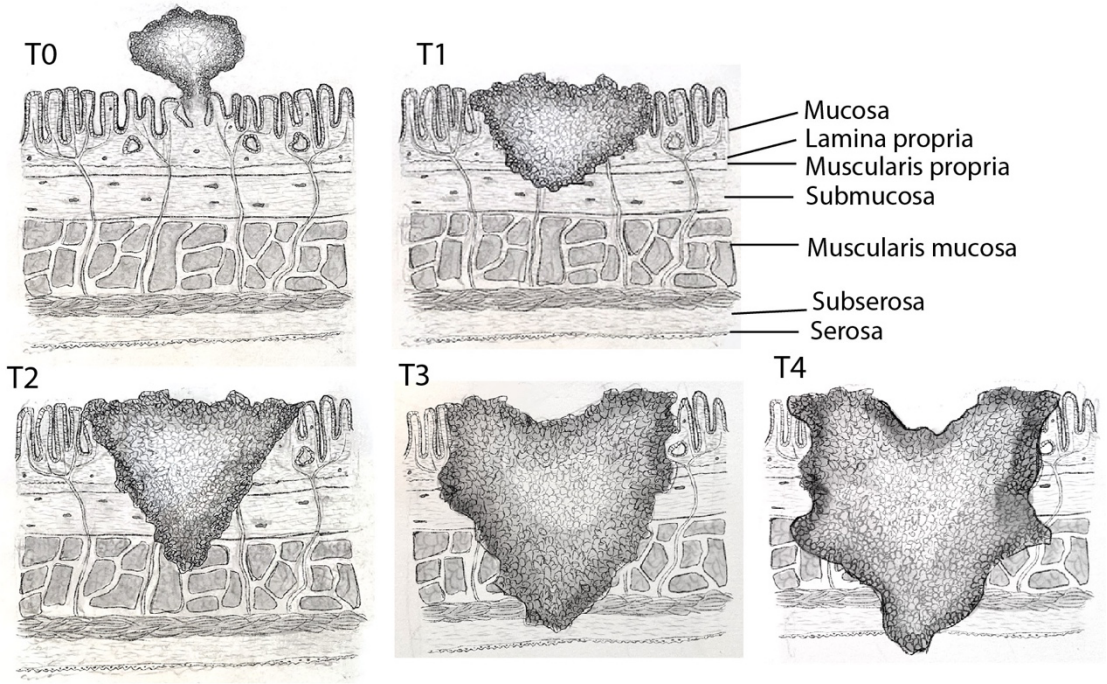

Figure 5. *Tumor stages according to the TNM eighth edition. Images drawn by Aletta Beilmann.*

Table 1. *Stage classification of colorectal tumors from the AJCC, eighth edition (2016).*

T – Primary tumor	
Tx	Cannot be assessed
T0	No evidence of primary tumor
T1	Carcinoma in situ, invasion of the lamina propria
T2	Tumor invades the submucosa
T3	Tumor invades the subserosa or into nonperitonealized pericolorectal tissues
T4	Tumor directly invades other organs or structures and/or perforates the visceral peritoneum
T4a	Tumor perforates the visceral peritoneum
T4b	Tumor directly invades other organs or structures
N – Regional lymph nodes	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1–3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2–3 regional lymph nodes
N1c	Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic tissues
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4–6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
M – Distant metastases	
M0	No distant metastases
Mx	Cannot be assessed
M1a	Metastasis in one organ without peritoneal metastasis
M1b	Metastasis in 2 or more organs without peritoneal metastasis
M1c	Peritoneal metastases alone or with other organ metastases

Stage	**T**	**N**	**M**	**Dukes**
0	Tis	N0	M0	
I	T1	N0	M0	A
	T2	N0	M0	A
IIA	T3	N0	M0	A
IIB	T4a	N0	M0	B
IIC	T4b	N0	M0	B
IIIA	T1–T2	N1/N1c	M0	C
	T1	N2a	M0	
IIIB	T3–T4a	N1/N1c	M0	C
	T2–T3	N2a	M0	
	T1–T2	N2b	M0	
IIIC	T4a	N2a	M0	C
	T3–T4a	N2b	M0	
	T4b	N1–N2		
IVA	AnyT	Any N	M1a	D
IVB	Any T	Any N	M1b	D
IVC	Any T	Any N	M1c	D

involvement of regional lymph nodes, and M represents distant metastases (Table 1). Tumors are divided into four American Joint Committee on Cancer (AJCC) stages based on the TNM classification (Table 1) (Brierley et al., 2017). The TNM stage is the most important prognostic factor among CRC patients. In the current TNM classification, tumor deposits, with a small tumor foci in pericolic, perirectal, or mesocolic fat, discontinuous with the primary tumor, are classified as N1c. Such tumors, even when no other lymph node metastases occur, are classified as stage III.

The Dukes classification, an older system for staging CRC, first published in 1932 for rectal cancer only, is the cornerstone for the management of CRC (Dukes, 1932). In Dukes stage A a tumor invades the submucosa or muscularis propria. In stage B a tumor invades through the bowel wall, and in stage C local lymph node metastases are present. Dukes reported that the prognosis depended upon the Dukes stage. Subsequently the staging system was modified and stage D was added for distant metastases, and the system was also adapted for colon cancers (Astler, 1954; Turnbull et al., 1967).

2.10 THE CONSENSUS MOLECULAR CRC SUBTYPES

Based on genetic mutations, CRC is divided into four consensus molecular subtypes (CMSes) (Guinney et al., 2015). CMS1 group is called MSI immune and comprises 14% of tumors. These tumors are commonly seen in female patients and in a proximal location. CMS1 tumors are characterized by a high number of MSI and *BRAF* mutations, and a high expression of several mutant forms of immunogenic proteins leading to high Th1 and CD8+ immune cell infiltration. CMS1 patients experience a poor survival rate following recurreence (Guinney et al., 2015).

The most common subgroup is the canonical or CMS2 group, comprising 37% of tumors frequently found in left-sided tumors. These are characterized by a high level of somatic copy number alterations (SCNAs), leading to chromosomal instability, with high copy number gains in oncogenes and high copy number deletions in suppressor genes. Hyperactivation of the classical WNT and MYC pathways is typical in these tumors. The CMS2 subtype is associated with the best survival following recurrence (Guinney et al., 2015).

The CMS3 group, also called the metabolic group, comprises 13% of tumors. These tumors are characterized by a chromosomal instability, but with a lower level of SCNAs. The high prevalence of a low CIMP status is common in these tumors, with a high number of *KRAS* mutations. One-third of CMS3 tumors are low/moderate MSI compared with CMS2 and CMS4 which are MSS tumors (Guinney et al., 2015).

The mesenchymal or CMS4 group comprises 23% of tumors, characterized by nonhypermutated MSS tumors with a chromosomal instability and the upregulation of epithelial–mesenchymal transition genes. Mesenchymal activation and the overexpression of proteins involved in extracellular matrix remodelling, stromal

infiltration, and angiogenesis characterize these tumors, which are commonly diagnosed at a more advanced stage. Overall survival and relapse-free survival are worse than among other CMS groups (Guinney et al., 2015).

Biomarkers such as the MSI status, *KRAS* and *BRAF* mutations, and CIMP status are used to plan CRC treatment (Phipps et al., 2015). However, as seen from the CMS descriptions, these specific biomarkers are insufficient for characterizing these heterogeneous tumors. The genomic profiling of CMS subtypes has not yet been adapted for clinical application. A robust immunohistochemical assessment of five biomarkers has been recommended for a simpler CMS classification, but does not allow for distinguishing between all CMS types, although a worse survival among CMS4–mecenchymal patients was validated (Trinh et al., 2017).

2.11 TREATMENT

2.11.1 Colon cancer surgery

Surgery is the standard curative treatment for local colon cancer, with the quality of surgery crucial to the outcome. However, an endoscopic resection and proper follow-up are acceptable for the treatment of invasive carcinoma in polyps (pT1) if no high-risk factors are present (Argiles et al., 2020; Hyöty et al., 2019).

The location of the tumor determines the type of surgery. A standard operation for right-sided tumors is a right hemicolectomy with removal of the mesocolon, high ligation of the ileocolic artery, and transection of the right branches from the middle colic artery, while the root of the middle colic artery is left intact. In an extended right hemicolectomy, used for hepatic flexure tumors, the middle mesenteric artery is transected from its root (Figure 6) (Hohenberger et al., 2002; Hyöty et al., 2019). For left-sided colon tumors, a left hemicolectomy serves as the standard procedure with the removal of the left mesocolon and ligation of the inferior mesocolic artery and vein (Hohenberger et al., 2002; Hyöty et al., 2019). Whether the inferior mesocolic artery should be ligated high or low remains debatable. A high ligation allows for a D3 dissection of the lymphatic tissue, but can predispose a patient to an anastomotic leakage and genitourinary dysfunction (Mari et al., 2018; Si et al., 2019). If nearby organs are involved, an en-bloc resection is preformed.

Increasingly the current method-of-choice in colon surgery is a complete mesocolon excision (CME), resembling total mesorectal excision (TME) techniques employed in rectal surgery (Hohenberger et al., 2009). CME involves dissection following the embryological planes and the lymphatic tissue remains intact within the mesenterium envelope covered with visceral fascia layers from both sides. High ligation of blood vessels allowing the apical lymph nodes (D3) from the vessel pedicle to be harvested with the specimen is part of CME technique (Hohenberger et al., 2009; Kim et al., 2016).

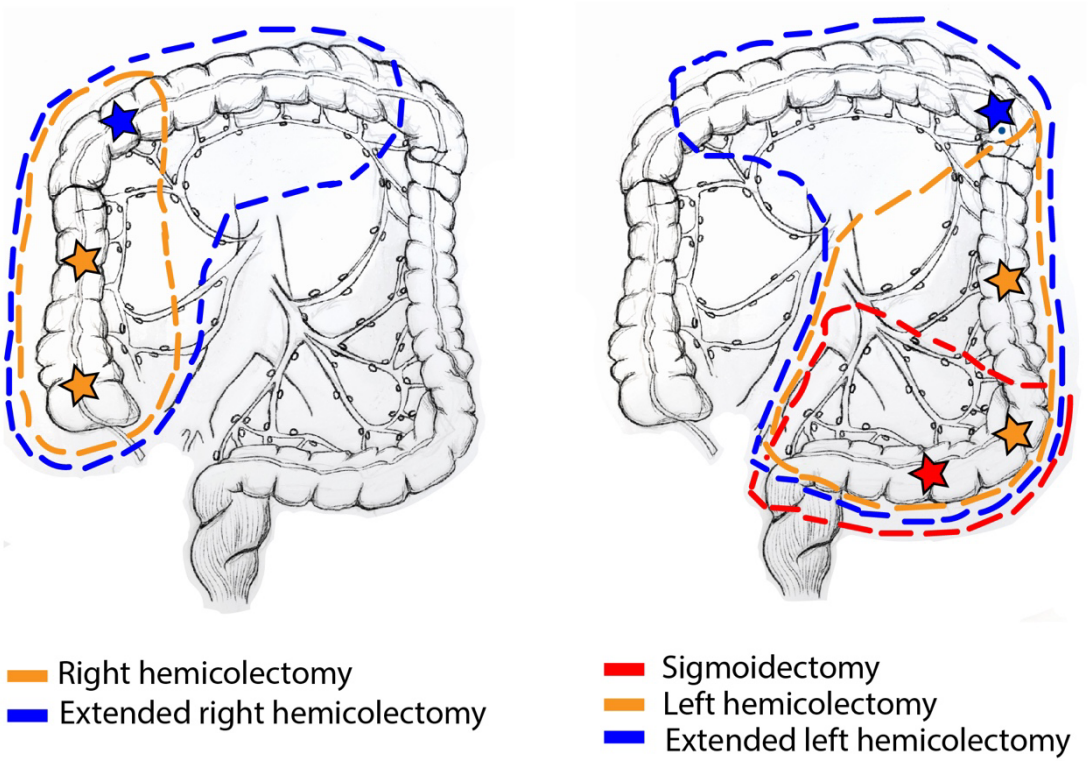

Figure 6. *Different surgical methods depending on the tumor site. Images drawn by Aletta Beilmann.*

Current discussions focus on whether the standard D2 or CME technique should be used. A better survival has been demonstrated for the CME technique in several studies (Alhassan et al., 2019), although randomized controlled trials remain lacking and CME has not yet been included in guidelines (Hyöty et al., 2019).

Laparoscopic colon surgery carries short-term advantages compared to open surgery, such as a shorter hospital stay, less postoperative pain, faster recovery of bowel motility, and a better quality of life. Interestingly, survival outcomes from laparoscopic surgery are similar to those from open surgery (Tanis et al., 2014; Wu et al., 2017).

2.11.2 Rectal cancer surgery

Multidisciplinary teams are the gold standard and the basis for rectal cancer care. Treatment strategies for rectal cancer patients, with both local and advanced disease, should be decided upon through multidisciplinary teamwork (Glynne-Jones et al., 2017; Prades et al., 2015).

A total mesorectal excision (TME) is the standard surgical technique based on a sharp dissection following the embryological planes and completely removing the rectal tumor along with the surrounding mesorectum and lymph nodes, enveloped in the intact mesorectal fascia (Knol and Keller, 2020). Middle and high rectum tumors are operated on using an anterior resection, while low rectum tumors rely on a very low anterior resection or abdominoperineal resection. Sphincter-preserving surgery yields a better

quality of life, particularly in relation to better sexual and urinary function outcomes, compared to abdominoperineal resection and with similar oncological outcomes (Kang et al., 2021). In the case of external sphincter involvement or levator invasion, an abdominoperineal resection should be chosen (Garcia-Henriquez et al., 2020). Temporary fecal diversion to avoid an anastomotic leak was previously routinely performed, but is now recommended for high-risk patients with a very low tumor and a sphincter-preserving resection (Chapman et al., 2019).

The circumferencial resection margin (CRM), defined as the closest radial distance between the dissection edge of the rectal resecate and the most invasive point of the tumor or lymph node, is the most important surgical prognostic factor of local recurrence (Liu et al., 2018; Nagtegaal and Quirke, 2008). Thus, a CRM ≥1 mm is recommended. The completeness of the TME specimen represents a good indication of the surgical quality and predicts recurrence (Song et al., 2018)

The advantages of the laparoscopic technique are similar in colon surgery with a shorter hospital stay, earlier bowel function, and less blood loss, but with a longer operative time (Bonjer et al., 2015; Fleshman et al., 2015; Stevenson et al., 2015). Interestingly, short- and long-term oncological outcomes are similar for laparoscopic and open surgeries (Fleshman et al., 2019; Stevenson et al., 2019). The TME specimen completeness is achieved in 87% of laparoscopically treated pateints and in 92% of pateints treated with open surgery. The CMR was clear in 93% of laparoscopic surgical patients and in 97% of open surgery patients. DFS at 2 years was 80% for the laparoscopic approach and 82% for open surgery. Robotic surgery may provide better results in terms of urinary and sexual function outcomes in rectal surgery with a conversion rate lower than that accompanying laparoscopy, although the duration of the procedure is longer (Prete et al., 2018).

Transanal total mesorectal excision (TaTME) is a new endoscopic transanal technique following the principles of TME (where the procedure begins from the distal margin of the resection), which could result in a better TME completeness and clean CRM in low rectal cancer. Local recurrence rates are similar to that achieved in an open and laparoscopic TME (Hol et al., 2019; Roodbeen et al., 2021), although the results from randomized controlled trials have yet to be published (Deijen et al., 2016; Kang et al., 2020). A complete or nearly complete TME specimen is achieved in 97% of cases and involvement of CRM exceeding 1 mm is seen in around 5% of patients. The local recurrence rate is around 3.8% at 2 years with an overall survival (OS) of almost 92% at 2 years (Hol et al., 2019; Roodbeen et al., 2021).

Local excision procedures, such as a transanal excision, transanal endoscopic miocrosurgery, and transanal minimally invasive surgery, are acceptable surgical techniques for some local rectal tumors even though such procedures do not provide the same advantages as TME and lack an N status (Erkan et al., 2018). Guidelines recommend local excision only for cT1N0 tumors without high-risk features (Glynne-Jones et al., 2017; Hyöty et al., 2019; Velde et al., 2014). If high-risk factors are present in the

pathology report after local excision, TME is recommended. The 5-year realtive survival rate is 93.4% for pT1 tumors treated with local excision and 95% for those treated with TME. For pT2 tumors, the survival rates are 88.2% and 92.9%, respectively, and for pT3–4 tumors 20.4% for local excision and 74.9% for TME (Verseveld et al., 2019). Local excision of cT1N0 tumors not considered low risk is recommended only for fragile patients or those who refuse more extensive surgery because of the possibility of morbidity.

Locally advanced tumors that invade nearby structures and organs require resection beyond the TME planes and often include multivisceral en block resection of nearby organs or even a total pelvic exenteration (Mohan et al., 2013; Yang et al., 2015). The 5-year OS after multivisceral resection is 52.8% for primary rectal cancer and 19.5% for recurrent disease. After pelvic exenteration, 5-year OS is 58.7% and 11.8%, respectively.

A wait-and-see policy without operative treatment can be considered for some select patients if a complete clinical response is observed following neoadjuvant treatment (Li 2016). Several doubts remain, however, and the appropriate time frame remains unclear for evaluating the complete response following neoadjuvant treatment. Patients who undergo a wait-and-see policy must undergo frequent follow-up screening. According to the data available, such patients exhibit a similar survival, but experience a higher local recurrence rate than those who undergo surgery after neoadjuvant treatment (Li 2016).

2.11.3 Metastatic disease surgery

Synchronous metastasized disease is diagnosed in almost 18% of CRC patients, and almost 20% of resected stage I–III patients develop metachronous metastases (Väyrynen et al., 2020). The liver is the most common site of metastases followed by the lungs. Advances in chemotherapy and surgical developments have widened the criteria for possible liver resection (Kopetz et al., 2009). Significant survival improvements can be achieved if metastases are treated with a curative intent (Adam et al., 2015; Villard et al., 2021). A patient's fitness for surgery is evaluated carefully and, alongside the technical possibility of resection, the characteristics of the future liver remnant must be calculated (Adam et al., 2012; Butler and Toogood, 2017). If major resection is planned and the remnant liver volume is estimated as too small, augmentation of the liver remnant is an option using portal vein embolization or two-stage hepatectomy (Zhang et al., 2014).

In synchronous metastatic disease, a liver-first principle may be used, but more commonly the primary tumor is operated on first, followed by chemotherapy for approximately 2–3 months, followed by liver resection if the disease has not progressed during chemotherapy (Wang et al., 2020). In some cases, chemotherapy is not administered between surgery on the primary tumor and the liver. The simultaneous surgical treatment of primary CRC and synchronous liver metastases is another option, although this option remains debatable since reports have yielded contradictory findings (Kleive et al., 2021). The 5-year OS among patients with resectable liver metastases is

45–48%, while among those with nonresectable liver metastases OS falls to 3% (Väyrynen et al., 2020; Villard et al., 2021).

Patients with primarly nonresectable metastatic disease in one or a few organs may become potentially treatable after receiving conversion chemotherapy with the goal of a maximum response rate and tumor shrinkage (Gruenberger et al., 2015; Stintzing et al., 2016). Around 10% of patients with primarily nonresectable liver metastases are suitable for conversion chemotherapy and 20–30% of them are suitable for resection (Nozawa et al., 2018; Villard et al., 2021). The median OS for patients who complete conversion chemotherapy and resection is 24 months compared with 44 months in patients resected after neoadjuvant chemotherapy and 14 months among patients who progress durging conversion chemotherapy (Villard et al., 2021).

In some select patients with unresectable liver metastases, liver transplantation may improve OS. The inclusion criteria for liver transplantation must be well defined and strict. Today, liver transplantation among patients with unresectable liver metastases is possible only among patients enrolled in trials. The development of oncological treatment options and the introduction of new immunosuppressants have resulted in possibilities for new trials in this field (Puia-Negulescu et al., 2021). Since data remain insufficient on liver transplantation among patients with CRC metastases, the criteria have yet to be defined (Dueland et al., 2020). Major ethical issues surround liver transplantation in metastasized CRC patients. In most countries, the waiting list for liver transplants is long and the number of available organs is limited (Puia-Negulescu et al., 2021).

Local treatment options may be used to achieve local control of metastases and do not rule out subsequent operative treatment (Cutsem et al., 2016). Thermal ablation (e.g., radiofrequency ablation), transarterial chemoembolization, selective internal radiation therapy, and high-dose rate brachytherapy all represent possible local treatment procedures well-tolerated by patients. Thermal ablation may be used during resection as an additional treatment for some lesions (Cutsem et al., 2016; Petre and Sofocleous, 2017).

The incidence of synchronous or metachronous peritoneal carcinosis reaches around 8.3–11.4%, with survival among such patients worse than among those with metastases in other locations (Bakkers et al., 2021; Segelman et al., 2012). Surgical cytoreduction followed by hyperthermic intraperitoneal chemotherapy (HIPEC) may be an option for patients with peritoneal metastases. First, all visible tumor tissue is removed surgically, followed by perfusion of the abdominal cavity with the heated (41–43°C) chemotherapeutic agent mitomycin or oxaliplatin (González-Moreno et al., 2010). Patients who receive cytoreductive surgery and HIPEC have a median OS of 36 months, which falls to only 1.8 months for those who receive the best supportive care and 12.2 months for those who receive palliative treatment (Bakkers et al., 2021).

In a recent randomized controlled trial (PRODIGE-7), adding HIPEC to cytoreductive surgery did not increase survival (Quénet et al. 2021). The median OS was 41 weeks for both cytoreductive surgery alone and when combined with HIPEC, demonstrating that

completing cytoreductive surgery is the most important factor for longer survival among patients with peritoneal colorectal metastases. Specifially, 1-year OS was 86.9%, while 5-year OS was 39.4% in the cytoreductive surgery group. Yet, in the subgroup of patients with a peritoneal cancer index of less than 16, OS was better among those who received HIPEC.

The peritoneal cancer index is the primary prognostic factor following cytoreductive surgery and HIPEC. An index between 12 and 17 is recommended, but no agreed upon cut-off score has been recommended globally (Faron et al., 2016). The presence of metastases at other sites, the general condition of the patient, and the response to neoadjuvant therapy should also be taken into account when choosing patients for cytoreductive surgery and HIPEC (Faron et al., 2016; Sommariva et al., 2021). In Finland, the HIPEC procedure is preformed only in Helsinki and Oulu (Lepistö, 2016).

2.11.4 Oncological treatment

Adjuvant therapy aims to reduce the recurrence risk and improve prognosis among patients undergoing surgery. This is based on assessing the risk of possible recurrence, since the benefit achieved from therapy must outweigh the possible complications (Argiles et al., 2020).

Adjuvant therapy is recommended to all stage III patients and to stage II patients with high-risk factors (lymph node sampling <12 and pT4 stage) or multiple minor risk factors (lower differentiation, lymphovascular and perineural invasion, emergency surgery, tumor budding, and a high preoperative serum CEA level) (Argiles et al., 2020; Benson et al., 2021; Hyöty et al., 2019).

Since the 1990s, adjuvant fluoropyrimidine-based chemotherapy has become the standard of care for stage III colon cancer patients based on the Moertel trial results, with a 41% overall reduction in the recurrence rate accompanying levamisole–fluorouracil therapy. DFS at 3.5 years was 63% for stage III patients treated with levamisole and fluorouracil compared with 47% for patients who were observed after surgery (Moertel et al., 1990). Combining oxaliplatin with fluoropyrimidine reduced the risk of recurrence by 24% among stage III colon cancer patients within 3 years of treatment compared with fluorouracil and folinic acid without oxaliplatin. The 3-year DFS was 72.2% and 65.3%, respectively (André et al., 2004). The 10-year OS for stage III patients treated with fluorouracil and folinic acid combined with oxaliplatin was 67.1% compared to 59.0% for the ones treated without oxaliplatin (André et al., 2015).

Current standard adjuvant therapy consists of fluorouracil (peroral capecitabine or intravenous 5-fluorouracil) combined with oxaliplatin starting at 3–6 weeks after surgery. CAPOX (capecitabine with oxaliplatin) is used for 3–6 months, depending on the risk of recurrence, and FOLFOX (5-fluorouracil with oxaliplatin) for 6 months (Grothey et al., 2018). Adverse effects may influence the duration of treatment and the dosage of agents.

If a patient does not tolerate oxaliplatin, monotherapy with capecitabine or 5-fluorouracil is selected (Argiles et al., 2020; Hyöty et al., 2019).

Neoadjuvant treatment is recommended for rectal cancer patients depending on the TNM stage, mesorectal fascia involvement, extramural vascular invasion, and sphincter infiltration (Valk et al., 2020; Velde et al., 2014). Early-stage tumors in the middle or high rectum (cT1–2, cT3a/b, cN0–1) with no extramural vascular invasion and far from the mesorectal fascia can be operated on without preoperative treatment. For almost all other rectal tumors, neoadjuvant therapy is recommended in order to achieve tumor shrinkage, improve resectability, and reduce the recurrence risk (Glynne-Jones et al., 2017; Hyöty et al., 2019; Valk et al., 2020). Short-course radiotherapy consists of a 25-Gy dose in 5-Gy fractions administered over 5 days just a few days before surgery (Glynne-Jones et al., 2017; Hyöty et al., 2019). In long-course chemoradiotherapy, 50–54 Gy is divided into smaller fractions administered for 5 days weekly over 5–6 weeks combined with fluorouracil-based chemotherapy followed by surgery after 6–8 weeks (Glynne-Jones et al., 2017; Hyöty et al., 2019; Velde et al., 2014).

Chemoradiotherapy is preferred to radiotherapy among cT3, N1–2 tumors and among patients with cT4N0–2 disease in order to achieve possible R0 margins. Neoadjuvant treatment leads to about a 50% reduction in local recurrence compared with surgery alone, although no clear impact on long-time survival has been reported (Rahbari et al., 2013). In locally advanced T3–T4, node-positive rectal cancer, at least a 40% reduction in local recurrence was achieved with chemoradiotherapy compared with radiotherapy (McCarthy et al., 2012). In T4 nonresectable carcinoma, R0 resection is achieved in 84% of patients treated with chemoradiotherapy compared with 68% treated with radiotherapy alone (Brændengen et al., 2008). If it is likely that a patient will not tolerate this option, radiotherapy is administred instead, followed by delayed surgery. Some patients with cT4N0–2 disease may remain unresectable (Glynne-Jones et al., 2017; Hyöty et al., 2019).

In locally advanced colon cancer (high-risk T3 and T4 tumors), neoadjuvant therapy might be beneficial by inducing tumor regression and downstaging, but lacking proof it is not included in current guidelines (Body et al., 2021). A trend indicative of falling recurrence among patients with at least T3 disease who receive neoadjuvant therapy has been reported, although long-term survival figures remain lacking (Seligmann and Group, 2020). There is a significant risk of overstaging and overtreatment among such patients (Body et al., 2021).

The *RAS* and *BRAF* mutation status is relevant in metastasized disease (Figure 7). Treatment combination choices are guided by the intent of treatment: Is a good treatment

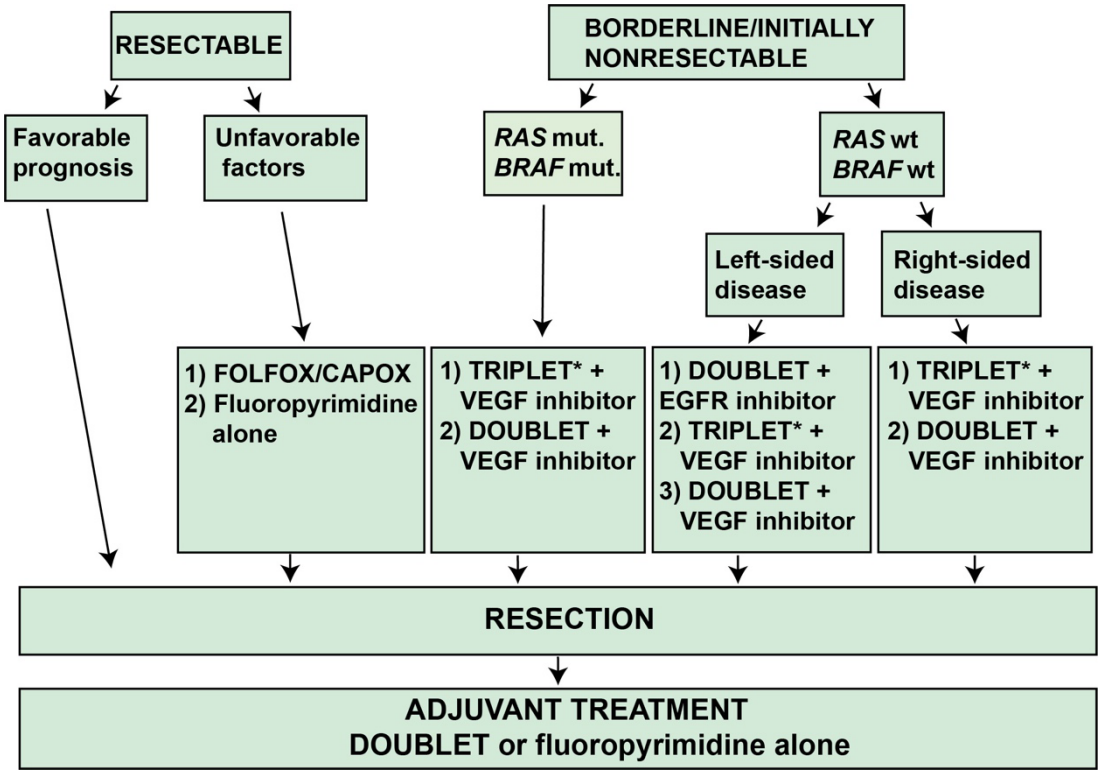

Figure 7. *Oncological treatment for metastasized resectable/potentially resectable colorectal cancer. Doublet: 5-fluorourasil/capecitabine + oxaliplatin (FOLFOX, CAPOX) or 5-fluorourasil/capecitabine + irinotecan (FOLFIRI, CAPIRI). Triplet: 5-fluorourasil + oxaliplatin + irinotecan (FOLFOXIRI). VEGF inhibitor–bevacizumab; EGFR inhibitor–cetuximab or panitumumab. Abbreviations: mut, mutation; wt, wild type. *Only for fit patients. Adapted from Cutsem et al. (2016) and Hyöty et al. (2019).*

response desired or is the goal to control disease in a palliative context? Patients with oligometastatic disease in one or a few organs may become potentially resectable following conversion chemotherapy even if primarily surgery for metastases is not possible (Gruenberger et al., 2015; Stintzing et al., 2016). Doublet and triplet chemotherapy combinations with immunotherapy are administered. The response to treatment is evaluated regularly to prevent overtreatment. Typically, treatment lasts 12–16 weeks, followed by surgery if tumor shrinkage is observed. Combining targeted therapy to the treatment program improves prognosis and resectability (Cutsem et al., 2016; Hyöty et al., 2019; Tomasello et al., 2017).

2.11.5 Palliative treatment

Palliative treatment includes symptom control, psycho-emotional support, and communication allowing for the best quality of residual life as the primary goal among patients with unresectable metastatic disease (Costi et al., 2014). Surgery on primary tumors in such asymptomatic patients is not recommended, although in symptomatic

cases surgical intervention may be necessary, leading to a solid relief of symptoms. Internal bypass, diversion, and resection are possible surgical procedures (Costi et al., 2014). In cases involving obstruction, the endoscopic application of a stent allows for faster initiation of chemotherapy with the same survival outcomes to that accompanying colectomy or diversion (Vogel et al., 2017). If a patient cannot tolerate palliative chemotherapy, the best possible supportive treatment is provided.

2.12 FOLLOW-UP

Follow-up aims to achieve early detection of treatable recurrence and possible new primary cancer (Argiles et al., 2020; Glynne-Jones et al., 2017). Clinical examination, CEA measurement, colonoscopy, and CT scans are included in follow-up protocols. Follow-up varies between countries and its intensity remains debated. In the COLOFOL randomized controlled trial, a more intense follow-up (5 CT scans and CEA measurements, respectively, instead of 2 during a 3-year period) did not improve OS or DFS among stage II and III CRC patients (Wille-Jørgensen et al., 2018). The intensity of follow-up should be based on each patient's risk assessment (Vera et al., 2019).

2.13 PROGNOSIS AND PROGNOSTIC FACTORS

The prognosis of CRC patients has substantially improved in recent decades given screening programs, earlier diagnosis, and surgical and oncological treatments (Brouwer et al., 2018; Favoriti et al., 2016). Recurrence occurs in 30–50% of all treated local colon cancer patients, with 80% of recurrence occurring during the first 3 years and 15% in 4–5 years from surgery. In addition, 17% of stage II and 36% of stage III patients experience recurrence within 5 years (Böckelman et al., 2014). Among stage II low-risk colon cancer patients, the 5-year recurrence risk is 6%, while for high-risk stage II patients the risk increases to 23%. Among stage III patients, the recurrence risk is 25% and 45%, respectevly (Osterman et al., 2021a).

The TNM stage at diagnosis is the most important prognostic factor among CRC patients. The 5-year survival rate for patients with local tumors is as high as 91%, 72% among those with lymph-node positive disease, and only 14% among patients who present with distant metastases (Howlader et al., 2021). Geographic variation in survival exists, even between high-income countries (Araghi et al., 2021). A survival paradox between stage IIB/C and stage IIIA patients also exists. For stage IIB patients, a 5-year OS of 52.4% and 55.6% for stage IIC patients has been reported, but for stage IIIA patients the survival is clearly better at 75.8% (Chu et al., 2016a; Li et al., 2020). Survival among stage IIB/C patients remains poorer even when comparing only those patients who received chemotherapy and had enough lymph nodes harvested (Chu et al., 2016b). In Finland, the 5-year OS among CRC patients is 66% (Pitkäniemi et al., 2015).

In addition to the TNM stage, several other factors impact prognosis and should be evaluated together in order to calculate the risk of recurrence (Osterman et al., 2021b). Retrieving less than the recommended 12 lymph nodes is a strong negative prognostic factor (Chu et al., 2016b). Examining more lymph nodes may also lead to stage migration and thus a better survival for both N0 and N+ patients (Akagi et al., 2013). Furthermore, CRM positivity is a strong independent prognostic factor of local recurrence, distal metastases, and survival among rectal cancer patients (Q. Liu et al., 2018; Nagtegaal and Quirke, 2008).

In addition, lymphovascular and perineural invasion strongly impact survival among CRC patients (Argiles et al., 2020; Glynne-Jones et al., 2017). Stage II patients with positive perineural invasion exhibit a worse prognosis than stage III patients without perineural invasion (Liebig et al., 2009). Moreover, the histologic type carries a prognostic impact since patients with a mucinous, serrated, and signet-ring cell carcinoma histologic type exhibit a poorer OS and cause-specific survival than adenocarcinoma histologic–type patients (Alburquerque-González et al., 2020; Xiaoli Wu et al., 2019).

Tumor budding, defined as single cancer cells or clusters of up to four tumor cells at the invasive front of a tumor, serves as an independent prognostic factor of a poorer prognosis in CRC and associates with a higher TNM stage, vascular invasion, and distant metastases (Lugli et al., 2021). Tumor budding also predicts lymph node metastases in pT1 CRC patients and a shorter DFS in stage II patients (Cappellesso et al., 2017; Ueno et al., 2019). Tumor budding is included in the structured pathological report of CRC specimens (Argiles et al., 2020; Lugli et al., 2017). Tumor buds are associated with epithelial–mesenchymal transition and represent a first step of invasion (Lugli et al., 2021).

Finding tumor deposits, small tumor foci in pericolic or perirectal fat, discontinuous with primary tumors, in patients otherwise classified as N0 is a strong negative prognostic factor and such patients should be treated as stage III patients. Tumor deposits associate with a worse prognosis among lymph node–positive patients as well (Cohen et al., 2021).

High MSI patients exhibit a better prognosis compared with low and stable MSI patients (Battaglin et al., 2018; Seppälä et al., 2015). A high MSI status serves as a marker of a better prognosis also in the presence of a *BRAF* mutation, although *BRAF* in MSS patients indicates a worse survival (Seppälä et al., 2015). Furthermore, mutations in the *RAS* genes associate with a worse survival than that in wild-type patients (Sanchez-Ibarra et al., 2020). As previously mentioned, patients with CMS1 tumors experience a poor survival after recurrence and OS and recurrence-free survival is worse in patients with CMS4 tumors compared to other CMS groups (Guinney et al., 2015).

Preoperatively elevated serum CEA associates with a worse prognosis. CEA values often normalize following treatment; however, a postoperatively persistently high serum CEA level predicts a poorer prognosis (Hall et al., 2019). Emergency presentation independently predicts local recurrence and a worse DFS and OS in CRC patients (Biondo et al., 2019; Hogan et al., 2015; Xu et al., 2017).

The prognostic value of various biomarkers not in clinical use has been studied in CRC. For example, elevated serum levels of carbohydrate antigen 125 (CA125), carbohydrate antigen 19-9 (CA19-9), carbohydrate antigen 242 (CA242), tumor-associated trypsin inhibitor (TATI), tumor-associated trypsin 2 (TAT2), matrix metalloproteinase 8 (MMP8), and tissue inhibitor of metalloproteinase 1 (TIMP1) all indicate a worse prognosis among CRC patients (Björkman et al., 2022, 2021; Böckelman et al., 2018).

Finally, liquid biopsies form a novel and promising field in cancer management (Lone et al., 2022). For example, patients with detected circulating tumor DNA after curative-intent treatment have higher risk of recurrence (Tie et al., 2016). Furthermore, circulating tumor DNA levels can be monitored to evaluate treatment response in metastatic disease (Tie et al., 2015). Assessing stage II CRC patients with T3 or T4 disease to adjuvant therapy according to circulating tumor DNA result spares unnecessary adjuvant therapy from half of patients that would receive it with similar 2-year recurrence free survival as in standard management (Tie et al., 2022).

2.14 CANCER AND INFLAMMATION

In recent decades, increasing evidence has demonstrated the role of inflammation in tumorigenesis and, currently, chronic inflammation is considered a risk factor in several malignancies. Chronic *Helicobacter pylori* infection associates with gastric carcinoma (Kumar et al., 2020), hepatitis B and C infection associates with hepatocellular carcinoma (Martel et al., 2015), and human papilloma virus infection associates with cervix and oral cavity cancers (Martel et al., 2017). An increased cancer risk is observed among immunosuppressed patients (Grulich et al., 2007). Chronic inflammatory diseases such as primary sclerosing cholangitis (Fung et al., 2019) and IBD are associated with an elevated cancer risk (Frosali et al., 2015). While chronic inflammation contributes to cancer development, acute inflammation may be antitumorigenic. For example, squamous cancer of the bladder is treated with induced acute inflammation through the administration of the *Mycobacterium bovis* strain bacillus Calmette–Guerin (BCG) inside the bladder (Askeland et al., 2012).

An inflammatory microenvironment is now known as a component of all tumors. Carcinogenic features such as tobacco and alcohol use and obesity promote tumorigenesis via inflammation. Furthermore, inflammation also impacts the host's immune response to tumors. Communication between the tumor and the host's immune system is complex, and the host's immune responses may have antitumorigenic and protumorigenic features (Grivennikov et al., 2010). The host's immune system consists of innate and adaptive immune responses, which work closely together. The cellular components of the immune system and their protumorigenic and antitumorigenic roles are summarized in Table 2.

Table 2. *Antitumorigenic and protumorigenic roles of different immune cells (Balta et al., 2021; Grecian et al., 2018; Grivennikov et al., 2010; Ostrand-Rosenberg and Fenselau, 2018).*

Immune cell	Antitumorigenic functions	Protumorigenic functions
Macrophage	M1: Release cytotoxic cytokines, antigen presentation to T cells	M2: Release reactive oxygen species, IL-10, TGF-β, supression CTLs
Dentritic cell	Direct cytotoxic effect, antigen presentation, release inflammatory cytokines IL-12, TNFα	Regulatory type immature dendritic cells produce IL-10 and TGF-β
Neutrophil	N1: Direct cytotoxic effect, release pro-inflammatory cytokines	N2: Promote invasion, angiogenesis, cancer cell proliferation
Mast cell		Promote angiogenesis, tumor growth, remodelling of tumor microenvironment
Myeloid-derived suppressor T cell		CTL suppression, induce M2, Treg, angiogenesis
Natural killer cell	Release cytotoxic cytokines, induces tumor cell apoptosis	
CD8+ CTL	Destroy cancer cells, produce cytotoxic cytokines	Release growth-promoting cytokines
CD4+ Th1 cell	Release inflammatory cytokines, stimulate CTLs, natural killer cells, M1 macrophages	Growth-promoting cytokines
CD4+ Th2 cell		Anti-inflammatory cytokines, suppress CTLs, stimulate Treg cells
CD4+ Th17 cell	Stimulate B cells and CTLs	IL-17 secretion, M2 polarization, angiogenesis
CD4+ Treg cell	Restore homeostasis, reduce chronic inflammation	Secrete inhibitory cytokines IL-10, TGF-β, suppress Bcells, CTLs, Th1 cells
B cell	Production of tumor-specific antibodies, antibody-related lysis	Regulatory-type B cells release protumorigenic cytokines, inhibit CTLs, natural killer cells

Abbreviations: CTL, cytotoxic T cell; IL, interleukin; TGF, transforming growth factor; Th1, T-helper type 1 cell; Th2, T-helper type 2 cell; Th17, T-helper type 17 cell.

2.14.1 Innate immunity

The activation of the innate immune system leads to recruiting various immune cells and the release of cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), and the tumor necrosis factor (TNF), which lead to inflammation and a microenvironment suitable for tumor development (Balta et al., 2021). Tumor-associated macrophages are the most abundant innate immunity cells seen in the tumor microenvironment. Macrophages fall into the antitumorigenic M1 subtype and the protumorigenic M2 subtype, which normally participate in wound healing and homeostasis with an anti-inflammatory potential (Belgiovine et al., 2016). Tumor-associated macrophages, primarily the M2 subtype, are activated by cytokines produced by both tumor cells and the host, which promote tumor growth, angiogenesis, and metastases by producing epidermal growth factor (EGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and immune-suppressive mediators such as IL-10 and transforming growth factor beta (TGF-β) (Balta et al., 2021; Belgiovine et al., 2016).

Similar to tumor-associated macrophages, tumor-associated neutrophils are dichotomized as antitumorigenic N1 and protumorigenic N2 neutrophils (Balta et al., 2021; Grecian et al., 2018). Factors in the tumor, especially TGF β, stimulates neutrophil activation in the N2 form and the production of protumorigenic cytokines and chemokines, leading to tumor proliferation, angiogenesis, and invasion (Grecian et al., 2018). Neutrophil extracellular traps, released during neutrophil death, may carry tumor cells and seed them in distant organs, thus contributing to metastases (Grecian et al., 2018; L. Wu et al., 2019). Interferon (IFN) I treatment blocks TGF-β and prevents N2 activation, while neutrophils are activated in antitumorigenic N1 form (Balta et al., 2021; Grecian et al., 2018; L. Wu et al., 2019).

In addition to phagocytic characteristics, dendritic cells connect innate and adaptive immune systems by maturing into antigen-presenting cells (Veglia and Gabrilovich, 2017). In tumors, dendritic cells are often immature and express inhibitory molecules, such as the programmed cell death 1 (PD1) protein, a well-known checkpoint protein. The tumor microenvironment may change dendritic cells into regulatory type suppressor cells, which produce IL-10 and TGF-β (Veglia and Gabrilovich, 2017).

Other innate cells in the tumor microenvironment are myeloid-derived suppressor cells and mast cells. VEGF and metalloproteinases produced by myeloid-derived suppressor cells induce angiogenesis, the epithelial-to-mesenchymal transition, and the preparation of a premetastatic niche, leading to tumor growth, invasion, and metastases (Balta et al., 2021; Ostrand-Rosenberg and Fenselau, 2018). Myeloid-derived suppressor cells polarize macrophages towards the M2 type, inhibit natural killer cell-mediated lysis, and promote the functioning of T-regulatory cells leading to immunosuppression. Programmed cell death ligand 1 (PD-L1) upregulation on myeloid-derived suppressor cells leads to T-cell exhaustion (Balta et al., 2021; Ostrand-Rosenberg and Fenselau, 2018). Furthermore, mast cells associate with an unfavorable prognosis in several cancers.

If mast cells are activated by tumor cells, they can promote tumor growth, angiogenesis, and remodeling of the tumor microenvironment (Derakhshani et al., 2019).

Finally, natural killer cells are primarily antitumorigenic, while recognizing that tumor cells lack major histocompatibility complex I (MHC I) molecules and induce the apoptosis of such affected cells. The presence of natural killer cells represents a favorable factor in malignancies, and modulating the tumor microenvironment to support the natural killer cell function could serve as a characteristic of targeted treatment (Balta et al., 2021).

2.14.2 Adaptive immunity

The adaptive immune system provides pathogen-specific immune responses to eliminate pathogens forming an immunological memory. Adaptive immune responses may be antitumorigenic or work in favor of cancer. The tumor microenvironment comprises adaptive immune cells, such as cytotoxic T cells (CTLs); T-helper type 1 (Th1), T-helper type 2 (Th2), and T-helper type 17 (Th-17) cells; regulatory T cells (Treg); and B lymphocytes (see Table 2) (Grivennikov et al., 2010)

The most prominent antitumorigenic adaptive immune cells are CTLs, with a direct antitumorigenic effect (Park and Lee, 2021). CTL infiltration in tumors is a known factor of a positive prognosis in CRC (Gunnarsson et al., 2020; Pagès et al., 2018). The *Immunoscore*®, formed from the intratumoral and invasive margin densities of CD3-positive T lymphocyte (CD3+) and CD8-positive T lymphocyte (CD8+) cells, is a reliable strong predictor of time until recurrence, DFS, and OS among stage I–III CRC patients (Pagès et al., 2018). High-risk stage II patients with a high *Immunoscore*® have a prognosis comparable to stage II patients without risk factors (Galon et al., 2019).

Th1 cells, activated by dendritic cells, promote the CTL capacity and the antitumoral effect of natural killer cells and macrophages by releasing proinflammatory cytokines such as interferon gamma (IFN-γ), tumor necrosis factor alpha (TNFα), and IL-2 (Disis, 2010; Gonzalez et al., 2018). Th2 cells, activated by tumor-associated macrophages and myeloid-derived suppressor T cells from the tumor microenvironment, have protumorigenic features, leading to the stimulation of Treg cells and an inhibited cytotoxic response (Disis, 2010; Gonzalez et al., 2018). In turn, Treg cells secrete inhibitory cytokines, suppress CTLs, Th1 cells, natural killer cells, macrophages, and B-cells.

In malignancies, B cells play important antitumorigenic roles by producing specific antibodies against tumor cells, but may also produce antibodies against autoantigens present on both tumor cells and host cells (Balta et al., 2021; Yuen et al., 2016). Circulating immune complexes formed by these autoantibodies lead to tumor progression through myeloid-derived suppressor T cell activation and induced angiogenesis following lymphotoxin secretion. In the tumor microenvironment, B cells become a more regulatory subtype and thus lead to the inhibition of natural killer cells and CTLs (Balta et al., 2021; Yuen et al., 2016).

Tumors have developed mechanisms to evade adaptive immune responses. Upon activation, T cells release IFNs to recruit neutrophils and natural killer cells, and express checkpoint proteins, such as PD1 to limit the overactivation of immune cells (Ribas, 2015). Tumor cells adapt to T cells by producing PD-L1 in response to IFNs and thus block the CTL function (Ribas, 2015). PD1/PD-L1 checkpoint blockade therapy with monoclonal antibodies is currently used in several cancers. For example, nivolumab and pembrolizumab are approved for unresectable or metastatic melanoma treatment and nivomulab as an adjuvant therapy for resected melanoma with metastatic disease or positive lymph nodes (Xiaomo Wu et al., 2019). In lung cancer, pembrolizumab is approved for previously treated advanced or metastatic PD-L1-positive non-small-cell lung cancer, while nivolumab is used to treat metastatic non-small-cell and small-cell lung cancer that has progressed after platinum-based chemotherapy. In CRC, pembrolizumab and nivolumab combined with low-dose ipilimumab are approved for a DNA mismatch repair deficiency or for a high-MSI unresectable or metastatic cancer that progresses through fluoropyrimidine, oxaliplatin, and irinotecan (Xiaomo Wu et al., 2019).

Cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) is another checkpoint protein and, thus, a negative regulator of adaptive immune responses. CTLA-4 reduces the activation of the adaptive immune system through self- and tumor antigens. Anti-CTLA-4 treatment has been used in stage III/IV melanoma treatment (Seidel et al., 2018).

2.14.3 Tumor microenvironment

According to the immunosurveillance theory, the immune system has the capacity to identify tumor-specific antigens and continuously check for defective and transformed cells in order to eliminate them. Evading this immunosurveillance is a known hallmark of cancer (Vesely et al., 2011). Tumor immunoediting occurs in three phases: elimination, equilibrium, and escape. Several tumor escape mechanisms are involved in immunoediting. If the immune system succeeds in detecting and eliminating tumor cells, no tumor progression follows. If the immune system cannot eliminate the tumor cells, they may remain functionally dormant in the equilibrium phase. If tumor cells succeed in immunoediting and immune system changes are induced by tumoral or host factors, the tumor may proceed to the escape phase (Vesely et al., 2011).

One characteristic feature of tumors is forming an environment that supports tumor growth, progression, and invasion. The tumor microenvironment plays a crucial role in the tumor escape mechanism (Vinay et al., 2015). In the tumor microenvironment, several cytokines produced by the tumor enhance the formation of tumor-derived Tregs that lead to the suppression of the immune response (Vinay et al., 2015). Another escape mechanism is the development of tumor cells deficient in co-stimulatory molecules, tumor antigens, and class I MHC complexes, leading to a T-cell inability to recognize them. Tumor cells may also produce immunosuppressive proteins leading to the inhibition

of T cells and the destruction of tumor-specific CD8+ cells by apoptosis (Vinay et al., 2015).

The tumor microenvironment is a complex network consisting of the extracellular matrix, stromal cells (fibroblasts, lymphovascular network, and pericytes), proliferating tumor cells, and immune cells from the innate and adaptive immune system (Anderson and Simon, 2020; Wang et al., 2017). The tumor stroma plays an important role in tumorigenesis. In normal physiology, fibroblasts are dormant, but are activated during wound healing through TGF-β signaling (Anderson and Simon, 2020; Wang et al., 2017). In normal colonic mucosa, fibroblasts synthesize components of the basal membrane (Colangelo et al., 2017). In tumors, stromal cells and cancer cells produce TGF-β, platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF2), IL-4, and IL-6, which induce fibroblast differentiation into cancer-associated fibroblasts (Anderson and Simon, 2020; Colangelo et al., 2017; Wang et al., 2017). Cancer-associated fibroblasts remain permanently activated and produce a wide range of immunomodulating cytokines, growth factors, metalloproteinases, and components of the extracellular matrix, which participate in angiogenesis, the reprogramming of immune cells and immunosuppression, remodeling of the extracellular matrix, and the epithelial–mesenchymal transition of epithelial cells, leading to tumor growth, invasion, and metastases (Anderson and Simon, 2020; Colangelo et al., 2017; Wang et al., 2017).

The extracellular matrix consists of collagen, fibronectin, elastin, and laminin, providing the structural frame for cells in the tumor microenvironment. Metalloproteinases, produced by cancer-associated fibroblasts, are capable of breaking the extracellular matrix down and remodelling it in favor of tumor progression and metastases. The extracellular matrix also deposits all of the growth factors and cytokines produced by tumor and stromal cells (Anderson and Simon, 2020; Wang et al., 2017). The tumor microenvironment differs from a normal environment, being hypoxic and characterized by a low pH. Most immune cells lose their antitumorigenic capacity in a hypoxic and acidic tumor microenvironment (Huber et al., 2017).

Cytokines and other characteristics of primary tumors begin preparing the premetastatic niche at the site of future metastases before the tumor cells arrive (Quail and Joyce, 2014). The tumor microenvironment in metastatic lesions differs from primary tumors. For example, in primary tumors the cells are more epithelial than mesenchymal in type, supporting the idea that epithelial–mesenchymal transition is necessary for invasion and metastases. In this trasition, primary tumor cells gain stem-like properties needed for metastases. It is thought that the reason why some patients develop recurrence after chemotherapy is the inability of therapeutic agents to attack the stem-cell type of tumor cells (Quail and Joyce, 2014). In circulation, platelets aggregate with tumor cells and help to escape immunosurveillance. These clusters attach to endothelial cells at the metastasis site (Colangelo et al., 2017; Quail and Joyce, 2014). Micrometastases in new locations may reamin dormant for a long time until they escape immunosurveillance and begin growing.

2.14.4 Subtyping according to the tumor microenvironment

Understanding the importance of the tumor microenvironment and crosstalk between tumor cells and other components of tumorigenesis in the prognosis of cancer patients has led to several attempts to classify tumors. CMS subtyping includes some of the microenvironment characteristics (discussed in 2.10). Thorsson et al. (2018) identified six immune subtypes of solid tumors in 2018 after analyzing over 10 000 tumor cases, including 33 different tumors, referred to as the wound healing (C1), IFN-γ dominant (C2), inflammatory (C3), lymphocyte depleted (C4), immunologically quiet (C5), and TGF-β dominant (C6) subtypes. The characteristics of these six immune subtypes are summarized in Table 3.

Soldevilla et al. (2019) analyzed the correlations between these immune subtypes and CMSes in CRC patients, finding that the immune phenotype more greatly impacts survival than the CMS. The C1 and C2 emerged as the most common immune subtypes among CRCs. The distribution of immune subtypes varied between CMSes with some overlap found between categories (Table 3). The worst prognosis was observed among the IFN-γ dominant C2 and inflammatory immune C3 subtypes, whereas patients falling in the TGF-β dominant C6 and wound healing C1 immune subtypes exhibited a better prognosis (Soldevilla et al., 2019). In CMS1 tumors, a greater infiltration of CD8+ cells explains the better prognosis whereas in CMS2 and CMS3 tumors there is almost no T-cell activity and the CMS4 subtype is characterized by an immunosuppressive tumor microenvironment rich in cancer-associated fibroblasts and TGF-β signaling, resulting in a worse prognosis (Colangelo et al. 2017).

In CRC, the benefit of immune checkpoint inhibitors has only appeared with the MSI phenotype, but not among those with MSS disease. In addition, the high number of DNA mutations, PD1/PD-L1 expression, and immune cell infiltration impact the response to treatment. Immunotherapies that repress tumor-associated macrophages have exhibited a minimal response as monotherapies (Binnewies et al. 2018). CMS1 tumors of the C1 subtype may be more resistant to immune checkpoint agents, whereas patients with a C1 type tumor in the CMS2–4 subgroups might benefit from immune checkpoint therapy (Soldevilla et al. 2019). Further profiling possibilities of tumors, particualarly the tumor immune microenvironment in addition to CMS subgrouping, would be beneficial in identifying patients who might benefit from specific treatments such as immune checkpoint inhibition.

Table 3. *Characteristics of six immune subtypes of solid tumors and their distribution in CMS subgroups (Soldevilla et al., 2019; Thorsson et al., 2018).*

Immune subtype	Characteristics of immune subtypes	Correlation to CMS subgroups of CRC
Wound healing (C1)	Expression of angiogenic genes, a high proliferation rate, and a Th2 shift in the adaptive immune infiltrate Widely seen among CRC patients	Dominant in CMS2 tumors (91%), present in 77–78% of CMS3 and CMS4 tumors, and least common in CMS1 tumors (46%)
IFN-γ dominant (C2)	High intratumoral heterogeneity, M1/M2 macrophage polarization, strong CD8+ T-cell signal, greatest T-cell receptor diversity, and high proliferation rate	Most common among CMS1 tumors (53%), mildly seen among CMS3 (11%) and CMS4 (13%) tumors, and barely represented among CMS2 (8%)
Inflammatory (C3)	Elevated Th17 and Th1 genes, a low to moderate tumor cell proliferation, lower levels of aneuploidy, and lower levels of somatic copy number alterations Associated with the most favorable prognosis	Seen in a small proportion of CMS3 (7%) and CMS4 (6%) tumors
Lymphocyte depleted (C4)	Moderate cell proliferation and intratumoral heterogeneity, suppressed Th1, and a high M2 response Associated with a worse outcome	Seen in some CMS3 tumors (4%)
Immunologically quiet (C5)	Lowest lymphocyte and highest macrophage responses, dominated by M2, and low rates of proliferation and heterogeneity	
TGF-β dominant (C6)	Displays the highest TGF-β signature and a high lymphocytic infiltrate with a balanced Th1:Th2 ratio Associated with the worst prognosis	Seen in a few CMS4 tumors (2.3%)

Abbreviations: CD8+, CD8-positive T cell; CMS, consensus molecular subtype, IFN, interferon; M1, type 1 macrophage; M2, type 2 macrophage; TGF-β, transforming growth factor beta; Th1, T-helper type 1 cell; Th17, T-helper type 17 cell.

2.14.5 Toll-like receptors

A key role in initiating innate immune responses to pathogen attacks is played by toll-like receptors (TLRs). TLRs are a family of transmembranous pattern-recognition receptors expressed on various immune cells, particularly on antigen-presenting cells (e.g., macrophages and dendritic cells), as well as on the epithelial cells of host–environment boundary tissues (Basith et al., 2012; Rakoff-Nahoum and Medzhitov, 2009). In total, 13 TLRs are known, 10 of which occur in humans (Kawasaki and Kawai, 2014). TLRs are activated by various viral, fungal, and bacterial pathogen-associated molecular patterns (PAMPs) (Basith et al., 2012). TLRs can also recognize host-derived endogenous damage-associated molecular patterns (DAMPs), which are released following tissue injury and nonphysiological cell death (Basith et al., 2012). According to their location, TLRs are divided into two groups. First, TLR1, TLR2, TLR4, TLR5, and TLR6 are located on cell membranes, and recognize microbial cell-surface components, such as lipopolysaccharides and lipoproteins (Kawasaki and Kawai, 2014; Vijay, 2018). Second, TLR3, TLR7, TLR8, and TLR9 are expressed intracellularly in endosomes and lysosomes, capable of detecting viral nucleic acids. This dichotomous model of TLR locations is challenged because cell surface TLRs, such as TLR2 and TLR4, may also signal intracellularly under certain conditions (Chen et al., 2007; Petnicki-Ocwieja et al., 2015; Uronen-Hansson et al., 2004).

Figure 8 illustrates the TLR signaling pathways. Upon ligand engagement all TLRs, except TLR3 activate the myeloid differentiation primary response gene 88 (MyD88) dependent pathway and use the MyD88 adaptor protein. In addition, TLR3 and TLR4 also signal through a MyD88-independent toll-interleukin-1 receptor- (TIR) related adaptor protein-inducing interferon (TRIF) pathway. Both MyD88-dependent and MyD88-independent pathways induce proinflammatory cascades, such as nuclear factor-κB (NF-κB), interferon-regulatory factor 3 (IRF3), and activator protein 1 (AP-1), which regulate the transcription of proinflammatory cytokines, as well as the proteins involved in cell proliferation, apoptosis, and angiogenesis (Fitzgerald and Kagan, 2020; Kawasaki and Kawai, 2014).

TLRs connect innate and adaptive immune systems since TLR activation induces the maturation of dendritic cells, leading to T-cell activation and differentiation into effector cells (Iwasaki and Medzhitov, 2004). Versatile immune responses follow TLR activation given that the responses depend on the activating stimulus, the activated TLRs, the cell types where TLRs are activated, and which pathways are activated. TLR expressions vary widely among different immune cell types and TLRs can be activated alone or in TLR complexes, potentially causing a wide range of responses (Vijay, 2018).

In addition, TLRs play a crucial role in tissue hemostasis by regulating cell death and participating in tissue repair and regeneration (Basith et al., 2012; Rakoff-Nahoum and Medzhitov, 2009). Under normal conditions, these TLR-induced responses are tightly controlled, although a dysfunction to the TLR signalling may lead to various diseases,

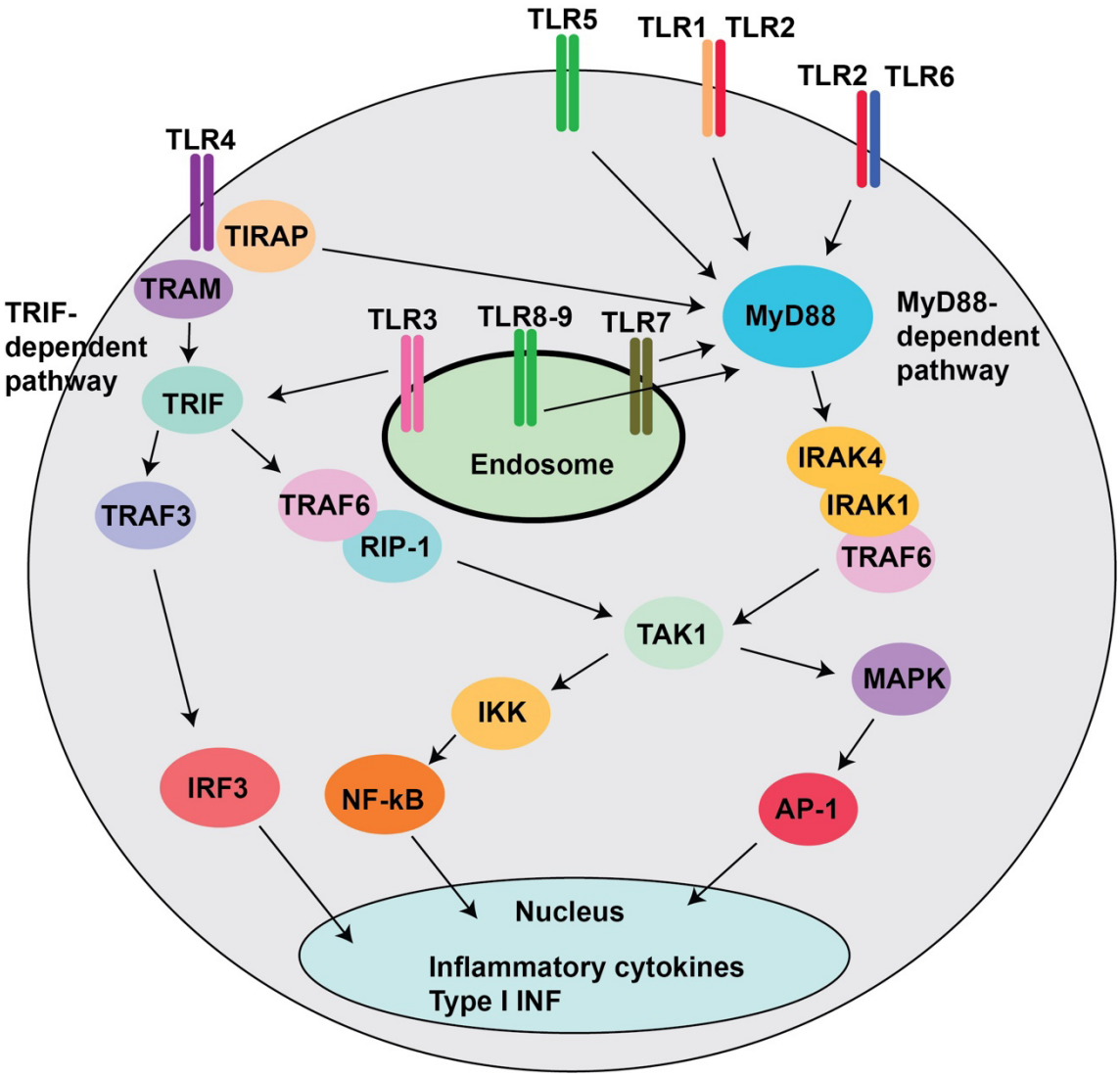

Figure 8. *Schematic representation of the TLR signaling pathways.*

including malignancies (Basith et al., 2012; Pradere et al., 2013). TLRs participate in the pathogenesis of several autoimmune and chronic inflammatory diseases including diabetes mellitus, asthma, rheumatoid arthritis, psoriasis, Crohn's disease, and ulcerous colitis (Chen et al., 2007; Duffy and O'Reilly, 2016; Xie et al., 2018).

2.14.6 Toll-like receptors in the development of colorectal cancer

TLRs have proved promising in efforts to reveal the mechanisms behind inflammation-associated cancer. Specifically, TLRs recognize both pathogenic and commensal microflora and participate in the maintenance of intestinal epithelial homeostasis. Deregulated TLR activation leads to an abnormal immune response to the normal enteric microflora and the development of chronic inflammation and IBD (Frosali et al., 2015). The expression pattern of TLRs changes in pathological situations. For example, in IBD

patients the expressions of TLR2 and TLR4 are upregulated and the expression of TLR5 is downregulated (Hug et al., 2018; Klampfer, 2017).

Tumor cells express functional TLRs. The activation of these TLRs, but also those of immune cells, releases immunosuppressive cytokines necessary in the formation of the tumor microenvironment, crucial for increased tumor cell proliferation, for resistance to apoptosis, for tumor progression, and for evading the host's immune response (Pradere et al., 2013). The role of TLRs in CRC development is not fully understood, particularly as TLRs may have both pro- and antitumorigenic features in the same tumor (Vijay, 2018). Possibly, during multistep development from the normal mucosa to dysplasia and cancer, the TLRs' behavior changes. For instance, TLR4 knockout mice did not develop premalignant polyps, but TLR4 upregulation was observed in colitis-associated tumors (Cammarota et al., 2010; Fukata et al., 2007). Patients who later developed CRC had a lower expression of TLR7 and TLR9 in their polyp tissues compared with those who did not develop CRC, although a higher TLR7 and TLR9 expression was observed in polyps compared with normal mucosa (Eiró et al., 2012). This finding may support the antitumorigenic potential of TLR7 and TLR9. Furthermore, TLR5 expression was not detected in the polyps of patients who developed CRC, indicating that TLR5 does not take part in primary carcinogenesis. Instead, TLR5 may play a role later during CRC progression (Eiró et al., 2012). In addition, TLR3 expression changes during CRC development, reaching its highest expression in normal colonic mucosa and decreasing from the polyps to CRC stages I–III (Xiang et al., 2012). Similarly, in the development of esophageal (Helminen et al., 2016; Huhta et al., 2016) and gastric cancers (Pimentel-Nunes et al., 2011), correlations between high TLR expressions and the development of mucosal lesions were reported.

Genetic TLR variations may alter the communication between the host and the microbiota and predispose individuals to chronic inflammation and cancer. TLR gene polymorphism leads to changes in the TLR expression, a malfunctioning of normal TLR signaling, an imbalanced homeostasis of the cell microenvironment, an enhanced local inflammation, and CRC development. Furthermore, a TLR polymorphism influences outcomes among CRC patients (Okazaki et al., 2017; Rutkowski et al., 2015).

2.14.7 Toll-like receptors as prognostic biomarkers in cancer

TLR4 is the most studied TLR in malignancies, including CRC. In CRC, a high TLR4 and MyD88 expression in tumor cells is associated with metastasized disease and a worse prognosis (Wang et al., 2010), while other studies have found a positive impact of TLR4 on survival among stage II patients (Eiró et al., 2014) or patients with proximal disease (Paarnio et al., 2017). In other malignancies, a high TLR4 expression associated with a worse outcome in breast cancer (González-Reyes et al., 2010; Ma et al., 2014) and hepatocellular carcinoma (Kairaluoma et al., 2021b), although in pancreatic ductal

adenocarcinoma patients, a strong TLR4 expression associated with a better prognosis among stage I–II patients (Lanki et al., 2018).

Only one study has demonstrated a prognostic role for TLR2 in CRC, with a high TLR2 expression linked to a worse survival among 24 CRC patients (Liu et al., 2018). In gastric cancer, a high TLR2 immunoexpression associated with a worse prognosis (Tye, 2012), but in pancreatic cancer a high tissue TLR2 expression associated with a favorable prognosis (Lanki et al., 2018).

Knowledge of the prognostic role of TLR5 in CRC remains lacking, but in gastric cancer, a high tissue TLR5 expression associated with a better prognosis (Kasurinen et al., 2019). In human papilloma virus-positive oropharyngeal squamous cell carcinoma (Jouhi et al., 2017), nasopharyngeal carcinoma (Ruuskanen et al., 2019), and hepatocellular carcinoma (Kairaluoma et al., 2021a), however, patients with a high TLR5 expression level experienced poorer outcomes.

Additionally, TLR7 has not been studied in CRC. That said, among stage III gastric cancer (Kasurinen et al., 2019), nasopharyngeal carcinoma (Ruuskanen et al., 2019), and human papilloma virus -positive oropharyngeal squamous cell carcinoma patients (Jouhi et al., 2017), a high TLR7 tissue immunoexpression associated with a better prognosis.

2.14.8 C-reactive protein (CRP) and systemic inflammatory response (SIR)

Some patients develop a systemic inflammatory response (SIR) to cancer, which can be evaluated by measuring circulating acute-phase proteins (Tuomisto et al., 2019). Generalized symptoms of cancer, such as a loss of appetite, fatigue, depression, and weight loss, are believed to at least partly result from chronic SIR (Roxburgh and McMillan, 2014).

CRP is an acute-phase protein synthesized in hepatocytes as a response to IL-6, released during infections and tissue damage (Sproston and Ashworth, 2018). CRP binds to damaged and apoptotic cells, activates a complement system, and induces phagocytosis by macrophages. In clinical work, CRP is primarily used to diagnose infections as well as to follow their course.

Epidemiological studies have demonstrated that an elevated CRP level indicates a predisposition to the development of future malignancies and serves as a marker of cancer (Allin and Nordestgaard, 2011). In patients with no infection or autoimmune disease, a preoperatively elevated CRP level, indicative of SIR, serves as an independent marker of an unfavorable prognosis in both primary and metastatic CRC (Kersten et al., 2013; Partl et al., 2020; Woo et al., 2015).

Since hypoalbuminemia is thought to appear as a secondary event following CRP elevation and decreased albumin levels associate with a poorer prognosis among CRC patients, CRP and albumin measurements are combined into the Glasgow prognostic

score (GPS) and a modified GPS (mGPS). A higher GPS and mGPS serve as negative prognostic markers in CRC patients (Liu et al., 2017; Lu et al., 2019; Woo et al., 2015).

The underlying mechanisms explaining the negative impact of SIR on survival remain poorly understood. On the one hand, SIR likely mirrors the more aggressive local features of the tumor (Woo et al., 2015), but it is also thought that SIR could lead to an imbalance between adaptive and innate immune responses, with the upregulation of the mechanisms of innate responses and participation in the premetastatic niche modifying the organs of future metastases (Køstner et al., 2016; Tuomisto et al., 2019).

3 AIMS OF THE STUDY

This study was designed to provide an evaluation of the tissue expression and prognostic role of toll-like receptors (TLRs) and their relationship to systemic and local adaptive immune response biomarkers in colorectal cancer (CRC).

The specific aims of the study were:

- To determine the associations between the immunoexpressions of TLR2, TLR4, TLR5, and TLR7 in tumor cells and clinicopathological parameters and their role as prognostic markers in CRC.

- To assess the relationship between the TLR2, TLR4, TLR5, and TLR7 tumor cell immunoexpression and systemic inflammatory response marker CRP in CRC.

- To determine the relationship between the TLR2, TLR4, TLR5, and TLR7 tumor cell immunoexpression and tumoral and stromal densities of CD3-positive and CD8-positive T cells in CRC.

4 PATIENTS AND METHODS

4.1 PATIENTS

In total, 1308 histologically verified consecutive colorectal cancer (CRC) patients were surgically treated in the Department of Surgery at Helsinki University Hospital, Finland, between 1982 and 2005. The first tissue microarray (TMA) series includes 825 patients who underwent surgery between 1982 and 2002, analyzed in studies I and II. The second TMA series includes 549 patients operated on between 1998 and 2005, comprising the material for studies III and IV. In this thesis, the TLR immunohistochemistry results from both series are combined and the TLR results from both cohorts are reported together.

The clinicopathological characteristics of both study populations are summarized in Table 4. In the study cohort, the median age at the time of surgery was 66.8 [interquartile range (IQR) 58.2–76.3], and the median follow-up time was 5.74 years (IQR 1.46–15.37). By the end of follow-up period, 967 (74.0%) patients had died, of whom 529 (40.5%) died from CRC. The 5-year OS for all patients was 54.5% [95% confidence interval (CI) 51.8–57.2%] and the 5-year disease-specific survival (DSS) reached 62.9% (95% CI 60.2–65.6%).

The clinical data were collected from patient medical records. The Digital and Population Data Service Agency (previously the Population Register Center of Finland) and Statistics Finland provided the survival statistics and the cause of death information for deceased patients

In the patients's records, the modified Dukes classification (Dukes, 1932) for CRC staging was used for the older cohort and the sixth edition of the TNM disease classification (Greene et al., 2001) for CRC staging among the more recent cohort. The stages were transformed to the same TNM staging system for better statistical analyses.

The study protocol was approved by the Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extensions 17 April 2013 and 16 June 2021), and the National Supervisory Authority of Health and Welfare (Valvira Dnro 10041/06.01.03.01/2012) granted permission to use the archived tissue and blood samples without requiring us to secure individual consent for these retrospective studies.

Table 4. *Patient characteristics for studies I–IV.*

Characteristic	n (%)	
	Entire cohort n = 1308	**Studies III–IV n = 549**
Age		
Median (IQR), in years	66.8 (58.2–76.3)	69.2 (59.2–77.4)
<65 years	545 (41.7	220 (40.1)
≥65 years	763 (58.3)	329 (59.9)
Gender		
Male	604 (46.2)	289 (52.6)
Female	704 (53.8)	260 (47.4)
Location		
Colon	685 (52.4)	281 (51.2)
Rectum	623 (47.6)	268 (48.8)
Histological type		
Adenocarcinoma	1170 (89.7)	432 (87.6)
Mucinous	129 (9.9)	61 (12.4)
Tumor stage		
I	202 (15.7)	110 (20.01)
II	427 (33.3)	155 (28.3)
III	394 (30.7)	197 (35.9)
IV	260 (20.3)	86 (15.7)
Tumor classification (pT)		
pT1–pT2	169 (24.9)	134 (24.8)
pT3–pT4	510 (75.1)	407 (75.2)
Lymph node metastasis (pN)		
pN0	344 (51.2)	276 (51.2)
pN1–2	328 (48.8)	263 (48.8)
Tumor grade (WHO)		
1–2	970 (77.6)	432 (87.6)
3–4	280 (22.4)	61 (12.4)
Systematic inflammatory response (CRP)		
≤8.7		287 (67.4)
>8.7		139 (32.6)

Abbreviations: IQR, interquartile range; WHO, World Health Organization; CRP, C-reactive protein.

4.2 TUMOR TISSUE SPECIMENS

Surgical tumor samples were stored in the archives of the Department of Pathology at Helsinki University Hospital after fixing them in a buffered 10% formalin solution and embedding them in paraffin. For anonymous analysis, each sample was given an identification number. An experienced pathologist from the research group marked representative tumor areas on hematoxylin and eosin–stained slides. A technical assistant punched three 1.0-mm cores from these representative tumor areas from the older cohort tumor samples (studies I–II) and four cores from the newer cohort samples (studies III–IV) and embedded in a new TMA paraffin block. A TMA Grand Master 3D instrument was used (Histech Ltd Budapest, Hungary) to construct the TMA blocks. Finally, 4-µm sections were cut from the TMA blocks for immunohistochemistry, fixed to slides, and dried at 37°C for 12–24 hours (Kallioniemi et al., 2001; Kononen et al., 1998).

4.3 IMMUNOHISTOCHEMISTRY

The same immunohistochemical staining protocol was used for each TLR. After deparaffinization in xylene (15 + 5 min), the 4-µm TMA sections were rehydrated in solutions containing a gradually decreasing concentration of ethanol, beginning with pure alcohol and ending with distilled water. The slides were prewarmed to 65°C in a PreTreatment module (Lab Vision UK Ltd, UK) and for antigen retrieval incubated for 20 min at 98ºC in a Tris-HCl buffer (pH 8.5). The Autostainer 480 (Lab Vision, Fremont, California, USA), with the REAL EnVision Detection System (peroxidase/DAB+, rabbit/mouse; Dako, Glostrup, Denmark) was used for staining the TMA slides at room temperature. Endogenous peroxidases were inactivated by incubating the slides in 0.3% hydrogen peroxide for 5 min. Slides were incubated with primary antibodies. Table 5 summarizes the antibodies, dilutions, and incubation times used. Finally, the samples were incubated with a peroxidase-conjugated Dako REAL EnVision/HRP, rabbit/mouse (ENV) secondary antibody for 30 min and visualized using the Dako REAL DAB+ Chromogen for 10 min. Between each step of staining, the slides were washed in a phosphate-buffered saline (PBS) 0.04% Tween20. Then, slides were counterstained with Meyer's hematoxylin, followed by washing for 10 min in tap water and mounted in Pertex Mounting (Histolab Products AB, Sweden). Tissues known to show a high immunoreactivity to the antigens were used as the positive controls (tonsillar, skin, and gums) and specimens processed without any primary antibody served as the negative controls. Since the different cohorts were stained at different points in time, the primary antibodies used for the first stainings were no longer available for later stainings, as summarized in Table 5. We have, however, stained a small cohort from study I with the new TLR4 mouse monoclonal antibody (sc-293072, Santa Cruz Biotechnology, Santa Cruz, CA, USA) to ensure that changing the antibody did not

Table 5. *Primary antibodies used for the immunohistochemistry.*

Antigen	Antibody	Antibody type	Manufacturer	Dilution	Incubation	Study
TLR2	H-175/sc-10739	Rabbit pAb	Santa Cruz Biotechnology, USA	1:50	1 h	I
TLR2	H-175/sc-10739	Rabbit pAb	Santa Cruz Biotechnology, USA	1:200	O/N	III/IV
TLR4	H-80/sc-10741	Rabbit pAb	Santa Cruz Biotechnology, USA	1:50	1 h	I
TLR4	25/sc-293072	Mouse mAb	Santa Cruz Biotechnology, USA	1:2000	1 h	III/IV
TLR5	IMG-664A	Mouse mAb	Imgenex/Novus Biologicals, CO, USA	1:200	1 h	II
TLR5	NBP2-24787	Mouse mAb	Novus Biologicals, USA	1:300	O/N	III/IV
TLR7	IMG-581A	Mouse mAb	Imgenex/Novus Biologicals, CO, USA	1:300	1 h	II
TLR7	NBP2-24906	Rabbit pAb	Novus Biologicals, USA	1:300	O/N	III/IV
TLR9	H-100/sc-25468	Rabbit pAb	Santa Cruz Biotechnology, USA	1:100	O/N	II
TLR3	H-125/sc-10740	Rabbit pAb	Santa Cruz Biotechnology, USA	1:100	1 h	II
CD3+	2GV6	Mouse mAb	Roche, Ventana Medical Systems Inc, USA	Ready-to-use	40'	IV
CD8+	4B11	Mouse mAb	Novocastra, USA,	1:50	40'	IV

Abbreviations: CD3+, CD3-positive T cell; CD8+, CD8-positive T cell; mAb, monoclonal antibody; O/N, overnight; pAb, polyclonal antibody; TLR, toll-like receptor.

alter the staining results. The immunoexpression of TLR4 using two different antibodies correlated (r_s= 0.721, $p < 0.001$, Spearman's rank correlation test).

An automatic Roche Ventana BenchMark ULTRA equipment (F. Hoffman-La-Roche AG, Basel, Switzerland) was used for CD3 and CD8 pretreatment and immunohistochemical staining. For pretreatment (deparaffinization, rehydration, and antigen retrieval) the slides were treated for 64 min in a Ventana Cell Conditioning (CC1) solution, followed by incubation with primary antibodies (Table 5). The detection and visualization of antibodies was performed by the Ventana Ultraview DAB detection kit. The slides were finally counterstained with Meyer's hematoxylin and washed in tap water. Tonsillar tissue was used as the positive control.

4.4 SAMPLE SCORING

The staining intensities from the TMA samples were scored independently by two assessors blinded to the clinical data. Differences in the scoring results were re-evaluated and discussed until reaching consensus. Three to four spots from each patient's sample were interpreted and the highest score was selected for statistical analysis.

TLR2 and TLR4 immunopositivity was defined as an even cytoplasmic brown color in the tumor cells, TLR5 immunopositivity as brown staining in the nuclear membranes of the tumor cells, and TLR7 immunopositivity as a brown granular cytoplasmic color in the tumor cells. Immunoreactivity was scored on a scale from 0 to 3: the absence of staining was scored as 0, weak positive immunopositivity as 1, moderate as 2, and strong staining as 3. Examples of TLR immunohistochemistry stainings appear in Figure 9.

CD3 and CD8 staining was indicated as a brown shading in the immune cells. The intratumoral CD3 and CD8 ($CD3^T$ and $CD8^T$) immunostainings were scored according to four categories: 0 indicated no positive cells were observed, 1 indicated a few solitary individual positive cells, 2 indicated that small positive cell clusters were visible (5% of cells positive), and 3 indicated abundant and organized staining with more than 10% positive cells. Stromal CD3 and CD8 ($CD3^S$ and $CD8^S$) immunostainings were categorized on a five-point scale: 0 indicated no positive cells, 1 indicated a few solitary positive cells, 2 indicated individual scattered cells and small clusters (5% of cells positive), 3 indicated medium clusters (10% of cells positive), and 4 indicated an extensive number of stained cells (over 20% of cells positive).

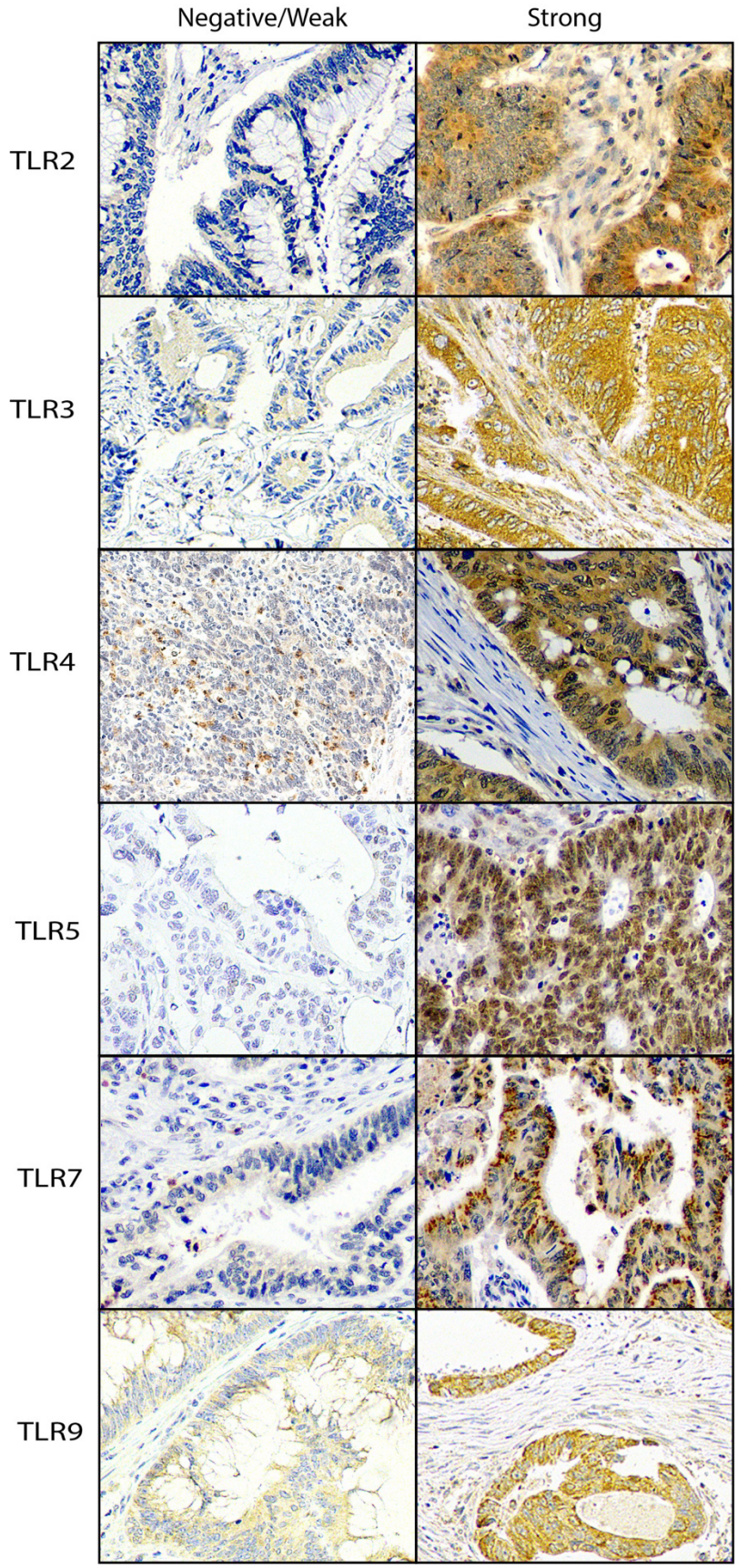

Figure 9. *Representative immunohistochemistry staining images from different toll-like receptors (TLRs) in colorectal cancer. Original magnification: 20x.*

4.5 BLOOD SAMPLES

Preoperative blood samples were available for 426 patients from the more recent cohort, while no blood samples were available for the older cohort. The blood samples were collected within 3 days before surgery in most cases (92.7%, range 0–30 days). After centrifuging, serum and plasma components were stored separately at –80°C. In study III, the plasma CRP levels were determined through a high-sensitivity method called time-resolved immunofluorometric assay (TR-IFMA) performed on microtitration plates with a monocolonal CRP antibody (anti-hCRP, code 6405, Medix Biochemica, Espoo, Finland) (Doumatey et al., 2014; Salmiheimo et al., 2016).

4.6 STATISTICAL ANALYSES

Variables were dichotomized for statistical analysis as described below. The Pearson's chi-square test was used to evaluate the associations between biomarker expression levels and clinicopathological variables and Spearman's correlation test for correlations. DSS was defined as the time from surgery until death due to CRC or the end of the follow-up period. At the time of their death, we censored patients who died for reasons other than CRC. Survival curves were constructed using the Kaplan–Meier method and the differences between groups were compared using the log-rank test. The 95% CIs were calculated for the survival rates. In study III, we used the maximum value for the Youden's index, obtained from receiver operating characteristic (ROC) curves (Youden, 1950) for the optimal cut-off value for the CRP level. For the univariate and multivariate survival analyses, we used the Cox proportional hazards model. Interaction terms were considered, although no significant interactions were identified. Age (continuous), gender (male/female), tumor stage (I/II/III/IV), WHO grade (1–2/3–4), and tumor location (colon/rectum) were used as independent covariates in the multivariate analysis, and tumor stage was processed as a categorical covariate. All tests were two-sided, and we considered $p < 0.05$ as statistically significant. The statistical analyses included in this thesis were performed using SPSS version 27.0 (IBM's SPSS Statistics, version 27.0 for Mac; SPSS, Inc., Chicago, IL, USA, an IBM Company), although earlier versions of SPSS were used for separate studies.

5 RESULTS

5.1 PILOT STAINING OF TLRS

The immunostainings for TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 were initially performed on a smaller series of 205 CRC patients. In this series, we successfully scored the TLR3 immunostaining in 198 samples (96.6%), TLR5 in 166 (81%), TLR7 in 199 (97.1%), and TLR9 in 196 samples (95.6%). In some cases, the scoring failed due to a missing representative cancer tissue and folding or overlapping spots.

In the pilot series, the immunoexpression of each TLR was grouped into low (scores 0–1) and high (scores 2–3) expression levels for the statistical analysis. In the Kaplan–Meier analysis of the pilot series, we detected no differences in prognosis between the different TLR2 (hazard ratio [HR] 0.61, 95% CI 0.37–1.01; p = 0.057), TLR3 (HR 0.91; 95% CI 0.55–1.51; p = 0.722), TLR4 (HR 0.91; 95% CI 0.57–1.45), TLR5 (HR 0.66; 95% CI 0.93–1.12; p = 0.126), TLR7 (HR 0.76; 95% CI 0.48–1.21; p = 0.245), and TLR9 (HR 1.38; 95% CI 0.432–4.37; p = 0.589) immunoexpression groups (data partly unpublished, Figure 10). The study was continued by investigating TLR2, TLR4, TLR5, and TLR7 expressions in the larger study population, setting TLR3 and TLR9 aside from further analysis. TLR4 was included because it was the most investigated TLR in CRC and other cancers for our knowledge.

5.2 IMMUNOSTAINING OF TLRS IN THE ENTIRE COHORT

The distribution of different TLRs scores for the entire cohort is shown in Table 6. From the 1308 TMA samples, the interpretation of the TLR2 immunoexpressions was successful in 1253 samples (95.8%), TLR4 in 1244 (95.1%), TLR5 in 1217 (93.0%), and TLR7 in 1243 (95.0%). TLR2 and TLR4 stained evenly in the cytoplasm, TLR7 showed a granular cytoplasmic staining, and a nuclear staining for TLR5 was observed.

Table 6. *Distribution of the immunoexpressions of TLR2, TLR4, TLR5, and TLR7 among 1308 colorectal cancer patients.*

	Negative (%)	Low (%)	Moderate (%)	High (%)
TLR2	62 (4.9)	258 (20.6)	537 (42.9)	396 (31.6)
TLR4	80 (6.4)	391 (31.4)	582 (46.8)	191 (15.4)
TLR5	211 (17.3)	255 (21.0)	395 (32.5)	356 (29.3)
TLR7	209 (16.8)	463 (37.2)	468 (37.7)	10 (8.3)

Abbreviations: TLR, toll-like receptor.

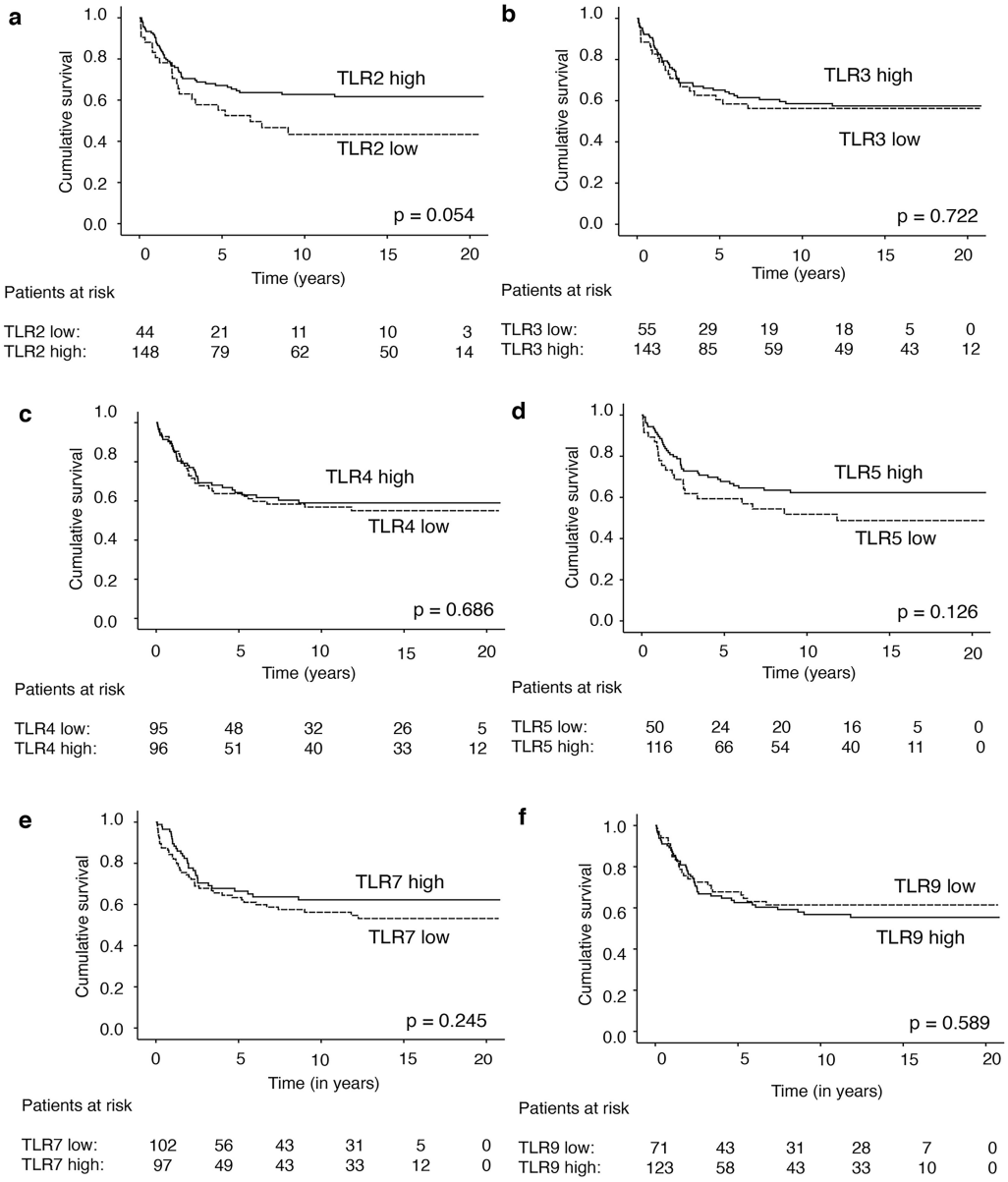

Figure 10. *Disease-specific survival for the pilot series baesd on the Kaplan–Meier method. The p values shown are based on the log-rank test. TLR, toll-like receptor.*

For a simplified statistical analysis and presentation of findings, the TLRs immunoexpression scores were dichotomized as follows: TLR2 low (scores 0–2) and high (score 3), TLR4 low (scores 0–1) and high (scores 2–3), TLR5 low (scores 0–2) and high (score 3), and TLR7 negative (score 0) and positive (scores 1–3) based on the Kaplan–Meier curves for all of the TLR scores.

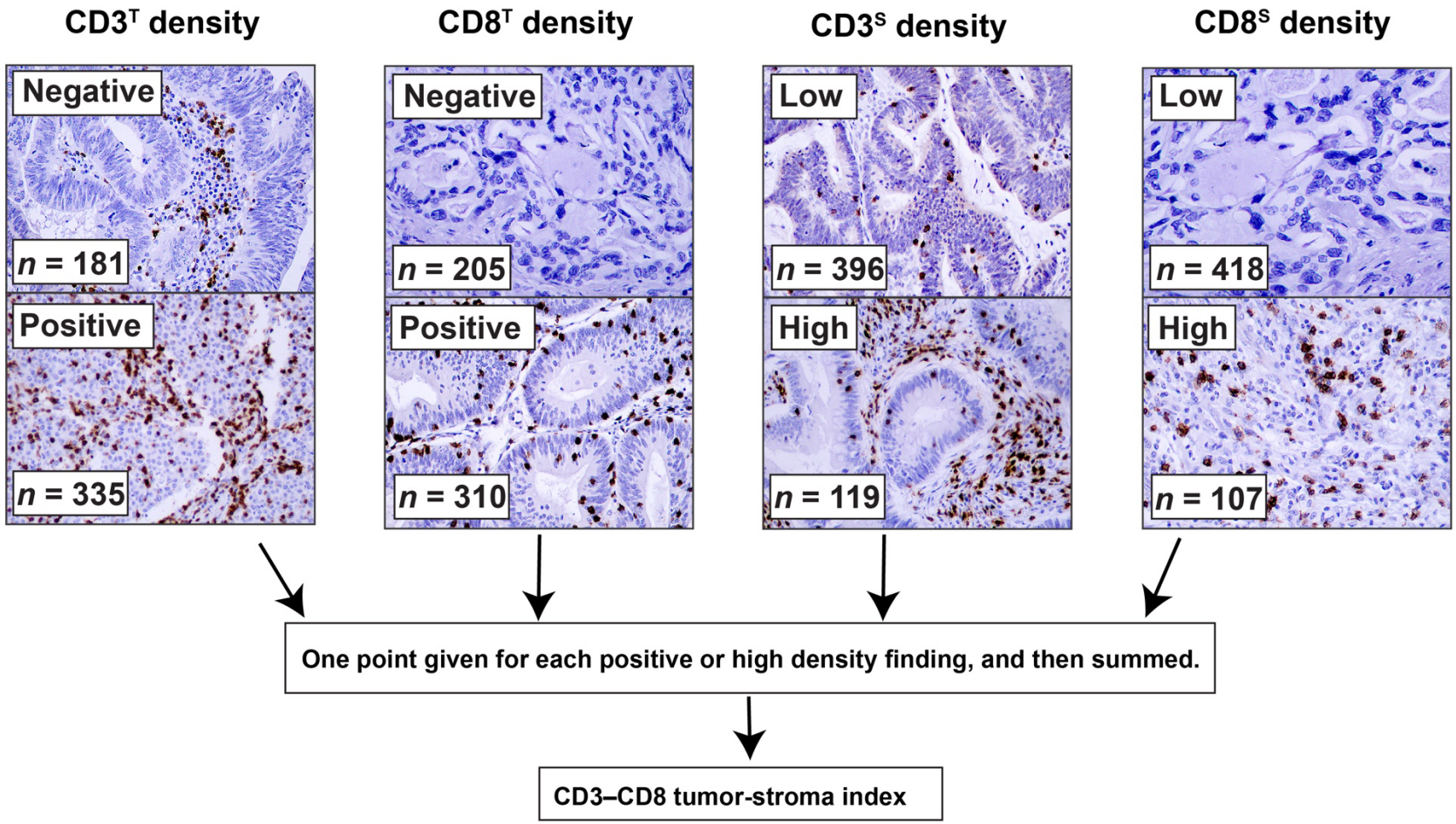

Figure 11. *Representative images of CD3 and CD8 immunostaining and flowchart of the CD3–CD8 tumor–stroma index formulation. Original magnification: 20x. Abbreviations: CD3, CD3-positive T cell; CD8, CD8-positive T cell.*

5.3 IMMUNOSTAINING FOR CD3 AND CD8

The scoring of $CD3^T$ was successful in 516 cases (94.0%), while $CD8^T$, $CD3^S$, and $CD8^S$ in 515 cases (93.8%). We dichotomized the $CD3^T$ and $CD8^T$ densities as negative (score 0) and positive (scores 1–3); $CD3^S$ and $CD8^S$ as low (score 0–3) and high (score 4). The dichotomies above were chosesn based on the Kaplan-Meier curves for all scores. Dichotomized $CD3^T$, $CD8^T$, $CD3^S$, and $CD8^S$ were used to generate a CD3–CD8 tumor–stroma index similar to the *Immunoscore*® (Kirilovsky et al., 2016). One point was given for every positive/high density, generating a four-point scale (points 0 to 4). The immunostaining examples of the CD3 and CD8 densities and scoring distribution appear in Figure 11.

5.4 ASSOCIATIONS BETWEEN TLRS AND CLINICOPATHOLOGICAL CHARACTERISTICS

Table 7 summarizes the relationship between the clinicopathological variables and tumor cell immunoexpression levels of different TLRs (unpublished data). The associations and correlations between different TLRs with each other appear in Tables 8 and 9 (unpublished data).

A low TLR2 immunoexpression level in cancer cells associated with a rectum location ($p < 0.001$), a higher pT classification ($p < 0.042$), lymph node–positive disease ($p = 0.006$), and with a higher WHO grade ($p = 0.008$). A high TLR4 immunoexpression level in cancer cells associated with a colon location ($p = 0.016$). In addition, a low TLR5 immunoexpression in the tumor cells associated with a higher tumor stage ($p < 0.001$), a higher pT classification ($p < 0.001$), lymph node positivity ($p = 0.004$), and a higher WHO grade ($p = 0.023$). A positive TLR7 immunoexpression level in the tumor cells associated with a rectum location ($p = 0.040$), a lower tumor stage ($p < 0.001$), a lower pT classification ($p < 0.024$), pN0 disease ($p < 0.001$), and a lower WHO grade ($p < 0.001$).

A low TLR2 immunoexpression level in the tumor cells associated with a low expression of TLR4 ($p < 0.001$), TLR5 ($p = 0.022$), and TLR7 in the tumor cells ($p < 0.001$), for which we observed weak or moderate positive correlations ($p < 0.001$ for all). A high TLR4 expression level in the cancer cells associated with a high TLR5 ($p = 0.005$) and positive TLR7 ($p < 0.001$) expression level in the tumor cells, for which the analyses revealed weak or moderate positive correlations here as well ($p < 0.001$ for both). A low TLR5 immunoexpression level in the tumor cells associated with a negative TLR7 immunoexpression in the cancer cells ($p = 0.006$), with a weak positive correlation ($p < 0.001$).

We identified no association between TLRs and CRP. A low CRP level, however, associated with a lower AJCC tumor stage and a lower T stage ($p < 0.001$ for both).

Table 7. *Associations between TLRs immunointensity and clinicopathological characteristics among 1308 colorectal cancer patients.*

	TLR2			TLR4			TLR5			TLR7		
	Low (%)	**High (%)**	**p value[1]**	**Low (%)**	**High (%)**	**p value[1]**	**Low (%)**	**High (%)**	**p value[1]**	**Negative (%)**	**Positive (%)**	**p value[1]**
Age												
<65	351 (67.5)	169 (32.5)	0.579	199 (38.7)	315 (61.3)	0.635	360 (71.0)	147 (29.0)	0.898	75 (14.5)	441 (85.5)	0.077
≥65	506 (69.0)	227 (31.0)		272 (37.3)	458 (62.7)		501 (70.6)	209 (29.4)		134 (18.4)	593 (81.6)	
Gender												
Male	465 (69.4)	205 (30.6)	0.429	218 (38.0)	356 (62.0)	0.952	457 (69.9)	197 (30.19	0.487	113 (16.8)	561 (83.2)	0.510
Female	392 (67.2)	191 (32.8)		253 (37.8)	417 (62.2)		404 (71.8)	159 (28.2)		96 (16.9)	473 (83.1)	
Location												
Colon	418 (63.1)	244 (36.9)	**<0.001**	230 (34.7)	432 (65.3)	**0.016**	459 (70.7)	190 (29.3)	0.518	124 (18.9)	532 (81.1)	**0.040**
Rectum	439 (74.3)	152 (25.7)		241 (41.4)	341 (58.6)		402 870.8)	166 (29.2)		85 (14.5)	502 (85.5)	
Tumor stage												
I	129 (68.3)	60 (31.7)	0.438	84 (43.3)	110 (56.7)	0.233	114 (61.0)	73 (39.0)	**<0.001**	21 (11.2)	167 (88.8)	**<0.001**
II	279 (67.4)	135 (32.6)		141 (34.6)	266 (65.4)		285 (70.4)	120 (29.6)		53 (12.9)	357 (87.1)	
III	255 (67.8)	121 (32.2)		141 (38.0)	230 (62.0)		268 (73.4)	97 (26.6)		73 (19.5)	302 (80.5)	
IV	182 (73.1)	67 (2.9)		91 (36.8)	156 863.2)		186 (79.1)	49 (20.9)		58 (23.7)	187 (76.3)	
Tumor classification (pT)												
pT1–pT2	88 (54.0)	75 (46.0)	**0.042**	64 (38.8)	101 (61.2)	0.926	72 (44.2)	89 (55.3)	**<0.001**	11 (6.6)	155 (93.4)	**0.024**
pT3–pT4	316 (63.2)	184 (36.8)		189 (38.3)	305 861.7)		315 (65.8)	164 (34.2)		66 (13.3)	432 (86.7)	
Lymph node metastasis (pN)												
pN0	191 (5.5)	147 (43.5)	**0.006**	123 (36.4)	215 (63.6)	0.228	183 (55.3)	148 (44.7)	**0.004**	20 (5.9)	320 (94.1)	**<0.001**
pN1–2	211 (66.4)	107 (33.6)		129 (41.4)	185 (58.9)		202 (66.4)	102 (33.6)		56 (17.7)	261 (82.3)	
Tumor grade (WHO)												
1–2	624 (66.9)	30 (33.1)	**0.008**	345 (37.1)	584 (62.9)	0.515	634 (69.6)	277 (30.4)	**0.023**	113 (12.2)	814 (87.8)	**<0.001**
3–4	200 (75.5)	65 (24.5)		102 (39.4)	157 (60.6)		194 (77.0)	58 (23.0)		86 (33.0)	175 (67.0)	

Abbreviations: TLR, toll-like receptor.

[1] Pearson's chi-square test.

Table 8. *Associations between TLR2, TLR4, TLR5, and TLR7 among 1308 colorectal cancer patients.*

	TLR2			TLR4			TLR5		
	Low (%)	**High (%)**	**p value[1]**	**Low (%)**	**High (%)**	**p value[1]**	**Low (%)**	**High (%)**	**p value[1]**
TLR4									
Low	373 (81.3)	86 (18.7)	**<0.001**						
High	457 (59.8)	307 (40.2)							
TLR5									
Low	585 (69.7)	254 (30.3)	**0.022**	327 (39.2)	508 (60.8)	**0.005**			
High	224 (62.9)	132 (37.1)		108 (30.4)	247 (69.6)				
TLR7									
Negative	168 (83.6)	33 (16.4)	**<0.001**	121 (62.1)	74 (37.9)	**<0.001**	150 (78.5)	41 (21.5)	**0.006**
Positive	663 (65.1)	356 (34.9)		336 (33.0)	681 (67.0)		685 (68.5)	315 (31.5)	

Abbreviations: TLR, Toll like receptor.

[1] Pearson's chi-square test.

Table 9. *Correlations among TLR2, TLR4, TLR5, and TLR7 levels in 1308 colorectal cancer patients.*

	TLR2		TLR4		TLR5	
	r_s	**p value**	**r_s**	**p value**	**r_s**	**p value**
TLR4	0.338	<0.001				
TLR5	0.165	<0.001	0.144	<0.001		
TLR7	0.299	<0.001	0.324	<0.001	0.103	<0.001

Abbreviations: TLR, toll-like receptor; r_s, Spearmans's correlation coefficient

5.5 ASSOCIATIONS AND CORRELATIONS BETWEEN TLRS AND CD3-POSITIVE AND CD8-POSITIVE T-CELL DENSITIES

High TLR2 and TLR4 immunoexpression levels in the tumor cells associated with positive $CD3^T$ ($p < 0.001$; $p = 0.013$) and positive $CD8^T$ ($p = 0.001$; $p = 0.025$) levels, and a higher CD3–CD8 tumor–stroma index ($p < 0.001$). Furthermore, weak positive correlations were observed between TLR2 and TLR4 immunoexpression levels and $CD3^T$ ($r_s = 0.175$, $p < 0.001$; $r_s = 0.135$; $p = 0.002$), $CD8^T$ ($r_s = 0.131$, $p = 0.003$; $r_s = 0.117$; $p = 0.008$), and the CD3–CD8 tumor–stroma index ($r_s = 0.157$, $p < 0.001$; $r_s = 0.098$; $p = 0.029$).

A low TLR5 immunoexpression level in the tumor cells associated with a negative $CD3^T$ ($p = 0.001$), low $CD3^S$ ($p = 0.001$) and negative $CD8^T$ ($p = 0.011$) levels, and a lower CD3–CD8 tumor–stroma index ($p = 0.003$), while a weak positive correlation was observed between a low TLR5 immunoexpression and $CD3^T$ ($r_s = 0.206$; $p < 0.001$), $CD3^S$ ($r_s = 0.137$; $p = 0.002$), $CD8^T$ ($r_s = 0.140$; $p = 0.002$), and the CD3–CD8 tumor–stroma index ($r_s = 0.203$; $p < 0.001$).

No association emerged between TLR7 immunoexpression levels in the tumor cells and CD3 or CD8 cell densities and the CD3–CD8 tumor–stroma index. A weak positive correlation was observed between the TLR7 immunoexpression and the $CD3^T$ density ($r_s = 0.095$; $p = 0.031$).

5.6 SURVIVAL ANALYSES

5.6.1 Univariate analyses

Patients with a high tumor cell TLR2 immunoexpression had a 5-year DSS of 68.4% (95% CI 63.7–73.1%) compared with 61.1% among low TLR2 immunoexpression patients (95% CI 57.6–64.6%; $p = 0.032$; Figure 12a). Among high tumor cell TLR5 immunoexpression patients, 5-year DSS was 72.2% (95% CI 67.3–77.1%) falling to 59.4% (95% CI 55.9–62.9%; $p < 0.001$; Figure 12c) among patients with a low TLR5 immunoexpression in the tumor cells. Patients with a low TLR7 immunoexpression in the tumor cells experienced a 5-year DSS of 55.1% (95% CI 48.0–62.2%), climbing to 64.9% (95% CI 61.8–68.0%; $p = 0.001$; Figure 12d) among those with a high TLR7. Ultimately, TLR4 tumor cell immunoexpression provided no prognostic value in the survival analysis across all patients (HR 0.94, 95% CI 0.79–1.13; $p = 0.516$; Figure 12b).

In the subgroup analyses, a high tumor cell TLR2 expression emerged as a favorable prognostic factor among female patients (HR 0.74, 95% CI 0.55–0.98; $p = 0.038$; Table 10), patients with stage III disease (HR 0.56, 95% CI 0.40–0.79; $p = 0.001$; Table 10 and Figure 13), and patients with a colon tumor location (HR 0.71,

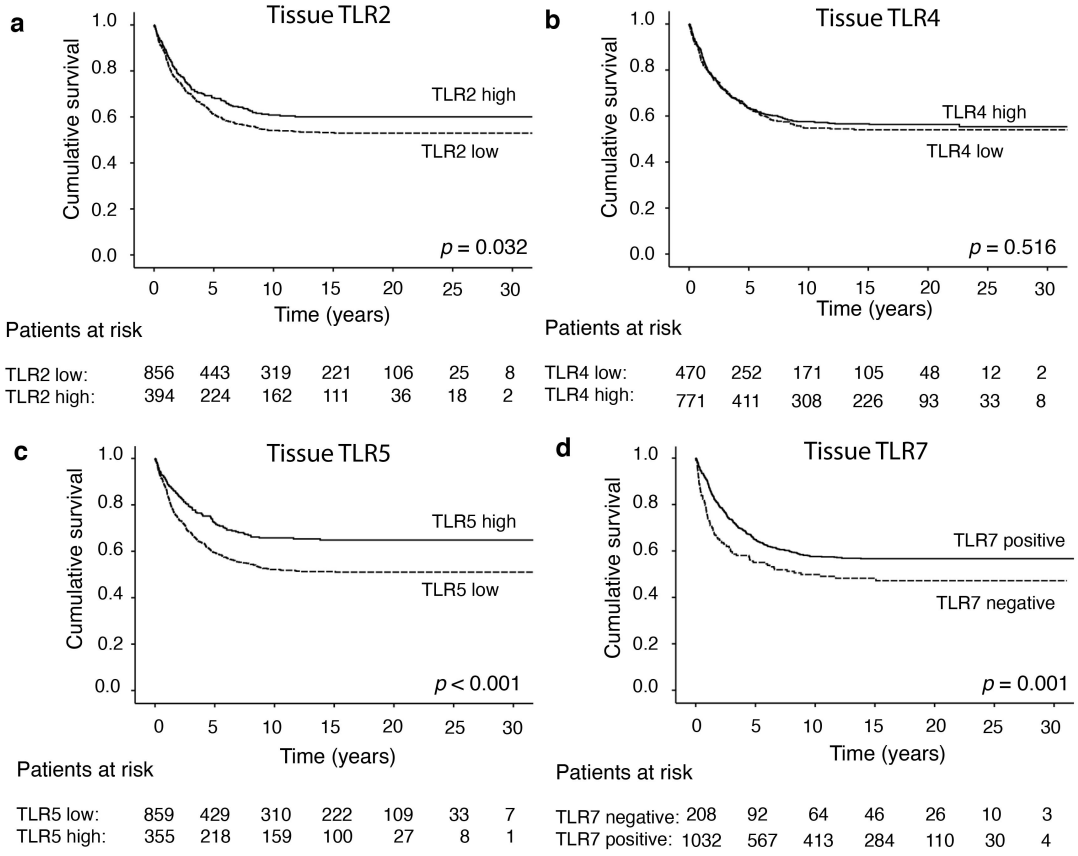

Figure 12. *Disease-specific survival for the entire cohort according to the Kaplan–Meier method. The p value for the log-rank test. Abbreviations: TLR, toll-like receptor.*

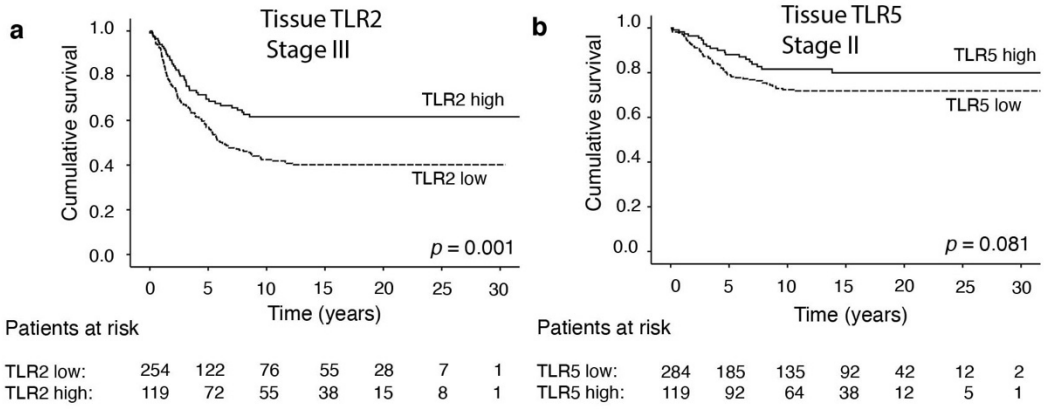

Figure 13. *Disease-specific survival analysis by subgroup using the Kaplan–Meier method. A high versus low TLR2 immunoexpression level among stage III patients (a) and a high versus low TLR5 immunoexpression among stage II patients (b). The p value for the log-rank test. TLR, toll-like receptor.*

Table 10. *Survival analysis by subgroups, comparing high versus low tumor cell TLR2 and TLR4 immunoexpression levels in 1308 colorectal cancer patients.*

	High vs. low TLR2			High vs. low TLR4		
	HR	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
Age						
<65 years	0.78	0.57–1.06	0.114	0.87	065–1.61	0.344
≥65 years	0.83	0.65–1.07	0.157	0.98	0.76–1.24	0.980
Gender						
Male	0.88	0.68–1.14	0.339	1.10	0.86–1.41	0.463
Female	0.74	0.55–0.98	**0.038**	0.79	0.61–1.03	0.084
Location						
Colon	0.71	0.54–0.92	**0.011**	0.80	0.62–1.04	0.092
Rectum	0.98	0.73–1.31	0.873	1.13	0.87–1.46	0.373
Tumor stage						
I	1.01	0.38–2.65	0.989	0.82	0.35–1.94	0.655
II	1.18	0.77–1.81	0.452	0.70	0.46–1.06	0.091
III	0.56	0.40–0.79	**0.001**	0.98	0.72–1.34	0.917
IV	1.04	0.77–1.41	0.799	1.03	0.77–1.37	0.854
Tumor classification (pT)						
pT1–pT2	0.61	0.25–1.51	0.282	0.68	0.29–1.61	0.385
pT3–pT4	0.81	0.60–1.07	0.129	0.95	0.72–1.25	0.069
Lymph node metastasis (pN)						
pN0	0.87	0.52–1.44	0.580	0.83	0.50–1.38	0.476
pN1–2	0.75	0.54–1.04	0.084	0.96	0.70–1.31	0.779
Tumor grade (WHO)						
1–2	0.88	0.70–1.10	0.249	0.93	0.67–1.31	0.688
3–4	0.75	0.49–1.15	0.186	0.91	0.37–2.22	0.837

Abbreviations: CI, confidence interval; HR, hazard ratio; TLR, toll-like receptor.

95% CI 0.54–0.92; p = 0.011; Table 10). In addition, patients younger than 65 years old and a high tumor cell TLR5 immunoexpression experienced a favorable prognosis (HR 0.79, 95% CI 0.49–0.99; p = 0.043; Table 10).

A high tumor cell TLR5 immunoexpression indicated a better prognosis for both male (HR 0.68, 95% CI 0.51–0.90, p = 0.006; Table 11) and female patients (HR 0.59, 95% CI 0.43–0.82; p = 0.002; Table 11) and among patients with disease in the colon (HR 0.72, 95% CI 0.54–0.96; p = 0.025; Table 11) and the rectum (HR 0.56, 95% CI 0.41–0.77; p < 0.001; Table 11). Furthermore, a high TLR5 tumor cell immunoexpression indicated a better prognosis among patients with a higher pT stage (HR 0.71, 95% CI 0.53–0.96, p = 0.028; Table 11), lymph node–negative disease (HR 0.58, 95% CI, 0.34–0.99, p = 0.043; Table 11), and a lower WHO grade (HR 0.65, 95% CI 0.50–0.83, p < 0.001; Table 11).

Among male patients (HR 0.66, 95% CI 0.49–0.89; p = 0.007; Table 11), older patients (HR 0.62, 95% CI 0.47–0.81, p < 0.001; Table 11), and those with disease in the colon (HR 0.71, 95% CI 0.53–0.96, p = 0.023; Table 11) or the rectum (HR 0.67, 95% CI 0.48–0.93, p = 0.016; Table 11), a high TLR7 immunoexpression emerged served as a positive prognostic factor. In addition, among patients with a higher tumor stage (HR 0.65, 95% CI 0.45–0.95, p = 0.025; Table 11) and a lower WHO grade (HR 0.77, 95% CI 0.62–0.97, p = 0.026; Table 11), a high TLR7 immunoexpression indicated a better prognosis. Moreover, the TLR4 immunoexpression in the tumor cells provided no prognostic value in the subgroup analyses.

In the cohort of 549 CRC patients in study III, patients with a high CRP level had a 5-year DSS of 64.3% (95% CI 54.6–72.2%) compared with 74.1% (95% CI 68.8–79.4%; p = 0.017; Figure 14f) for those with a low CRP level. Among pateints with a low CRP level, the ones with a high TLR2 immunoexpression (HR 0.53; 95% CI 0.35–0.80; p = 0.002; Figure 14a), a high TLR5 immunoexpression (HR 0.059; 95% CI 0.37–0.92; p = 0.021; Figure 14c), and a positive TLR7 immunoexpression in the tumor cells (HR 0.53; 95% CI 0.28–1.00; p = 0.049; Figure 14e) exhibited a better prognosis, while a high tumor cell TLR4 immunoexpression indicated worse prognosis among high CRP level patients (HR 2.04; 95% CI 1.04–4.00; p = 0.038; Figure 14b).

From the cohort of 549 CRC patients in study IV, among high TLR2 tumor cell immunoexpression patients, those with a high $CD3^S$ (HR 0.40; 95% CI 0.24–0.65; p < 0.001), a high $CD8^S$ (HR 0.55; 95% CI 0.34–0.88; p = 0.013), a positive $CD8^T$ (HR 0.38; 95% CI 0.27–0.52; p < 0.001), and a positive $CD3^T$ value (HR 0.40; 95% CI 0.29–0.55; p < 0.001) exhibited a better prognosis. A positive $CD3^T$ value emerged as a positive prognostic factor among low TLR2 immunoexpression level patients as well (HR 0.36; 95% CI 0.16–0.79; p = 0.012).

Table 11. *Survival analysis by subgroups, among high versus low tumor cell TLR5 immunoexpression and positive versus negative tumor cell TLR7 immmunoexpression levels in 1308 colorectal cancer patients.*

	High vs. low TLR5			Positive vs. negative TLR7		
	HR	**95% CI**	**p value**	**HR**	**95% CI**	**p value**
Age						
<65 years	0.79	0.49–0.99	**0.043**	0.89	0.60–1.13	0.892
≥65 years	0.84	0.63–1.12	0.231	0.62	0.47–0.81	**<0.001**
Gender						
Male	0.68	0.51–0.90	**0.006**	0.66	0.49–0.89	**0.007**
Female	0.59	0.43–0.82	**0.002**	0.73	0.52–1.01	0.058
Location						
Colon	0.72	0.54–0.96	**0.025**	0.71	0.53–0.96	**0.023**
Rectum	0.56	0.41–0.77	**<0.001**	0.67	0.48–0.93	**0.016**
Tumor stage						
I	0.49	0.28–1.33	0.161	0.76	0.22–2.60	0.665
II	0.64	0.39–1.06	0.081	1.09	0.58–2.04	0.800
III	0.83	0.58–1.18	0.293	0.76	0.53–1.11	0.155
IV	0.94	0.66–1.34	0.728	0.87	0.63–1.23	0.419
Tumor classification (pT)						
pT1–pT2	0.80	0.34–1.89	0.615	0.50	0.12–2.13	0.346
pT3–pT4	0.71	0.53–0.96	**0.028**	0.65	0.45–0.95	**0.025**
Lymph node metastasis (pN)						
pN0	0.58	0.34–0.99	**0.043**	0.77	0.43–1.41	0.399
pN1–2	0.79	0.55–1.09	0.147	0.74	0.54–1.01	0.056
Tumor grade (WHO)						
1–2	0.65	0.50–0.83	**<0.001**	0.77	0.62–0.97	**0.026**
3–4	0.82	0.42–1.27	0.372	0.90	0.65–1.25	0.529

Abbreviations: CI, confidence interval; HR, hazard ratio; TLR, toll-like receptor.

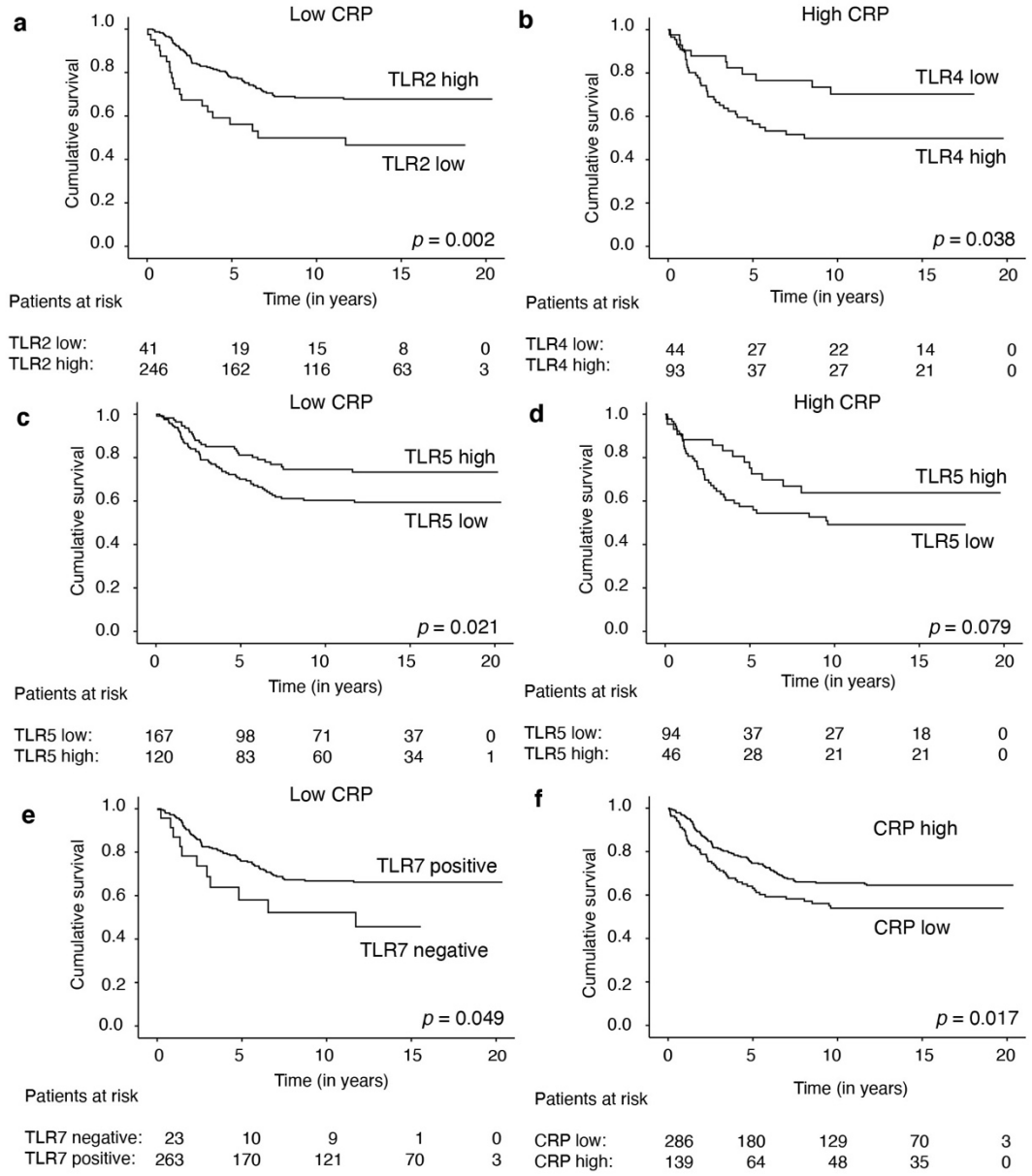

Figure 14. *Disease-specific analysis of 549 CRC patients according to the Kaplan–Meier method. A high versus low TLR2 among patients with a low CRP (a), a high versus low TLR4 among patients with a high CRP (b), a high versus low TLR5 among patients with a low CRP (c) and a high CRP (d), a positive versus negative TLR7 among patients with a low CRP (e), and DSS among low CRP and high CRP patients (f). p value for the log-rank test. TLR, toll-like receptor; CRP, C-reactive protein.*

In the high TLR4 tumor cell immunoexpression subgroup, those with a positive $CD3^T$ (HR 0.35; 95% CI 0.24–0.51; p < 0.001), a positive $CD8^T$ (HR 0.38; 95% CI 0.27–0.52; p < 0.001), a high $CD3^S$ (HR 0.35; 95% CI 0.19–0.62; p < 0.001), and a high $CD8^S$ value (HR 0.36; 95% CI 0.19–0.67; p = 0.001) exhibited better outcomes. Furthermore, a positive $CD3^T$ (HR 0.45; 95% CI 0.27–0.72; p = 0.001) and a positive $CD8^T$ value (HR 0.59; 95% CI 0.36–0.95; p = 0.029) indicated a better prognosis among the low TLR4 imunnoexpression subgroup as well.

Moreover, a positive $CD3^T$ value indicated a better prognosis among both low TLR5 (HR 0.38; 95% CI 0.26–0.54; p < 0.001) and high TLR5 tumor cell immunoexpression patients (HR 0.45; 95% CI 0.26–0.76; p = 0.003). This was similar to positive $CD8^T$ (HR 0.51; 95% CI 0.36–0.74; p < 0.001 and HR 0.25; 95% CI 0.15–0.43; p < 0.001, respectively) and high $CD3^S$ values (HR 0.46; 95% CI 0.26–0.81; p = 0.008 and HR 0.38; 95% CI 0.19–0.78; p = 0.008, respectively). A high $CD8^S$ value indicated a better prognosis only among those with a low TLR5 tumor cell immunoexpression (HR 0.53; 95% CI 0.31–0.91; p = 0.021).

Among high TLR7 tumor cell immunoexpression patients, those with a positive $CD3^T$ (HR 0.34; 95% CI 0.24–0.47; p < 0.001), a positive $CD8^T$ (HR 0.32; 95% CI 0.23–0.45; p < 0.001), a high $CD3^S$ (HR 0.32; 95% CI 0.19–0.54; p < 0.001), and a high $CD8^S$ value (HR 0.46; 95% CI 0.27–0.76; p = 0.003) exhibited a significantly better outcome. We observed no prognostic impact among low TLR7 immunoexpression patients.

A low CD3–CD8 tumor–stroma index served as a negative prognostic factor among all TLR subgroups, except the negative TLR7 immunoexpression subgroup. Those with the highest CD3–CD8 tumor–stroma index exhibited a 5-year DSS of 82.2% (95% CI 70.2–94.2%) compared with 51.8% (95% CI 40.6–63.0%; p < 0.001; Figure 15) among patients with an index of 0 among the high TLR2 immunoexpression subgroup. Among patients with the lowest CD3–CD8 tumor–stroma index, the 5-year DSS reached only 39.7% (95% CI 26.4–53.0%) compared with 88.9% (95% CI 77.1–100.0; p < 0.001; Figure 15) among those with the highest CD3–CD8 tumor–stroma index in the high TLR4 immunoexpression subgroup. Among patients with a high TLR5 tumor cell immunoexpression, the 5-year DSS was 50.3% (95% CI 31.9–68.7%) among those with a tumor–stroma index of 0 and reached 81.8% (95% CI 65.7–97.9%; p < 0.001; Figure 15) among those with the highest CD3–CD8 tumor–stroma index. In the positive TLR7 tumor cell immunoexpression subgroup, patients with a CD3–CD8 tumor–stroma index of 4 had a 5-year DSS of 87.9% (95% CI 76.7–99.1%) compared with 48.0% (95% CI 37.4–58.6%; p < 0.001; Figure 15) among patients with a CD3–CD8 tumor–stroma of 0.

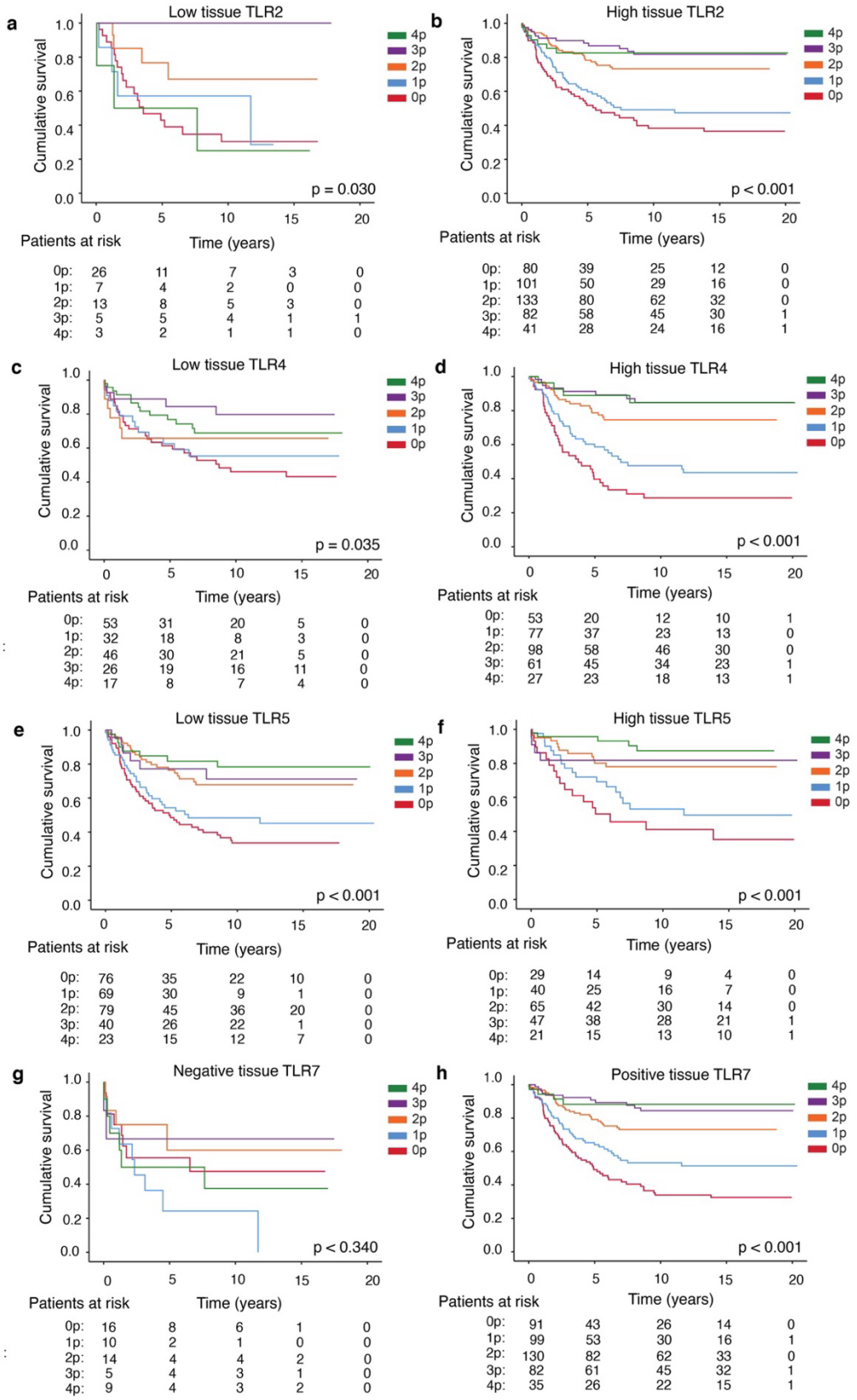

Figure 15. *Disease-specific survival analysis according to the Kaplan–Meier method. CD3-CD8 index among low and high TLR2 (a, b), TLR4 (c, d), and TLR5 expression patients (e, f), and negative and positive TLR7 expression patients (g, h). The log-rank test was used.*

5.6.2 Multivariate analyses

In the Cox multivariate survival analysis for the entire cohort, none of the tumor cell immoexpressions among the TLRs analyzed emerged as independent prognostic factors for DSS alongside age, sex, tumor stage, and WHO grade, even though when analyzed in the smaller cohort, lower TLR2 tumor cell immunoexpression (study I) emerged as an independent negative prognostic factor among patients with lymph node metastases and a high TLR5 tumor cell immunoexpression (study II) served as an independent positive prognostic factor.

6 DISCUSSION

6.1 TLR2, TLR4, TLR5, AND TLR7 IN COLORECTAL CANCER

The findings from this study demonstrate that high TLR2, TLR5 and TLR7 immunoexpression levels in tumor cells could identify CRC patients likely to experience a better prognosis. Furthermore, among stage III patients, those with a high tumor cell TLR2 immunoexpression experienced a better prognosis. TLR4 tumor cell immunoexpression did not appear to impact survival across the entire cohort, although among patients with a high CRP level, those with a high TLR4 immunoexpression experienced a worse prognosis.

No previous studies are available regarding the prognostic role of TLR5 and TLR7 in CRC, while only a few exist on TLR2 and TLR4. Only one research group has reported a possible prognostic value for TLR2. Contrary to our findings, they observed a poorer survival among CRC patients with a high TLR2 expression (Liu et al., 2018). In their study, they assessed the immunohistochemistry from the tissue samples of 24 CRC patients, a too-small cohort for definitive conclusions (Liu et al., 2018). Yet, they found that TLR2 expression associated with an MSI/CpG island methylation CIMP status. In cell lines and in a mice model, TLR2 signalling promoted the growth, migration, and invasion of CRC cells through Akt and NF-κB pathways, leading to the upregulation of several antiapoptotic genes (Liu et al., 2018).

In another study investigating TLR2 and TLR4 among 118 CRC patients, a strong TLR4 expression along the invasive front associated with a better DSS among patients with proximal disease, but TLR2 expression did not impact survival (Paarnio et al., 2017). Their results demonstrated that heterogeneity inside the same tumor exists, whereby TLR4 expression was stronger in the tumor front compared to the tumor bulk (Paarnio et al., 2017). In our study, however, TLR4 expression was assessed only in the tumor bulk, which may explain why no association between survival and TLR4 was found in the entire cohort.

Eiró et al. (2013) found that patients with a higher TLR4 expression in the tumor cells had a lower recurrence rate, while expression in the cancer associated fibroblasts associated with a high rate of tumor recurrence. This demonstrates that the role of the TLR depends on the cell type expressing it. In another study, the same group found that stage II CRC patients with a high TLR4 expression exhibited a better OS, a lower recurrence rate, and a lower rate of distant metastases and carcinomatosis development (Eiro et al., 2019). Thus, their findings did not agree with our findings, whereby patients with a high CRP level and with a high TLR4 expression experienced a worse prognosis.

Nihon-Yanagi et al. (2012) measured TLR2 and TLR4 protein expressions using real-time PCR protein analysis, finding that TLR2 is upregulated in tumors compared

with normal tissue, but no TLR4 upregulation was observed. Furthermore, TLR2 expression was higher in cancer samples at all stages compared with the levels in the normal mucosa. TLR4 expression remained similar in the normal mucosa and cancer tissue at different stages (Nihon-Yanagi et al., 2012). They found that TLR2 expressed at higher levels in stage II and III tumors, possibly demonstrating that at different cancer stages the role of TLR2 changes. In our cohort, however, among stage III patients, those with lymph node–positive disease without distal metastases with a high TLR2 immunoexpression exhibitied a better prognosis.

Studies of TLR2 and TLR4 expression in other gastrointestinal malignancies have reported contradictory findings. In esophageal adenocarcinoma (Huhta et al., 2016) and hepatocellular carcinoma (Kairaluoma et al., 2021b), patients with a high TLR4 expression exhibited a poorer prognosis, which agreed with findings among the high CRP subgroup in our study. Contrary to our findings, however, in gastric cancer upregulated TLR2 associates with a poorer survival (Tye et al., 2012), while in another study no impact on survival was observed (Kasurinen et al., 2019). In pancreatic ductal adenocarcinoma, among patients with smaller size tumors, a higher TLR2 expression served as a positive prognostic factor (Lanki et al., 2018). Furthermore, among stage I–II patients, both high TLR2 and TLR4 expressions associated with a better DSS (Lanki et al., 2018).

In our study on CRC, patients with high TLR5 and TLR7 expressions experienced a significantly better prognosis. Other studies regarding the prognostic role of TLR5 and TLR7 tissue immunoexpression in CRC remain lacking. Contradictory findings are available, however, for other malignancies. Similar to these CRC results, gastric cancer patients with a high tissue TLR5 immunoexpression exhibited a better prognosis (Kasurinen et al., 2019) and a high TLR7 immunoexpression associated with better outcomes among stage III gastric cancer patients (Kasurinen et al., 2019). However, in oropharyngeal squamous cell carcinoma (Jouhi et al., 2017), squamous cell carcinoma of the tongue (Kauppila et al., 2013), and nonendemic nasopharyngeal carcinoma (Ruuskanen et al., 2019) patients with a high TLR5 expression experienced a worse prognosis.

The upregulation of TLRs in CRC has been observed in several studies (Fukata et al., 2007; Liu et al., 2018; Lu, 2020; Semlali et al., 2016). TLRs participate in the multistep development from normal mucosa to adenoma and carcinoma. For insteance, Wang et al. (2010) demonstrated that in normal mucosa and adenomas, TLR4 and adaptor molecule MyD88 immunoexpression were negative or very low, but high in CRC tissue samples. Furthermore, they reported a worse survival among high TLR4 and MyD88 expression patients. This agrees with the findings from our study among patients with a high CRP level. In one study, TLR2, TLR3, TLR4, and TLR5 were not observed in polyps at all, but the expression of TLR7 and TLR9 was higher in polyps than in normal tissue (Eiró et al., 2012). Interestingly, patients who

subsequently developed CRC exhibited lower levels of TLR7 and TLR9 in polyps than those who did not develop CRC (Eiró et al., 2012).

Although it is assumed that TLRs participate in the development of CRC, the mechanisms remain unexplained. As seen from the studies described above, TLRs play versatile roles in different malignancies. Furthermore, in the same malignancy TLRs may lead to variable responses among different individuals. The activating ligand may represent one possible explanation. In tumors, unprogrammed cell death releases DAMPS, which may activate TLR-mediated aberrant cascades leading to tumor progression through angiogenesis, forming the protumorigenic tumor microenvironment, and immunosuppressive functions (Sato et al., 2009).

An imbalance in commensal microbiota serves as another explanation of TLR involvement in tumorigenesis, since TLRs may be activated by microbial ligands that mediate additional protumorigenic responses (Fukata et al., 2007). It may be that common CRC risk factors such as obesity, smoking, and alcohol use change the normal microbiota and thus promote tumorigenesis. Different microbiota may also partly explain versatile TLR responses between different tumors and the variable results reported across different studies. While genetic variations may alter the intestinal homeostasis, polymorphism within the TLR genes quite commonly lead to changes in the TLR expressions and alter the detection of microbiota and subsequent inflammatory responses (Okazaki et al., 2017).

Moreover, tumor cells also express TLRs and their activation may overcompete with the antitumorigenic roles of TLRs expressed in tumor-infiltrating lymphocytes (TILs) and lead to the activation of the TLR-mediated antiapoptotic responses of tumor cells. TLRs expressed in tumor cells recruit immune cells, whose functions are modified to produce cytokines that promote the formation of the protumorigenic tumor microenvironment (Sato et al., 2009).

6.2 RELATIONSHIP BETWEEN TLRS AND CRP

To evaluate the relationship between the local innate and systemic inflammatory response, we evaluated the tumor cell immunoexpression of TLRs and plasma CRP. We found that a high TLR2, TLR5, and TLR7 immunoexpression in the tumor cells can identify patients with a better prognosis among those with a low preoperative CRP value. Among patients with a high CRP, a high tumor cell TLR4 expression indicated a worse prognosis. Patients with an elevated preoperative CRP exhibited a worse survival than those with a low CRP level.

The negative prognostic role of an elevated preoperative CRP level in CRC was previously demonstrated (Kersten et al., 2013; Køstner et al., 2016; Partl et al., 2020; Woo et al., 2015). Our findings confirm this, since patients with a low preoperative plasma CRP experienced a better prognosis than those with a high CRP. Furthermore,

CRP associated with the TNM stage and tumor invasiveness. Only a few studies investigating local and systemic inflammation together in CRC exist and the relationship between local TLR expression and CRP has not been previously examined.

In this study, DSS was best among patients with a low CRP value and a high immunoexpression of TLR2, TLR5, or TLR7. Furthermore, patients with a low TLR4 immunoexpression in the low CRP subgroup also experienced a better prognosis than those with a high TLR4 immunoexpression. Similarly, other studies have found the best survival among patients with a low systemic and a high local antitumorigenic inflammatory response, even though somewhat different markers of local and/or systemic inflammatory response were investigated. The findings available which investigated local and systemic inflammation in CRC primarily concentrated on local immune cell infiltration to image the local inflammatory response. For example, Roxburgh et al. (2009) found that the best survival occurred among stage II and stage III CRC patients with mGPS 0 and a high Klintrup grade.

In another study among 498 stage II CRC patients, the local chronic immune cell density (lymphocytes, plasma cells, and macrophages) and systemic inflammatory response (neutrophil-to-lymphocyte ratio) were assessed. Similar to the findings from our study, the best survival was observed among patients with a low neutrophil-to-lymphocyte ratio and a high immune cell density (Turner et al., 2015). They observed this among high-risk stage II patients as well. Furthermore, they found that only 10% of patients had both a high local and systemic response, which could indicate that the local and systemic immune responses are rather independent from each other. This agrees with our findings, whereby no association or correlation was observed between the immunoexpressions of different TLRs and plasma CRP.

In addition, Paarnio et al. (2019) examined the CRP and circulating serum TLR2 and TLR4 among CRC patients, finding that those with an undetectable serum TLR2 level had higher CRP values. Unfortunately, they did not assess the association between the tissue TLR expression and systemic inflammation as we did here. 75% of patients had undetectable serum TLR2 levels and 15% undetectable or low TLR4 levels, which were comparable to the control group. Patients with mGPS 0 (normal CRP and albumin levels) had higher TLR serum levels than mGPS 1 patients, and no mGPS 2 patients were identified in the measurable serum TLR2 group. Comparing these results to our study is tricky, however. Specifically, since serum TLR levels were not measured in our study and it is debatable if the serum TLR levels of the patients in the study by Paarnio et al. are connected with colorectal tumors of these patients as the serum levels of TLR2 and TLR4 did not correlate with the tissue TLR2 and TLR4 immunoexpression levels in tumor or lymph node metastases (Paarnio et al. 2019).

The results here suggest that the local and systemic inflammatory response are rather independent, since no associations or correlations between CRP and the TLRs examined were observed. Other studies found associations between local

inflammation and SIR, given that a low tumor CD4+ T-cell infiltration associated with a high CRP in one study (Canna et al., 2005) and a high FOXP3+ infiltration and elevated CRP negatively correlated in another (Gunnarsson et al., 2020). In CRC, FOXP3 cells associate with a better survival, in contrast to several other cancers. It remains debatable if CRP mirrors a nonspecific response to tumor expansion, tumor necrosis, and tissue damage or if CRP itself drives tumor progression by secreting proinflammatory cytokines, upregulating chronic protumorigenic inflammatory responses, and inducing the expression of adhesions on the endothelial cells which promote metastases (Läubli and Borsig, 2010; Tuomisto et al., 2019; Woo et al., 2015).

6.3 RELATIONSHIP BETWEEN TLRS AND TISSUE-INFILTRATING T CELLS

To examine the relationship between the innate and adaptive local immune responses, the TLR tissue expressions alongside CD3+ and CD8+ T-cell densities were assessed. High expressions of tumoral and stromal CD3+ and CD8+ T cells were associated with high expressions of TLR2, TLR4, and TLR5. In addition, a high CD3–CD8 tumor–stroma index served as a positive prognostic factor among all TLR subgroups, except for the negative TLR7 subgroup.

A high density of TILs, especially CD3+ and CD8+ T cells served as a positive prognostic factor in CRC (Pagès et al., 2018). Our research group previously demonstrated similar findings (Kasurinen et al., 2022). In current study, high tumoral and stromal densities of CD3+ and CD8+ T cells indicated a better prognosis in different TLR subgroups, but found no prognostic value in some subgroups, indicating that TLRs have an impact on the prognostic value of TILs. TLRs are present in various immune cells. For instance, dendritic cells and natural killer cells express a variety of functional TLRs depending on their subtype. Furthermore, dendritic cells and natural killer cells continuously engage in crosstalk between the innate and adaptive immune systems, and TLRs are strongly involved in this process (Guo and Zhang, 2012; Qiu et al., 2011). TLRs induce dendritic cell maturation, T-cell priming, and a tumor-specific cytotoxic T-cell response (Park et al., 2019; Ramakrishna et al., 2007). Furthermore, different TLRs together may have a synergistic or negative effect on the cytotoxic T-cell response (Nourizadeh et al., 2014). This might explain why CD3+ and CD8+ T cells have a different prognostic impact among various TLR subgroups in our study.

The findings of this study support a strong connection between TLR-induced dendritic cells and CD3+ and CD8+ T cells since high intratumoral CD3+ and CD8+ T-cell densities associated and correlated with high expressions of TLR2, TLR4, and TLR5. In addition, a high CD3+ T-cell intratumoral density also correlated with a high

TLR7 expression. Similarly, Väyrynen et al. (2013) investigated the relationship between a wide range of innate and adaptive immune cells, finding that mature CD83+ dendritic cells were strongly clustered to T cells (CD3+, CD8+, and FOXP3+) and particularly associated with CD3+ T cells. Furthermore, they were present in both the tumor core and along the invasive front. But, in hierarchical clustering, immature CD1+ dendritic cells clustered far away from other cells.

The TLR-induced response depends on the cell type in which they are expressed and on the activating ligand, which also explains the different prognostic impact of CD3+ and CD8+ T cells in various TLR subgroups. Stimulating dendritic cells through most TLRs leads to the release of Th1-type proinflammatory cytokines. Yet, some TLRs induce dendritic cells to produce IL-10, leading naïve T cells to differentiate in a Th2 and Treg manner (Jin et al., 2012). The effector result depends on the stimulus that activates TLRs, as well as the duration of the stimulus, since the chronic long-lasting stimulus leads to Th2 and Treg responses, even if the TLRs would otherwise promote Th1 responses to the same stimulus (Jin et al., 2012).

TLR-mediated NF-κB-directed signaling leads to M1-type macrophage differentiation, the expression of co-stimulatory molecules and immunostimulatory cytokines, followed by natural killer cell activation and CTL stimulation (Liu et al., 2020; Müller et al., 2015). In addition to CTL activation through co-stimulation, even activation in the absence of antigen presenting cells remains possible, since TLRs are also expressed in the T cells (Jin et al., 2012). Again, this result depends on the specific TLR and stimulus. For example, TLR2 and TLR7 activation leads to the suppression of Treg cell responses, but TLR5 and TLR4 stimulate Treg immunosuppressive responses (Jin et al., 2012).

The findings here support the idea that evaluating only CD3+ T-cell densities instead of a combination with CD8+ T cells might yield a similar prognostic value and could be more easily used in clinical practice. We found no TLR subgroups in which CD8 densities had a prognostic value while CD3 densities did not. The prognostic significance of the CD3–CD8 tumor–stroma index was comparable to $CD3^T$. Kasurinen et al. (2022) similiarly suggested using the CD3 index in clinical practice. Furthermore, combined with innate markers such as TLRs could improve the prognostic evaluation of CRC patients, although further studies are necessary in order to clarify the biological mechanisms and prognostic value.

6.4 STRENGTHS AND LIMITATIONS OF THIS STUDY

This study examined a large cohort, consisting of 1308 CRC patients. The possibility of observing the more natural course of disease represents an advantage to relying on an older cohort. Less frequently used neoadjuvant treatment grants us the opportunity to investigate the natural primary tumor and the tumor microenvironment. Another

benefit to reyling on an older cohort is the longer follow-up time period and the possibility of identifying patients who are truly cured of disease. Some patients may develop recurrence late, several years following treatment, when the normal follow-up of patients has ended, although this is not that common for CRC.

Using a cohort where the oldest patients were operated on more than 30 years ago also carries limitations. During that long follow-up period, long-term changes in medical care, the conditions of care, and improvements in surgical techniques may have occurred, all of which positively impact long-term outcomes, particularly among more recent surgical patients. In addition, the Dukes classification was used to classify the older cohort of patients treated in our clinic, while the TNM classification was applied to more recent patients. Thus, combining these cohorts into the same stage classification system may have introduced some bias. Furthermore, stage migration is possible due to the improved quality of structured pathological reports. Surgical and oncological treatments were administered in different hospitals belonging to the Helsinki University Hospital system with nonunified patient records. Unfortunately, the records for all patients receiving chemotherapy were not available and some of those records available were insufficient or incomplete

It remains debatable whether old archival tissue samples may tend towards weaker staining patterns compared with more recent tissue blocks, possibly resulting in a bias in the immunostaining-based analysis. Such a tendency in older tissue samples was not observed, since in separate studies the prognostic role of the immunoexpressions of different TLRs in older (studies I and II) and more recent (study III) tissue samples were evaluated, resulting in similar findings.

Given that the different cohorts were stained at different time points, many of the primary antibodies used for the initial stainings were no longer available for subsequent stainings, as demonstrated in Table 5 (see page 59). Yet, the specificities of all antibodies used in our laboratory were tested before use in order to verify the reliability of the results. Validation stainings for TLR4 comparing the two different antibodies were performed, resulting in similar staining expression levels. Furthermore, our research group has used the same antibodies for several studies on other cancers.

Using TMA slides instead of whole-tissue samples has been debated since one TMA spot only allows us to investigate a small area of a tumor. Punching areas are carefully chosen and more than one spot from different areas of the tumor are included in the TMA block (Kyndi, 2008). One advantage to the TMA technique is the opportunity to analyze larger cohorts at less expense while sparing valuable tumor tissue for future studies.

In the subgroup analyses, we did not adjust the p values for multiple testing, and the results from this exploratory study introduced the use of raw p values, serving as a limitation to this study. Given that several subgroup analyses have also relied on raw p values, there is a possibility that family-wise errors occurred. Another limitation to

this study is the lack of a prospective power analysis. However, we report the effect sizes with confidence intervals, leaving the conclusions to the reader. The large cohort in this study increases the strength of our findings, although in some subgroup analyses the smaller number of patients may decrease the reliability of our findings. No established cut-offs exist for the TLR expressions in CRC. Given the limited knowledge on TLR biology in CRC, an *a priori* cut-off determination was not agreed upon, which might also be considered a limitation. The cut-off for TLR immunoexpressions was decided separately for each study according to the distribution of expressions, thereby allowing for a better statistical evaluation. Moreover, the single-center setting and the lack of an independent validation cohort limits the generalizability of our results. Thus, in the future these findings should be confirmed using another well-defined patient cohort, possibly in a multicenter setting.

Despite some concerns about using a rather old cohort, the advantages were considered as sufficiently valuable, whereby we decided to examine TLRs using this cohort.

6.5 CONCLUDING REMARKS AND FUTURE PROSPECTS

We investigated the innate immune response in CRC patients by assessing the tissue immunoexpression of different TLRs, finding that high tissue expressions of TLR2, TLR5, and TLR7 can identify which patients are likely to experience a favorable prognosis, also among the ones with low preoperative CRP level. A low TLR4 immunoexpression can identify patients with a better prognosis among those with a high preoperative CRP level. Accordingly, TLR2, TLR5, TLR7, and possibly also TLR4 are applicable biomarkers to assess prognosis among CRC patients. Since this study was the first to report a positive prognostic role of TLR2, TLR5, and TLR7 in CRC, further studies are needed to validate the findings. Additionally, combining TLRs with other prognostic factors, such as CRP or immune cell density, may be an even better tool to identify patients with the best or worst prognoses. The TLRs studied could be interesting targets for immunotherapy in the future.

The immune system is programmed to respond to nonself components. Cancer cells, originating from the host's own cells, create a challenge to our immune system. The components of a highly immunosuppressive microenvironment directly inactivate the host's immune system and modulate it to work in favor of a malignant tumor (Schreiber et al., 2011). It is important to identify which mechanisms are involved in evading immune destruction and how we can use this knowledge in cancer therapy. For personalized targeted cancer therapy, we need new biomarkers for the further subtyping of heterogenic tumors in order to identify which patients are likely to benefit from adjuvant treatment and to determine which treatment would be most effective for certain individuals.

Since TLRs are expressed in various immune cells in the tumor microenvironment, TLR agonist immunotherapy represents a promising field in modulating the immune response in an antitumorigenic direction. To date, the topical TLR agonist imiquimod has been approved for superficial basal cell carcinoma, actinic keratosis, and genital/perianal external wraths; BCG (TLR2/4 agonist) for treating bladder cancer; and *Salmonella Minnesota* LPS derivative MPL as an adjuvant in a prophylactic vaccine against human papilloma virus (Pahlavanneshan et al., 2021). Several preclinical and clinical phase I/II trials are ongoing in CRC as well. In addition to monotherapy, TLR agonists appear promising when used in combination with other immunotherapy agents, such as PD1 and CTLA-4 blockers or IL-2. Furthermore, radiotherapy and chemotherapy have been added to the list of combination therapies. Such treatment enables a synergistic effect by stimulating immune responses and reducting side effects (Pahlavanneshan et al., 2021; Schölch et al., 2015; Shi et al., 2020).

In conclusion, TLR2, TLR4, TLR5, and TLR7 may be useful as prognostic markers in colorectal cancer. Furthermore, we can speculate that possibly in the future assessing TLRs from preoperative primary tumor colonoscopy biopsies or surgical tissue specimens could prove beneficial to evaluating the efficiency of TLR immunotherapy for specific patients. It would be beneficial to study TLRs in CRC metastases as well to determine if the prognostic pattern is similar to that of the primary tumor. In addition, more studies which focus on assessing the association of circulating TLR with survival and the tissue TLR expressions might yield beneficial results.

7 CONCLUSIONS

According to the four studies summarized here and the combined statistical analyses in this thesis, we conclude as follows:

- Tissue TLR immunoexpressions may serve as a positive prognostic factor among CRC patients. Patients with high TLR2, TLR5, and TLR7 immunoexpressions in the tumor cells exhibit a better prognosis. A high TLR2 expression also indicates a better prognosis among stage III CRC patients.

- An elevated preoperative plasma CRP value indicates a worse prognosis among CRC patients. Combining plasma CRP and the immunoexpression of TLR2, TLR4, TLR5, and TLR7 in the tumor cells may serve as a tool to identify patients with better or worse outcomes. Among patients with a low CRP value, a high TLR2, TLR5, and TLR7 immunoexpression and a low TLR4 immunoexpression indicate a better prognosis.

- High densities of CD3-positive and CD8-positive T cells associate with high TLR2, TLR4, and TLR5 tumor cell immunoexpressions. A high CD3–CD8 tumor–stroma index indicates a better prognosis in all TLR subgroups, except in the negative TLR7 subgroup. Combining TLR2, TLR4, TLR5, and TLR7 tissue expressions and the densities of CD3-positive and CD8-positive T cells may be useful in assessing prognosis among CRC patients.

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ERRATA LIST

Page 13, Line 12
Word “huonompi” must be replaced with word “parempi”.
Correction: Korkean CRP:n alaryhmässä oli parempi ennuste niillä potilailla, joilla oli matala TLR4-ilmentymä.

Page 25, 2.6 Screening, Line 8
Zero is missing behind number 2.
Correction: With one time testing, the sensitivity of FIT for detecting cancer is around 80% and 20–30% for detecting advanced dysplastic lesions.

Page 29, Figure 5
Correction: Bowel wall layer names “muscularis mucosa” and “muscularis propria” should be located the other way round.

Page 30, Table 1
Tis is missing from the first column resulting in wrong definitions for T1 and T2.
Correction:
Tis – Carcinoma in situ, invasion of lamina propria
T1 – Tumor invades submucosa
T2 – Tumor invades muscularis propria