Predictive and prognostic markers in localized colon cancer

with emphasis on the biomarkers maspin, mismatch repair deficiency, CDX2, tumor grade, PD-L1, and tumor-infiltrating lymphocytes.

Kjersti Elvestad Hestetun

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

The research presented in this thesis was performed while the candidate was employed by the Department of Clinical Science, Faculty of Medicine, University of Bergen and the Department of Oncology and Medical Physics, Haukeland University Hospital, Bergen. It has been carried out by the research group headed by Professor Olav Dahl. Mette Pernille Myklebust has been main supervisor. Professor Olav Dahl and Professor Halfdan Sørbye have been co-supervisors. Financial support, including the Ph.D. grant, was received from the University of Bergen. Additional financial support was received from The Norwegian Cancer Society and Health Region West. The laboratory work was performed at Mohn Cancer Research Laboratory.

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Abstract

Colon cancer is one of the most common cancers worldwide. Predicting disease outcomes is challenging. Around 15-20% of patients will experience disease relapse. At the same time, a substantial fraction of patients will receive unnecessary treatment with chemotherapy after primary surgery with the risk of developing long-term adverse effects. Therefore, there is an urgent need for specialized biomarkers to improve patient survival and avoid over-treatment.

We aimed to examine biomarkers with potential prognostic and/or predictive value in colon cancer. This thesis covers the investigation of the biomarkers maspin (SERPINB5), caudal type homeobox 2 (CDX2), tumor grade, mismatch repair (MMR) deficiency, programmed death-ligand 1 (PD-L1), and tumor-infiltrating lymphocytes.

Two different cohorts of patients were included in this study. The NGICG cohort consists of patients with colon or rectal cancer stage II and III randomized to receive fluorouracil after surgery or to surgery alone. The HDH cohort consists of patients with colon cancer stage I-III treated according to existing guidelines. Detailed clinicopathological data and follow-up data were available from both cohorts. Immunohistochemistry was performed for maspin, CDX2, CD3, CD8, PD-L1, MLH1, MSH2, MSH6, and PMS2. Information about tumor grade was retrieved from the initial pathology assessment.

In ***paper I***, we show that a low expression of nuclear maspin predicted the effect of adjuvant fluorouracil/levamisole in the randomized cohort. Neither nuclear nor cytoplasmic expression of maspin was associated with prognosis. Nuclear maspin expression was not associated with any clinicopathological variables.

Paper II demonstrates the association between low CDX2 expression and deficient mismatch repair, high tumor grade, and right-sided primary in stage II-III colon cancer. Patients with low CDX2 expression combined with proficient MMR (pMMR) had a very poor prognosis. Cases with pMMR and high tumor grade had a poor prognosis when treated with surgery only. High tumor grade did not convey a poor prognosis for

pMMR patients randomized to receive adjuvant chemotherapy, indicating that these patients respond well to adjuvant fluorouracil-based chemotherapy.

In ***paper III***, deficient MMR (dMMR) was associated with a poor prognosis in stage III colon cancer, compared to pMMR cases. dMMR stage II patients had an improved prognosis, suggesting the presence of a prognostic shift in localized dMMR colon cancer. Our multivariate models demonstrated a significant statistical interaction between MMR phenotype and stage. Density of tumor-infiltrating lymphocytes was an independent prognostic marker with higher density associated with improved prognosis. PD-L1 expression was not associated with prognosis. The prognostic shift demonstrated in the multivariate models was significant also when adjusted for the influence of PD-L1 expression, CDX2 expression, chemotherapy, and TIL density.

In conclusion, a low expression of maspin might predict the effect of adjuvant chemotherapy. Still, there is no consensus in the present literature regarding the predictive and prognostic value of maspin. Currently, the maspin biomarker is not ready for clinical implementation. According to our findings, the combination of pMMR and low CDX2 expression identifies a group of stage II and III patients with a high risk of recurrence. We believe that CDX2 can become an important marker in the treatment stratification of stage II and III pMMR colon cancer. High tumor grade is acknowledged as a *minor* risk factor in stage II colon cancer, and, standing alone, it does not warrant treatment with adjuvant chemotherapy. As pMMR patients with high tumor grade seem to respond well to chemotherapy, the benefit of adjuvant chemotherapy for this group should be re-assessed. Our study demonstrates a poor prognosis for dMMR stage III colon cancer patients. Few studies assess the prognosis of dMMR stage III colon cancers separately, and our results call for validation in larger cohorts. If confirmed, these results may impact the clinical recommendations for dMMR stage III colon cancer as this tumor phenotype responds well to immunotherapy.

List of Publications

The thesis is based on the following papers, referred to by their roman numerals in the text:

I. **Hestetun KE**, Brydøy M, Myklebust MP, Dahl O. Nuclear maspin expression as a predictive marker for fluorouracil treatment response in colon cancer. *Acta Oncol.* 2015;54:470-9.

II. **Hestetun KE**, Aasebø K, Rosenlund NB, Müller Y, Dahl O, Myklebust MP. Mismatch repair phenotype determines the implications of tumor grade and CDX2 expression in stage II-III colon cancer. *Mod. Pathol.* 2021;34:161-70.

III. **Hestetun KE,** Rosenlund NB, Stanisavljević L, Dahl O, Myklebust MP. Stage-dependent prognostic shift in mismatch repair-deficient tumors: assessing patient outcomes in stage II and III colon cancer. Manuscript.

Abbreviations

5FU	Fluorouracil
5FU/Lev	Fluorouracil/Levamisole
AKT	AKT serine/threonine kinase
APC	Adenomatous Polyposis Coli
ASCO	American Society of Clinical Oncology
BAX	BCL2 Associated X
BRAF	B-Raf Proto-oncogene
CASP5	Caspase 5
CDX2	Caudal Type Homeobox 2
CHEK1	Checkpoint Kinase 1
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CME	Complete Mesocolic Excision
CMS	Consensus Molecular Subtypes
COX-2	Cyclooxygenase-2
CRC	Colorectal Cancer
CSS	Cancer-specific Survival
CT	Computed Tomography
ctDNA	Circulating Tumor DNA
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CyTOF	Cytometry by Time of Flight
DFS	Disease-free Survival
dMMR	Deficient Mismatch Repair
DNA	Deoxyribonucleic Acid
DPD	Dihydropyrimidine Dehydrogenase
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
ERK	Extracellular Signal-Related Kinases
ESMO	European Society for Medical Oncology
FFPE	Formalin-Fixed Paraffin-Embedded
H&E	Hematoxylin and Eosin
HDH	Haraldsplass Deaconal Hospital
HR	Hazard Ratio
ICI	Immune Checkpoint Inhibitor
IGF2R	Insulin-like Growth Factor 2 Receptor
IHC	Immunohistochemistry
KRAS	KRAS Proto-oncogen
MAPK	Mitogen-activated Protein (MAP) Kinase
Maspin	Serpin B5
mCRC	Metastatic Colorectal Cancer

MLH-1	MutL Homolog 1
MMR	Mismatch Repair
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MSI	Microsatellite Instability
MSI-H	Microsatellite Instability- High
MSS	Microsatellite Stable
mTOR	Mechanistic Target of Rapamycin Kinase
NGICG	Norwegian Gastrointestinal Cancer Group
NordiQC	The Nordic Immunohistochemical Quality Control
NOS	Not Otherwise Specified
NRAS	Neuroblastoma-RAS
OS	Overall Survival
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1 Receptor
PD-L1	Programmed Death-Ligand 1
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
pMMR	Proficient Mismatch Repair
PMS2	PMS1 Homolog 2
POLD1	DNA Polymerase Delta 1
POLE	DNA Polymerase Epsilon
PRAS40	Proline-Rich AKT Substrate of 40 kDa
PTEN	Phosphatase and Tensin homolog
RAS	Ras Oncogene
REMARK	Reporting Recommendation for Tumor Marker Prognostic Studies
RS	Relative Survival
S6K	S6 kinase
SEER	Surveillance, Epidemiology, and End Results
SMAD2	SMAD Family Member 2
SMAD4	SMAD Family Member 4
TCR	T Cell Receptor
TGFβR2	Transforming Growth Factor Beta Receptor 2
TILs	Tumor-infiltrating Lymphocytes
TMA	Tissue Microarrays
TMB	Tumor Mutational Burden
TNM	Tumor Node Metastasis
TP53	Tumor Protein p53
TPS	Tumor Proportion Score
UICC	Union for International Cancer Control
UL	Ultrasound
VEGF	Vascular Endothelial Growth Factor
WISP3	WNT1 Inducible Signaling Pathway Protein 3

Contents

1. Introduction

This thesis focuses on biomarkers in colon cancer stage II and III. Rectal cancer will therefore not be covered in detail. Still, as these entities share the same epidemiological traits and risk factors, they will be covered together in several chapters.

1.1 Epidemiology

Colorectal cancer (CRC) is a major health issue and the second most prevalent cancer worldwide (1). It is the second most commonly diagnosed cancer in women (after breast cancer) and the third most commonly diagnosed in men (after prostate cancer and lung cancer). In Norway, colon cancer median age at diagnosis is 73 years for men and 75 years for women (2). The incidence of colon cancer in Norway has increased since the 1950s but has remained stable for the last six years (Figure 1). Still, Norway has one of the highest colon cancer incidence rates globally (Figure 2) (2, 3).

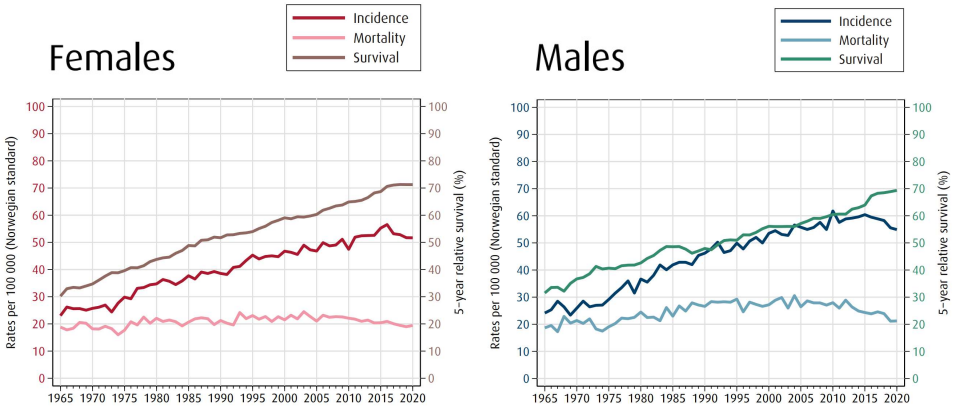


Figure 1. Incidence, mortality, and survival of colon cancer in Norway 1965-2020 (3). Reproduced with permission from the Cancer Registry of Norway.

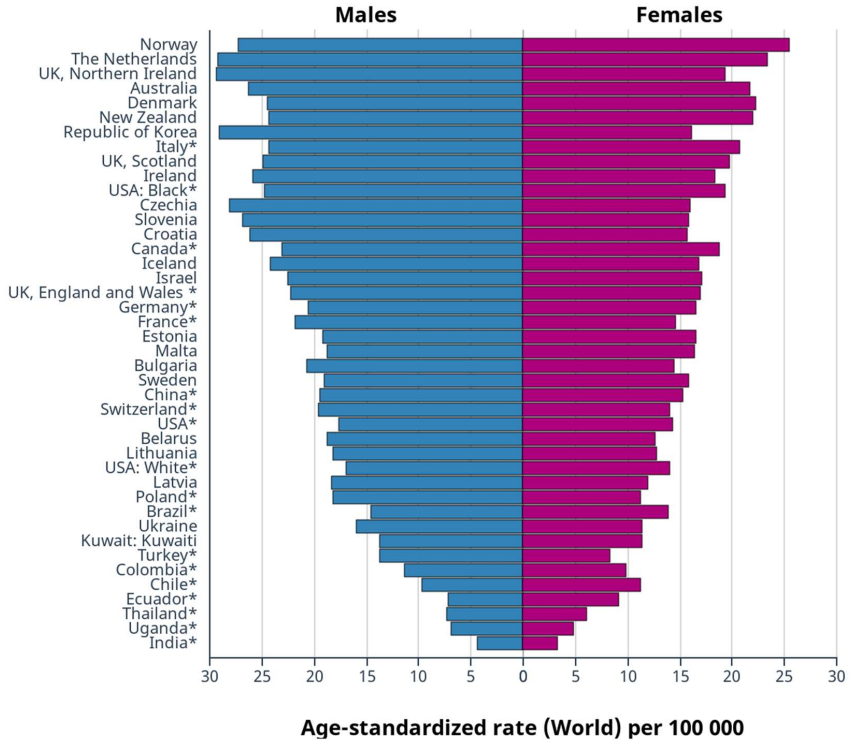


*Figure 2. Colon cancer, incidence, 2012. *Subnational data. Adapted with permission from the International Agency for Research on Cancer (WHO) (1, 4).*

1.2 Risk factors

Strong evidence exists connecting consumption of red meat, processed meat, alcoholic drinks (>2 units/day), being overweight/obese, and being tall to increased risk of colon cancer (5). Cigarette smoking also increases the risk of CRC (6). The associations between lifestyle and CRC development are reflected in the global incidence patterns, with higher rates in high-income countries (7). Economic growth and industrialization of former low-income countries tend to be followed by increasing incidence of colorectal cancer, probably due to the adaptation of lifestyle factors associated with both wealth and colorectal cancer. Inflammatory bowel disease is an established risk factor for CRC (8). Diabetes type 2 seems to increase the risk of CRC per se, also when correcting for lifestyle factors associated with both diseases.

The link between the composition of gut microbiota and cancer has gained increased attention during the past decade. However, separating between changes in microbiota causing cancer and changes occurring as a consequence of cancer is challenging. High levels of *Fusobacterium nucleatum* and/or *Bacteroides fragilis* are strongly associated with colorectal cancer (9).

A positive family history of colorectal cancer is described in 10-30% of patients diagnosed with CRC (7, 8, 10), but established high-risk hereditary cancer syndromes are present in only 5% of CRC patients (10). The most common hereditary cancer syndromes in CRC are Lynch syndrome (further described in section 1.11.3) and familial adenomatous polyposis. The most frequent cause of familial adenomatous polyposis is an inherited defect in the adenomatous polyposis coli (APC) gene (11).

1.3 Prevention

There is strong evidence linking consumption of whole grains, foods with dietary fiber, dairy products or/and calcium supplements with reduced risk of developing colorectal cancer (5). The use of aspirin is associated with a reduced risk of CRC, but the advantage of usage must be weighed against the increased risk of gastrointestinal bleeding (12, 13). Both endogenously produced estrogen and postmenopausal hormone replacement therapy protect against CRC development in women (14).

1.4 Symptoms and presentation

The most common symptoms associated with CRC are often subtle (15). They are not specific for colorectal cancer and depend on the localization of the tumor. Occult bleeding and anemia are most common in proximal tumors (16). Tumors in the rectum and sigmoid may present with fresh, visible bleeding. Symptoms of obstruction (changes in bowel habits, abdominal pain) are more common in distal tumors. Other symptoms include weight loss and fatigue. Hospital emergency admittance for ileus, major bleeding, or colonic perforation is the first presentation of colorectal cancer in 15-25%

of patients (16). In some patients, symptoms and findings related to metastatic disease will lead to the diagnosis (15).

1.5 Screening and diagnosis

Colonoscopy with biopsy is the gold standard for diagnosing colorectal cancer and also allows for polyp removal (10, 15). Colonoscopy can be utilized as a singular screening- or diagnostic procedure or to follow up occult blood detected in stool tests or pathological findings from flexible sigmoidoscopy.

Screening for colorectal cancer can reduce mortality both by detecting non-symptomatic cancer at an early stage and by discovering premalignant lesions (10). Screening in individuals with a risk of developing hereditary CRC and patients with inflammatory bowel disease involves regular colonoscopies (11, 17). Screening methods and -implementation for average-risk adults vary to a great extent across Europe (18). In many countries with long-standing nationwide screening, incidence rates have decreased between 2000-2016. In many countries without screening programs (including Norway), incidences have risen during this period. Implemented screening methods include combinations of stool tests for occult blood (Fecal immunohistochemical test or Guaiac-based Fecal Occult Blood test), flexible sigmoidoscopy, and colonoscopy (10). In Norway, screening of average-risk individuals from the age of 55 will be gradually implemented towards the year 2024 (19, 20).

1.6 Statistical measures of Cancer survival

Different survival measures are used to describe the prognosis of colorectal cancer. **Overall Survival (OS)**: Time from a defined start point (often the time of surgery) until death of any cause (21). **Overall Survival rate** measures the percentage of people still alive after a defined period. **Cancer-Specific Survival (CSS):** Time from a defined start point until death of the predefined cancer type or treatment complications. Other deaths are censored. **Cancer-specific survival rate**: The percentage of people who have not died from the predefined cancer or treatment complications after a defined period.

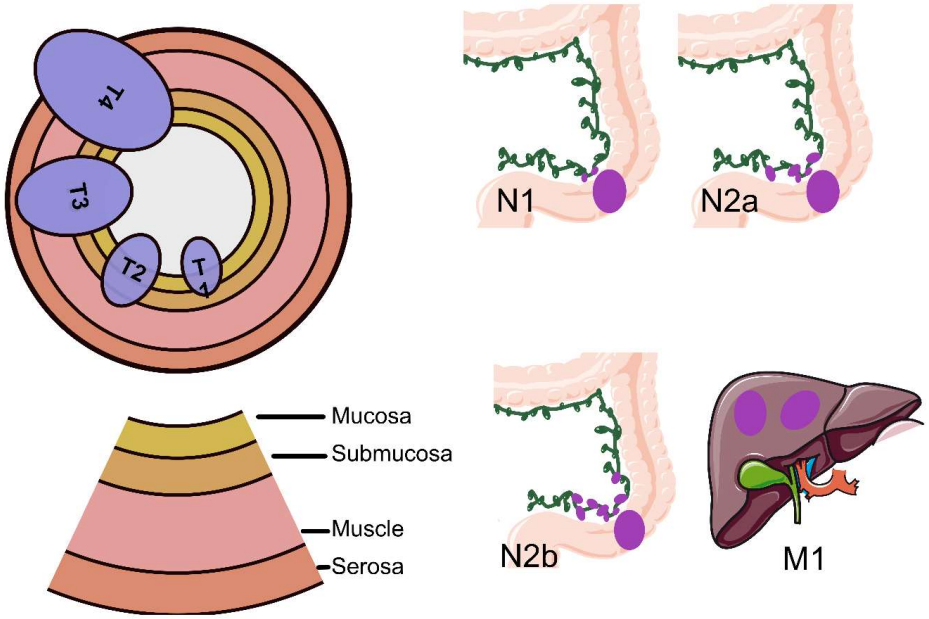
Relative survival (RS) rate: Relative survival rates compare the observed survival of people with cancer with the expected survival of comparable individuals in the whole population (22). Commonly used in large epidemiological studies, it determines the chance of surviving cancer without needing extensive follow-up data that include cause of death. **Disease-free Survival (DFS):** Time from a defined start point until relapse (local or advanced) of the predefined cancer type (21). **Disease-free survival rate**: The percentage of people who have not experienced relapse from the predefined cancer type after a defined period.

1.7 Staging

The current recommended staging for colorectal cancer follows the TNM 8 Classification of Malignant Tumours by the Union for International Cancer Control (UICC) (Table 1, Figure 3) (23).

Table 1. TNM Clinical Classification, Colon and Rectum. Adapted from UICC, TNM classification of malignant tumors, eighth edition (23).

T – Primary tumor	N – Regional lymph nodes	M – Metastases
TX Primary tumor cannot be assessed **T0** No evidence of primary tumor **Tis** Carcinoma in situ: invasion of lamina propria **T1** Tumor invades submucosa **T2** Tumor invades muscularis propria **T3** Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues. **T4** Tumor directly invades other organs or structures and/or perforates visceral peritoneum **T4a** Tumor perforates visceral peritoneum **T4b** Tumor directly invades other organs or structures	**NX** Regional lymph nodes cannot be assessed **N0** No regional lymph node metastasis **N1** Metastasis in 1 to 3 regional lymph nodes **N1a** Metastasis in 1 regional lymph node **N1b** Metastasis in 2 to 3 regional lymph nodes **N1c** Tumor deposit(s), i.e., satellites in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue without regional lymph node metastasis **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	**M0** No distant metastasis **M1** Distant metastasis **M1a** Metastasis confined to one organ (liver, lung, ovary, non-regional lymph node(s)) without peritoneal metastases **M1b** Metastasis in more than one organ



Stage I	Stage II			Stage III			Stage IV
	IIA	***IIB***	***IIC***	***IIIA***	***IIIB***	***IIIC***	
T1/T2	T3	T4a	T4b	T1/T2 + N1 T1 + N2a	T1/T2 + N2b T2/T3 + N2a T3/T4a + N1	T3/T4a + N2b T4a + N2a T4b + N1/N2	Any T Any M
N0, M0	N0, M0	N0, M0	N0, M0	M0	M0	M0	M1

Figure 3. TNM staging. Adapted from UICC, TNM classification of malignant tumours, eighth edition (23). The figure includes components from smart.servier.com (CCBY3.0).

1.8 Prognosis

Stage I-II

Stage I colon cancer has an excellent prognosis with a five-year DFS of ~95% after surgical resection alone (24). The 2020 report from the cancer registry of Norway does not separate between stage I and stage II; five-year RS for localized (stage I + stage II)

colon cancer was 97.1-98.2% (3). In the SEER database, five-year RS for stage I + stage II colon cancer was 90.9% (years 2011-2017) (25).

The prognosis of stage II colon cancer is heterogeneous. For stage II in general, reported OS rates are 86.8-90.0% (26-28) and 70.0-84.2% for DFS (26-30). Stage IIB and stage IIC colon cancer represent a minority of stage II tumors (31), and few studies are available for prognostic assessment. Present guidelines recommend adjuvant chemotherapy for stage IIB and IIC (15, 32), but as these recommendations have been implemented in recent years, differences in the use of chemotherapy might influence the reported survival for this group. Still, it is worth noticing that the prognosis of the most advanced stage II colon tumors (T4N0) is worse than for the least advanced stage III tumors (T1-2N1) (31).

Stage III

As adjuvant chemotherapy is recommended treatment for stage III colon cancer patients, current survival data include patients who have received chemotherapy. Data for survival after surgery only are therefore from older studies and may not reflect the results after the surgical treatment of today. For patients treated with oxaliplatin-based chemotherapy, studies report OS 72.9%-77.6 % (26, 28, 33) and DFS 64.4%-73.3% (26, 28, 30, 33). For patients with N1 disease, 83.3% OS and 71.4% DFS (26) is registered and 64.5% OS and 52.3% DFS for N2 disease (26). In the recent IDEA study, 3-year DFS after receiving chemotherapy was 83% for T1-3N1 (defined as low-risk stage III cancer) versus 62.7-64.4% for T4N2 (34). The SEER database's RS for stage III colon cancer is 71.9% (2011-2017) (25). In Norway, registered survival for stage III colon cancer has improved during the past decade. For 2011-15, five-year RS was 82.4% and for 2016-2020: 84.9% (estimated) (3). In comparison, five-year RS was 77.6% in 2006-2010 and 71.6% in 2001-2005.

Stage IV

When first diagnosed with colorectal cancer, 20-30% of patients have metastatic disease (synchronous metastases) (15, 25, 35). Relative five-year survival for stage IV disease is 13.9% for colon cancer in the SEER database (25) and 14.1-16.3% for colon cancer

in Norway (3). Median overall survival for patients with metastatic colorectal is for the general population of mCRC patients 12-14 months, whereas for patients selected for clinical trial enrollment, it varies between 21-30 months (36-39). Clinical trial patients are selected patients with younger age and without comorbidity, whereas a substantial number of elderly patients or patients with poor performance status are given the best supportive care alone without palliative chemotherapy.

1.9 Treatment

1.9.1 Localized colon cancer

Surgery

The main treatment for localized colon cancer is resection. Some T1 cancers may be removed by endoscopic resection (8, 15). Surgery is often performed laparoscopically and involves resection of the affected colonic segment with margins on either side of the tumor (15, 16). The extent of resection in the transcolonic plane depends on tumor localization and the anatomy of the local blood vessels. In the mesocolic plane, complete mesocolic excision (CME) is the standard technique in Europe (40). This involves separating the tumor with the visceral fascia from the retroperitoneal fascia *en bloc, i.e.*, in one piece without breaching the visceral fascia. The goal is to achieve an R0 dissection — a resection with a negative microscopic margin. This procedure permits central vascular ligation for a maximal harvest of regional lymph nodes. Resecting less than 12 lymph nodes is an important adverse risk factor in colon cancer (15).

Chemotherapy

Adjuvant chemotherapy is administered to eradicate potential microscopic residual disease after surgery. The 1990 Moertel trial showed that adjuvant fluorouracil (5FU)/levamisole improved outcome for stage III colon cancer patients compared to surgery alone (41), and this finding was later confirmed in several other studies (29, 42, 43). Patients with stage II colon cancer (42, 44) or rectal cancer (29, 42) had minor or no benefit. No effect was demonstrated for levamisole alone (41). Conversely, 5FU+folinate was proven more effective than 5FU alone, and levamisole was later replaced by calciumfolinate (45). Fluoropyrimidines still represent the main components

of adjuvant chemotherapy for colon cancer. Adjuvant treatment with 5FU/folinate increases DFS by 4-20% and OS by 7-8% (45). As the enzyme dihydropyrimidine dehydrogenase (DPD) is essential in the metabolism of fluoropyrimidines, testing for DPD-insufficiency should be performed before initiating treatment (15).

The Mosaic trial assessed the effect of adding oxaliplatin to fluoropyrimidines (28). This increased five-year DFS from 67.4% to 73.3% and six-year OS from 76% to 78.5% compared to 5FU/calciumfolinate alone in stage III. Similar DFS improvements were also reported in the NSABP C-07 and XELOXA trials (30, 33). The CAPOX/FOLFOX regimens are used in today's adjuvant treatment. The CAPOX regimen consists of the oral 5FU prodrug capecitabine, combined with intravenous oxaliplatin administered every 3rd week. The FOLFOX regimen contains 5FU/calciumfolinate and oxaliplatin administered intravenously every 2nd week.

Current adjuvant chemotherapy stage III

The 2018 IDEA study was a pooled analysis of six randomized phase 3 studies designed to assess whether three months of adjuvant FOLFOX or CAPOX was non-inferior to current six-month regimes (34). Although non-inferiority was not reached in the overall study population, the difference in DFS was small (three-year DFS: 74.6% in the three-month group and 75.5% in the six months-group). Subgroup analyses showed non-inferiority for three months of CAPOX for T1N1, T2N1, and T3N1 disease, now defined as low-risk stage III patients. For patients with T4 and/or N2 disease, now defined as high-risk stage III patients, six months treatment was superior to three months treatment. The variation between FOLFOX and CAPOX in this study was unexpected, and the reason is unknown as the regimens were not randomized (46). The difference in frequency and dosing of oxaliplatin might have contributed to these findings.

Notably, the "high-risk" and "low-risk" stage III groups defined from the IDEA study do not comply with the TNM 8 staging. Patients defined as "high-risk" will be represented in both stage IIIA, IIIB, and IIIC disease and "low-risk" patients in IIIA and IIIB (Figure 2, (23)).

The Norwegian 2021, ASCO 2019, and ESMO 2020 guidelines for adjuvant chemotherapy in stage III colon cancer are summarized in Figure 3. The ESMO guidelines recommend a six-month duration of treatment if FOLFOX is used, but the ASCO and the Norwegian guidelines present three months of adjuvant FOLFOX as an alternative (15, 16, 47). The ASCO guidelines emphasize a shared decision-making approach for the duration of oxaliplatin-based adjuvant chemotherapy for stage III colon cancer in general (47).

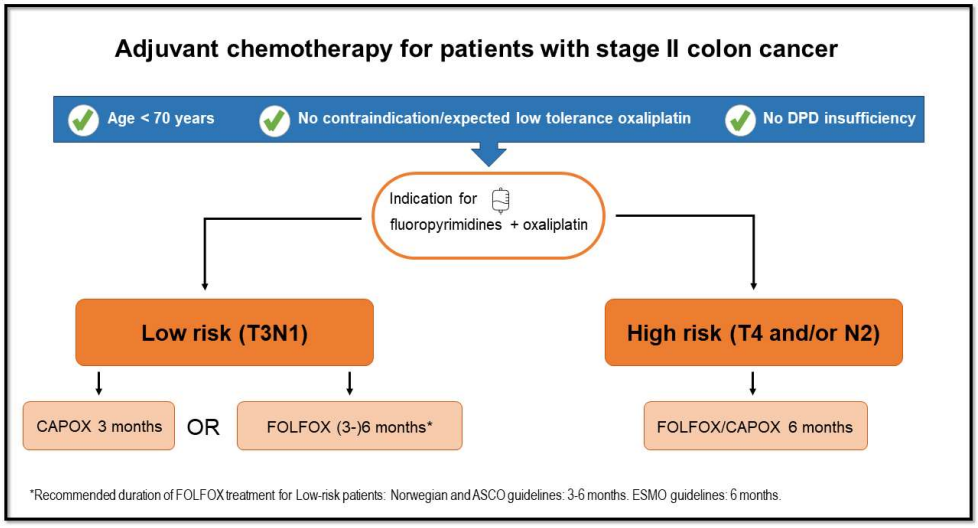


Figure 4. Adjuvant chemotherapy stage III colon cancer. Adapted from Norwegian-, ESMO- and ASCO guidelines (15, 16, 47).

Current adjuvant chemotherapy stage II

For stage II patients, many treatment recommendations are based on indirect evidence from trials performed for or including stage III patients, and the evidence to guide adjuvant treatment in stage II is weaker than for stage III patients (48). This might explain the variation in recommendations in the ESMO 2020, ASCO 2021, and Norwegian 2021 treatment guidelines (15, 16, 32). Common for all guidelines is that adjuvant chemotherapy is not recommended routinely for stage II colon cancer. In addition, patients with microsatellite instability-high (MSI-H) tumors have limited benefit from monotherapy with fluoropyrimidines and should be offered doublet chemotherapy with oxaliplatin if adjuvant therapy is advised. Having a T4 tumor (stage

IIB or stage IIC disease), tumor perforation, and/or less than 12 lymph nodes sampled are considered as major risk factors for disease recurrence. High tumor grade, lymphatic, perineural, or vascular invasion, high preoperative CEA levels, and cancer presenting with obstruction are considered minor risk factors, but the ASCO update emphasizes that high tumor grade is a risk factor in microsatellite stable (MSS) tumors only (15). ESMO guidelines recommendations are summarized in Figure 4.

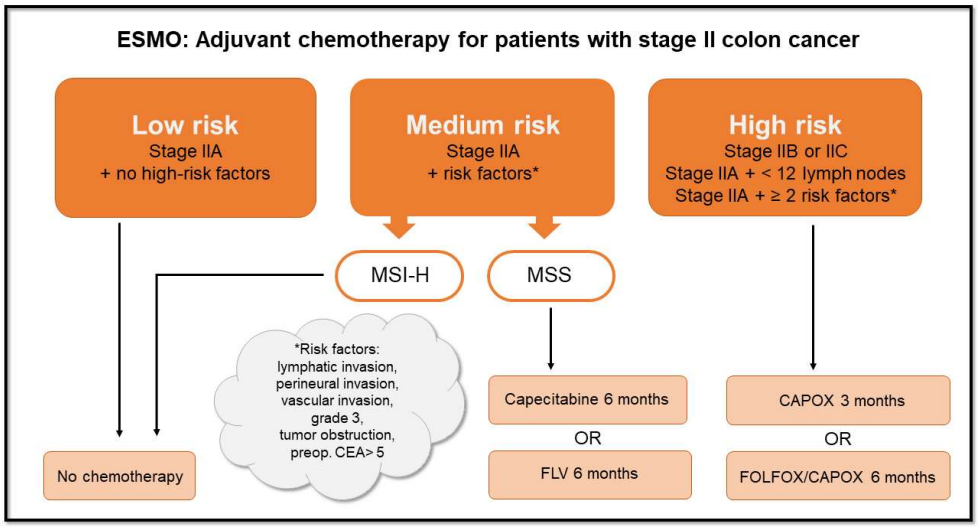


Figure 5. ESMO recommendations for adjuvant chemotherapy for stage II colon cancer. Adapted from (15).

Elderly patients

Clinical trials often have stringent inclusion criteria that prevent elderly patients from entering, causing treatment advice for this group to be based on analyses from a limited number of patients (48). There seems to be underuse of adjuvant therapy in the elderly population of CRC patients (49, 50). In Norway, patients over 70 years of age are recommended to receive monotherapy with fluoropyrimidines if adjuvant therapy is indicated (51, 52). Individual assessment is recommended for patients over the age of 75.

1.9.2 Metastatic colorectal cancer.

Patients with metastatic colorectal cancer are not included in this thesis, and this topic is therefore not covered in detail here. In brief, both local and systemic treatment options are available for metastatic (stage IV) colorectal cancer. **Local treatment** involves surgery of metastases, local ablation of lung- or liver metastases, palliative surgery of primary lesion, and palliative radiation treatment, including stereotactic radiation (53). **Systemic treatment** involves palliative chemotherapy, neoadjuvant therapy for down-sizing metastases before surgery, and palliative targeted therapy. The liver is the most common metastatic site. Liver resection is an option for ~20-30% of patients with liver metastasis with a 30-40% five-year OS but only 20% DFS five years post-surgery (54, 55). Liver transplant studies have shown promising results (56, 57). Cytoreductive surgery followed by hyperthermic intraperitoneal chemotherapy is a treatment option for localized peritoneal carcinomatosis with a five-year DFS of 20-25% (58). Surgical resection of lung metastases may increase survival in selected patients (59). Palliative systemic treatment mainly includes combinations of fluoropyrimidines, oxaliplatin, and irinotecan, with or without the addition of bevacizumab (anti-vascular endothelial growth factor (VEGF)) or, for patients with *RAS* wildtype cancer, anti-epidermal growth factor receptor (EGFR) (panitumumab or cetuximab) (16, 35). Regorafenib (multi-kinase inhibitor) and TAS-102 (a combination of a thymidine analog and a thymidine phosphorylase inhibitor) are also available agents but seldom used due to limited survival benefit and regorafenib toxicity profile. Encorafenib (small molecule BRAF inhibitor) plus cetuximab is a new treatment option for patients with *BRAF*V600E-mutated tumors (60). Immunotherapy with checkpoint inhibitors pembrolizumab or nivolumab, alone or combined with ipilimumab, is the recommended first-line therapy for patients with microsatellite instability-high (MSI-H) mCRC (53).

1.10 Localized colon cancer – current challenges

Current treatment with adjuvant therapy leads to overtreatment of colon cancer stage III at the cost of serious adverse effects in a large proportion of patients. Adjuvant FOLFOX increases survival by 12% and is recommended to all eligible stage III colon cancer

patients (34, 61), although >50% is cured by surgery alone (46). Administration of oxaliplatin can lead to long-term adverse effects. For patients in the IDEA study, oxaliplatin-related sensory neuropathy was registered in ~45% of patients receiving six months of CAPOX/FOLFOX (34). A neoadjuvant chemotherapy strategy might reduce overtreating by reducing the total chemotherapy dose for some patients. This is the principle behind the Nordic Neocol study (NCT01918527) and the FOxTROT study (NCT00647530) (62, 63). Administering limited cycles of chemotherapy before surgery allows for examining the surgical specimen before deciding if postoperative chemotherapy is necessary.

At the same time, 15-20% of CRC patients treated with adjuvant chemotherapy will eventually develop metastatic disease (2, 16). To optimize the adjuvant treatment for CRC, specific biomarkers are highly warranted. The identification of clinically relevant biomarkers involves studying the molecular pathways of colorectal cancer development, the histopathology of CRC, and the role of the tumor microenvironment

1.11 Molecular pathways and pathogenesis in colon cancer

The development of colorectal cancer is a multistep process, transforming normal colon mucosa to premalignant precursor lesions and, ultimately, invasive cancer. This evolution is driven by changes in the tumor cell and interactions between the tumor and the tumor microenvironment. These changes enable important biological abilities of cancerous tumors described as the Hallmarks of Cancer (64), Figure 6.

Cancerous tumors have a higher number of mutations than other cells, and they continue to evolve and accumulate more mutations throughout cancer progression (65). Baseline spontaneous cell mutation rates are not sufficient to form all the mutations observed in cancerous cells. Cancers must have a fundamental genomic instability, described as a mutator phenotype to gain their increased mutation rate. At least three molecular pathways forming genomic instability have been identified in colorectal cancer — the Chromosomal instability (CIN), CpG Island Methylator Phenotype (CIMP), and

microsatellite instability (MSI) pathways. These pathways are not mutually exclusive (66).

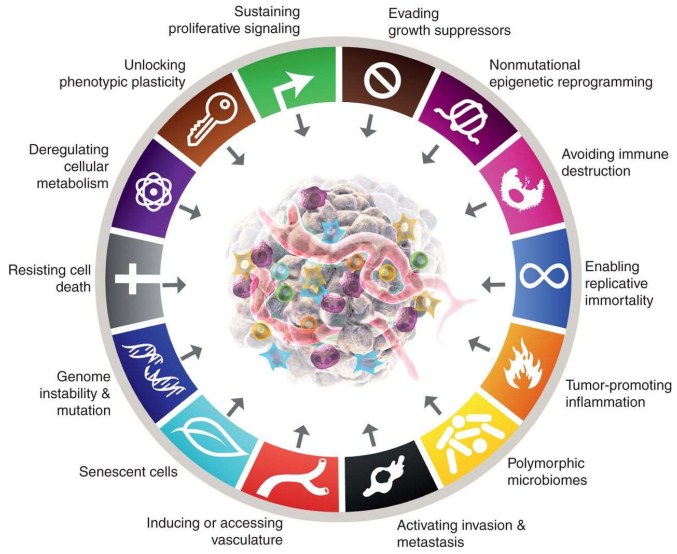


Figure 6. The Hallmarks of Cancer 2022 (64). Reprinted with permission from AACR journals.

1.11.1 Chromosomal instability (CIN)

Around 65-85% of CRCs develop through the chromosomal instability (CIN)-pathway described by Fearon and Vogelstein in 1990 (67-69). The characteristic karyotypic pattern in tumors developing through this pathway is gains or losses of whole chromosomes or large parts of chromosomes, causing cell-to-cell differences in chromosome number (aneuploidy). This results in a high frequency of loss of heterozygosity and sub-chromosomal genomic amplifications. The karyotypic abnormalities are accompanied by mutations in several specific tumor suppressor genes and oncogenes (66). The classical adenoma-carcinoma sequence for colorectal cancer development is highly correlated with the CIN pathway (Figure 7).

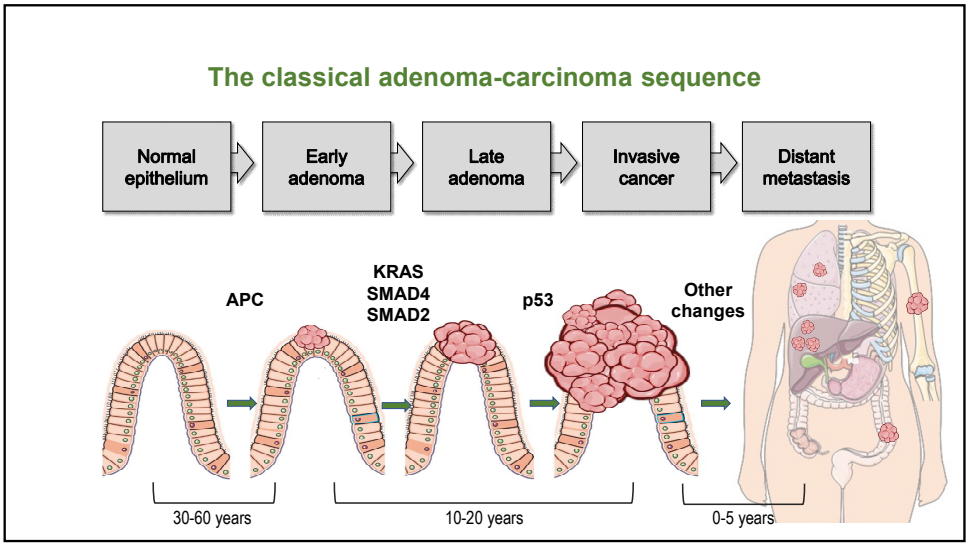


Figure 7. The classical adenoma-carcinoma sequence (7, 69, 70). The figure includes components from smart.servier.com (CCBY3.0).

Colorectal cancer is thought to be initiated in cells with stem cell capabilities residing at the base of colonic crypts (8, 70). The first cancer-initiating step in CIN is the mutation of the APC-gene. Structural changes in the colonic mucosa are gradual. An aberrant crypt focus evolves into a polyp and after that, an adenoma. Adenomas are polyps with dysplastic features that form tumors not invading the submucosa. These neoplastic precursor lesions may evolve to invasive cancer. Mutations in *KRAS*, *TP53*, *SMAD2,* and *SMAD4*, overexpression of COX-2, and 18q allelic loss are common in the CIN pathway (66). The most relevant pathways in CIN are the MAPK pathway and the Wnt pathway (71). In **invasive colorectal cancer**, cancerous cells have protruded through the basement membrane, through the muscularis mucosa, and into the submucosa (23). In sporadic cancers, the evolution through these phases spans several decades. However, many hereditary cancer syndromes are characterized by accelerated carcinogenesis (72).

1.11.2 CpG Island Methylator Phenotype (CIMP)

Epigenetic alterations modify gene expression without changing the DNA sequence. The main forms of epigenetic alterations are DNA methylation and histone modifications. In addition, non-coding RNAs such as long non-coding RNAs and microRNAs can act as epigenetic regulators (68). Changes in DNA methylation patterns

include DNA hypo- and hypermethylation. DNA hypermethylation mainly occurs at CpG islands.

CpG islands are regions in the DNA where there is a high frequency of CpG sites that are not methylated. CpG islands are common in gene promoters of tumor suppressor genes. The CpG island methylator phenotype **(CIMP)** involves simultaneous hypermethylation of CpG islands. In CRC, this causes reduced transcription and therefore reduced activity of several involved tumor suppressor genes (68).

The CIMP pathway is responsible for 10-15% of CRCs and strongly associated with development through the serrated pathway (7). Here, normal epithelial cells progress to a hyperplastic polyp, then a sessile serrated adenoma before becoming invasive CRC. The gene promotor of the mismatch repair (MMR) protein MutL homolog 1 (*MLH1*) is frequently methylated in CIMP (73). CIMP is also strongly associated with mutations of the B-Raf proto-oncogene (*BRAF*) in colorectal cancer (74).

1.11.3 Mismatch repair and microsatellite instability

The microsatellite instability (MSI)-pathway is another recognized molecular pathway in CRC. MSI is a symptom of defective mismatch repair (75). Mismatch repair deficiency is central in ***paper II*** and ***paper III*** and therefore a topic of increased attention in this thesis.

The mismatch repair (MMR) system repairs errors in the DNA replication system (in addition to the DNA proofreading system) (75). The main proteins in the MMR system are **MLH1**, MutS protein homolog 2 (**MSH2**), MutS homolog 6 (**MSH6**), and PMS1 homolog 2 (**PMS2**). The MMR proteins form the **MutS heterodimers** (MSH2/MSH6 and MSH2/MutS protein homologue 3 (MSH3)) and the **MutL heterodimers** (MLH1/PMS2, MLH1/PMS homologue 1 (PMS1) and MLH1/MutL homologue 3 (MLH3)). The MutS heterodimers recognize indels and mispaired nucleotides and start repairing (76). They recruit the MutL heterodimers to catalyze the excision of the faulty strand and for resynthesizing the correct sequence.

A microsatellite is a DNA sequence in which a DNA motif (of one to six base pairs) is repeated many times (75). Microsatellites are present throughout the whole genome and are prone to mutations during replication due to DNA polymerase slippage. Defective MMR will cause altered length of microsatellite regions, a phenomenon known as microsatellite instability. When the MMR system is defective, it will leave genes with repeating base sequences vulnerable to mutations. Several tumor suppressor genes have such sequences, including genes regulating cell proliferation (*TGFβR2*, *WISP3*, *IGFI2R*), cell cycle or apoptosis (*BAX*, *CASP5*, *PTEN*), and DNA repair (*CHEK1*) (77).

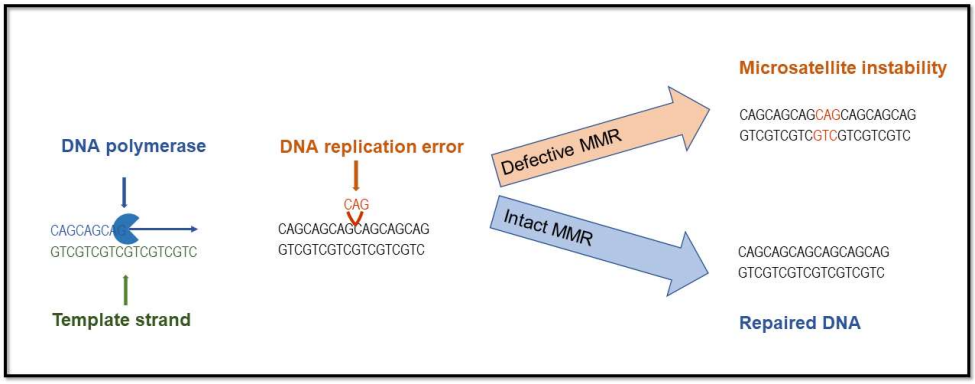


Figure 8: Mechanism of microsatellite instability. Adapted from (78).

Terminology in this thesis

A microsatellite instability-high (MSI-H) tumor is a tumor with instability in microsatellite repeats analyzed by polymerase chain reaction (PCR) (75). dMMR refers to defective mismatch repair as detected by immunohistochemistry. In this thesis, presuming the methodology is not central to the interpretation of the study, the term dMMR will be used to cover both these entities.

Lynch Syndrome

Hereditary mismatch repair deficiency was described many years prior to knowing the molecular cause. The first paper characterizing what later became known as Lynch syndrome was published by Aldred S Warthin in 1913 (79). He studied the pedigree of his seamstress, who had lost several family members to different cancers. He discovered that the distribution of cancers in her family complied with autosomal dominant

Mendelian inheritance (80). In 1966, Henry Lynch and Marjorie Shaw introduced the "cancer family syndrome," later named "Lynch Syndrome" (81). In 1993, the first studies linking Lynch Syndrome to MSI were published by Aaltonen (82), Ionov (83), and Thibodeau (84). Soon after that, it was demonstrated that Lynch Syndrome cancer cells had features consistent with defective MMR (85).

Lynch Syndrome is the most prevalent hereditary colorectal cancer condition and is characterized by increased lifetime risk of several cancers and accelerated carcinogenesis (72, 86). In Lynch Syndrome-associated cancers, an adenoma can transform to cancer in 2-3 years, compared to 6-10 years in sporadic CRC. The most common Lynch Syndrome-associated cancers are colorectal cancer and endometrial cancer (80). In Lynch Syndrome, patients have either a germline monoallelic mutation in one of the MMR genes *MLH1*, *MSH2*, *MSH6*, or *PMS2* or a germline deletion at the 3'end of the *EPCAM* gene, which causes silencing of the downstream gene *MSH2* (87). Cancer forms after the damage of the remaining functional allele, following Knudson's two-hit hypothesis (88). Patients with double germline MMR gene mutations develop a rare condition called constitutional MMR deficiency which causes pediatric-onset cancers (89).

Non-germline dMMR

The majority of dMMR cases are not hereditary. In colon cancer, ~83% of dMMR cases have a sporadic origin (90). The most common somatic cause of defective MMR is epigenetic silencing of the *MLH1* gene (91). This phenomenon is strongly associated with CIMP and often includes concurrent *BRAF* mutation (92). Double somatic MMR mutations account for ~3% of dMMR cases in CRC (77, 93).

dMMR epidemiology and histopathology

The frequency of dMMR in colon cancer depends on stage. In patients included in clinical studies, it is detected in approximately 13-18% of stage II colon cancers (94, 95), 7- 14% of stage III colon cancers (94, 96, 97), and 3.5-5% of metastatic colorectal cancer (98, 99). Although dMMR colorectal tumors are more common in early TNM stage, they are also associated with a higher T stage and larger tumor transverse diameter

compared to pMMR tumors (96, 100, 101). dMMR tumors are associated with mucinous adenocarcinomas, poor differentiation, female sex, proximal tumor location, and older age (96, 100, 101). In rectal cancer, dMMR is present in 2-3% of tumors only (100, 102).

1.12 Prognostic markers and predictive markers

The previously described CIN, CIMP, and MSI pathways are models explaining the molecular changes that drive the transformation of normal colonic epithelium into invasive cancer. The next step in cancer development is the formation of metastasis. Metastasis is the most common cause of death in cancer patients with solid tumors (103), and the metastatic potential of individual cancers can be difficult to foresee. An important goal in the field of cancer biomarker research is to clarify the individual risk of metastatic disease for each patient. Another goal is to predict the most effective treatment for each type of tumor. A common definition of a **biomarker** is "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (104). Further, "A **prognostic biomarker** provides information about the patient's overall cancer outcome, regardless of therapy, while a **predictive biomarker** gives information about the effect of a therapeutic intervention" (105). The use of prognostic and predictive biomarkers facilitates individualized and effective therapy to reduce the risk of metastasis and, at the same time, avoid unnecessary treatment.

This thesis involves the study of biomarkers that reflect different characteristics of colorectal cancer development. The following chapters will introduce selected aspects of the following:

- histopathology of CRC (marker in this thesis: tumor grading)
- tumor microenvironment of CRC (markers in this thesis: CD3, CD8, PD-L1)
- molecular markers in CRC (markers in this thesis: maspin, CDX2)

1.13 Histopathology of CRC

Over 90% of carcinomas in the colon and rectum are adenocarcinomas, *i.e.*, cancer deriving from the epithelial cells of the colon mucosal lining (106, 107). Other (rare) types of carcinomas in the colon and rectum include spindle cell carcinomas, neuroendocrine carcinomas, and squamous cell carcinomas. This thesis focuses on adenocarcinomas only. Most CRC adenocarcinomas are defined as adenocarcinoma Not Otherwise Specified (NOS). Other types of CRC adenocarcinomas include signet ring, mucinous, and medullary adenocarcinomas; these three subtypes are all associated with dMMR (106, 108). In mucinous adenocarcinomas, >50% of the tumor volume consists of extracellular mucin (106). In signet ring cell cancers, >50% of cells have intracytoplasmic mucin that pushes the nucleus to the periphery. Medullary adenocarcinomas have sheets of malignant cells, are often heavily infiltrated by lymphocytes, and have a good prognosis.

1.13.1 Stage

TNM stage is undoubtedly the most important **predictive** and **prognostic** factor in CRC (8, 15, 23, 107). It is used to select patients for adjuvant chemotherapy. In addition, several histopathological risk factors are also used to select patients for chemotherapy in stage II colon cancer, as mentioned in chapter 1.9.1. These are established prognostic markers that indicate an increased risk of relapse, but the **predictive** power of these high-risk markers, *i.e.*, the ability to select patients for whom therapy will be effective, is not clear (32).

1.13.2 Tumor grade

Histological tumor grading is only applied to adenocarcinoma NOS. Tumor grading reflects the degree of tumor glandular formation (107). Well-differentiated tumors have the highest degree of gland formation and include 10% of CRC tumors, whereas 70% of tumors are moderately differentiated and 20% are poorly differentiated. Grading should be based on the least differentiated component (106). Poorly differentiated clusters and signs of tumor budding at the invasion front should not be considered when grading the tumor but be reported separately. The term “undifferentiated carcinoma”

refers to a histological subtype and is not a part of the tumor grading system. The two-tiered grading system combines well and moderately differentiated tumors into low grade. Poorly differentiated tumors are defined as high grade. High tumor grade is a marker of poor prognosis in colorectal cancer (15). Tumor grade is discussed in ***paper II***.

1.13.3 Tumor budding

Tumor budding is an emerging marker of poor prognosis in CRC. Tumor budding refers to buds of single tumor cells or small clusters of cells in the tumor center or at the tumor invasion front (109). These cells seem to have a higher invasive and migratory potential than other cancer cells, and the budding process is believed to be a morphological manifestation of epithelial-mesenchymal transition (EMT) (106). EMT is the process where cells lose epithelial traits and gain mesenchymal traits. This phenomenon is an important part of normal physiology, *e.g.*, embryonic development, but also essential in metastasizing as the process reduces intercellular adhesion and allows cancerous cells to migrate (110, 111).

1.14 Tumor microenvironment

The interplay between the tumor and the host immune response is an important regulator of tumor progression. Human tumors evoke immune surveillance, but the immunogenicity varies between tumors (112). When tumor-related antigens are exposed, the immune system can activate both the innate immune system and the adaptive immune system (113). The adaptive immune response requires that the tumor cell effectively expresses adequate levels of unique antigens in a way that leads to immune activation and not immune tolerance (112). Cancer cells may also develop traits that allow them to escape immune surveillance, *e.g.*, downregulating of antigen processing or expression of dominant antigens. Tumors can also create a hostile microenvironment by recruiting suppressive immune cells and secreting immunosuppressive mediators. In addition, increased interstitial fluid pressure and dense extracellular matrix make it harder for immune cells to reach tumor cells. The

Elimination, Equilibrium, and Escape-model is a commonly used model for describing the phases of cancer immunoediting (114).

The adaptive anti-tumor immune response is mainly mediated by T cells, and the most important are the cytotoxic T cells: CD8 positive cells. To trigger the CD8 cells, antigen-presenting cells (APC) first bind neoantigens to their major histocompatibility complex type I, migrate and become mature dendritic cells that present the antigen to the T cell receptor (TCR) on the CD8 cell in the regional lymph nodes (113). The activation of CD8 cells requires one more step — the binding of the CD28 receptor on the T cell to a B7 ligand on the dendritic cell (Figure 9). James Allison and colleagues studied the function of another protein that was generated in all T cells upon activation — the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (115). This protein is highly homologous to CD28 and is also a ligand to B7 molecules. CTLA-4 turned out to be an inhibitory protein that opposed CD28 co-stimulation (116). The group continued to develop an anti-CTLA4 antibody that led to impressive tumor responses in mice upon administration (115, 117). This laid the foundation for immune checkpoint inhibitor (ICI) treatment in human cancers, where blockade of different T-cell brakes (immune checkpoints) lead to the unleashing of T cell responses and the attack on tumor cells (Figure 9) (113, 115).

Discoveries by Tasuku Honjo's group led to the development of antibodies binding to the programmed cell death protein 1 (PD-1) or one of its ligands, programmed death-ligand 1 (PD-L1) (118). Antibodies targeting other immune checkpoints are being developed and tested. Still, despite inducing durable responses in a selection of patients, the majority of patients treated with ICI will not respond to this treatment, highlighting the need for markers predicting ICI response (119).

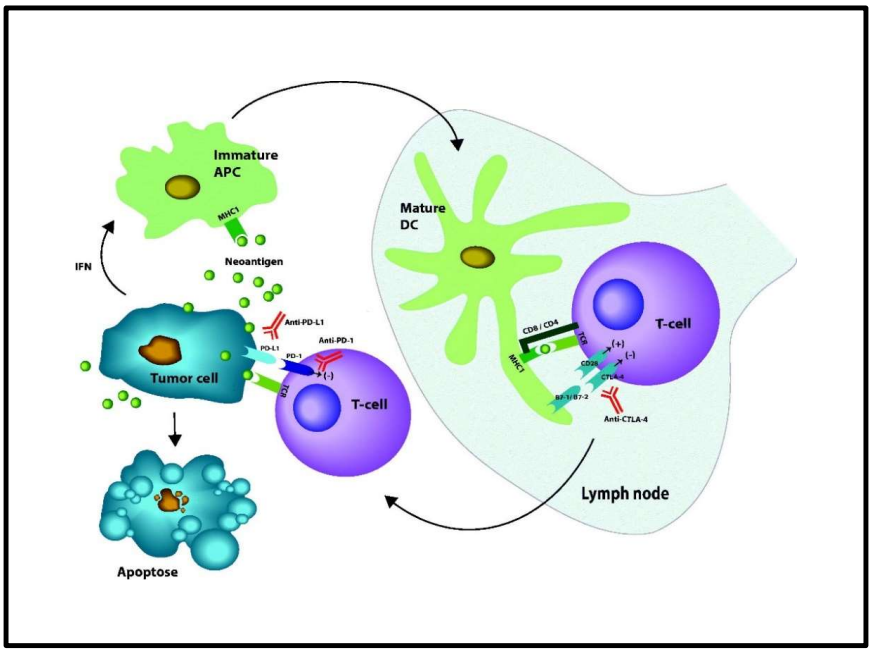


Figure 9. Mechanisms for CD8 T cell activation. Reprinted with permission from Taylor and Francis publishing (113).

1.14.1 Tumor mutational burden

Tumor mutational burden (TMB) represents the number of non-synonymous somatic mutations of a tumor (119). The highest numbers of somatic mutations are observed in melanoma, lung squamous carcinoma, and lung adenocarcinomas (120). The mutational burden can also vary within a tumor type, and fractions of tumors with high TMB are observed in almost all cancer types (121). In CRC, 16% of tumors are hypermutated (122). High TMB is mainly observed in dMMR cancers and cancers with mutations in DNA polymerase genes *POLD1* or *POLE* (121).

TMB is a promising **predictive** marker of immunotherapy-response in several tumor types (123, 124), including within dMMR mCRC patients (125). In addition, small subsets of pMMR tumors also have high TMB and might benefit from anti-PD-1/PD-L1 treatment (126). TMB is a potential **prognostic** marker in pMMR mCRC (127).

1.14.2 PD-L1

Programmed cell death protein 1 (PD-1) is an inhibitory surface receptor expressed on T lymphocytes (128). PD-L1 and PD-L2 are the ligands of PD-1 and signaling via PD-1/PD-L1 dampens the CD8 T cell response. Tumors may exploit this system by expressing PD-L1 on their cell surface. In theory, PD-L1 tumor expression should be an accurate predictor of the effect of anti-PD-1 therapy, and the initial Keynote/Checkmate studies suggested a better response of pembrolizumab and nivolumab in patients with PD-L1 immunoreactivity (119). PD-L1 is still the most used and validated biomarker to select patients for ICI, but the **predictive** impact is disputable and has limitations (129, 130). In most solid tumors, PD-L1 expression is associated with a poor prognosis (131), but no clear **prognostic effect** of PD-L1 expression has been established for CRC (132).

1.14.3 Tumor-infiltrating lymphocytes

One of the primary histopathological hallmarks of a tumor immune response is infiltration of T cells within the tumor, called tumor-infiltrating lymphocytes (TILs) (133). A high number of TILs has long been associated with dMMR, and TIL counts on hematoxylin eosin-stained (H&E) slides have previously been used to select patients for MSI testing (134). A high density of TILs is associated with high TMB (135) and PD-L1 expression (128).

In the last decade, the **prognostic** value of TILs has gained increased attention. The Immunoscore® developed by Galon *et al.* is the most acknowledged system for TIL scoring and developed for colorectal cancer prognostication (136). Originally, it involved the scoring of the density of CD8 (cytotoxic T cells) and CD45RO (memory T cells) positive cells in the invasive margin and central tumor by immunohistochemistry (IHC), but CD45RO was replaced by CD3 (pan T cell marker) based on antibody performance and the fact that CD3 density also has prognostic value (133).

An international consortium has validated the prognostic impact of the Immunoscore® in a large, combined cohort of patients with localized CRC (137). Bruni *et al.* examined the prognostic impact of several immune parameters in solid cancers (138). For CRC,

infiltration of CD8 positive TILs was associated with longer DFS or OS in all the 20 included studies.

High density of TILs has been associated with the effect of ICI in malignant melanoma and non-small cell lung cancer (119). In localized colon cancer, the density of TILs with co-expression of CD8 and PD-1 predicted the effect of neoadjuvant ICI treatment in pMMR tumors (139). High density of TILs might predict the effect of ICI in patients with dMMR mCRC, but it is not clear if the TILs themselves are predictors of effect or if this phenomenon is due to the association between high TIL density and high TMB (135).

1.14.4 MMR

MMR phenotype is a **predictive** biomarker impacting the effect of several different agents. The benefit of **fluorouracil** for dMMR patients has been subject to long-term controversy. Leading articles by Sargent and Ribic *et al.* have described a detrimental effect of adjuvant fluoropyrimidines for patients with dMMR (95, 140). In vitro studies have suggested that dMMR is a mechanism of fluorouracil resistance due to reduced detection of the DNA damage caused by fluorouracil treatment (141, 142). A recent pooled analysis state that stage II dMMR colon cancer patients do not benefit from fluorouracil monotherapy due to the improved prognosis associated with dMMR, but no detrimental effect of fluorouracil treatment was demonstrated (32).

Oxaliplatin forms platinum adducts with DNA that tumors with dMMR cannot repair (143). Therefore, some studies conclude that colon cancers with dMMR might have an increased benefit of oxaliplatin-containing adjuvant therapy (144). A pooled analysis recently demonstrated the benefit of adding oxaliplatin to fluorouracil when treating stage III dMMR patients (145). If colon cancer stage II dMMR patients are recommended adjuvant therapy, it should contain oxaliplatin (32).

The pivotal 2015 study by Le *et al.* demonstrated that dMMR tumors are sensitive to treatment with the anti-PD1 drug pembrolizumab (146), and dMMR has since become a tumor agnostic marker for the effect of **anti-PD1** antibodies (147).

dMMR is an acknowledged **prognostic marker** in colon cancer. In stage II colon cancer, dMMR is a marker of improved prognosis (90, 101). The frequent frameshift mutations in coding sequences of dMMR tumors result in an increased number of neoantigens and increased immunogenicity (75), which leads to a reduced metastatic potential in stage II dMMR tumors (148). The dMMR phenotype is associated with a high density of several T cell subtypes, high Immunoscore®, and increased PD-L1 expression (149). In contrast, dMMR CRCs seem to have a poor prognosis after recurrence (101, 150, 151). The prognostic impact of dMMR in stage III colon cancer is controversial and the topic of ***paper III***.

1.15 Molecular markers

1.15.1 KRAS, BRAF, and the MAPK/ERK pathway

The MAPK/ERK pathway deserves special attention as it contains two of the most studied oncogenes and established markers in colorectal cancer — the KRAS proto-oncogene (*KRAS*) (152, 153) and the B-Raf proto-oncogene (*BRAF*) (154). *KRAS* functions like a molecular switch and activates a cascade of transcription factors. The result is cell proliferation and gene transcription, including altered transcription of important cell cycle genes (155) (Figure 10). The anti-EGFR monoclonal antibodies cetuximab and panitumumab can block the signaling cascade by binding to the extracellular domain of EGFR (156, 157).

KRAS/NRAS/HRAS

Activating mutations of *KRAS* are reported in 35-45% of both localized and metastatic CRC (158-162). The most common *KRAS* mutations in CRC are G→A transitions at the second base of codons 12 or 13, which result in G12D or G13D mutations, and G→T transversion at the second base of codon 12, making G12V (163). *KRAS* mutant CRC tumors are more often low grade, MSS, and right-sided (158, 162, 164). *NRAS* mutations (165) are detected in ~3% of CRCs (159, 160). Mutations of the *HRAS* gene (166) are very rare in CRC (167).

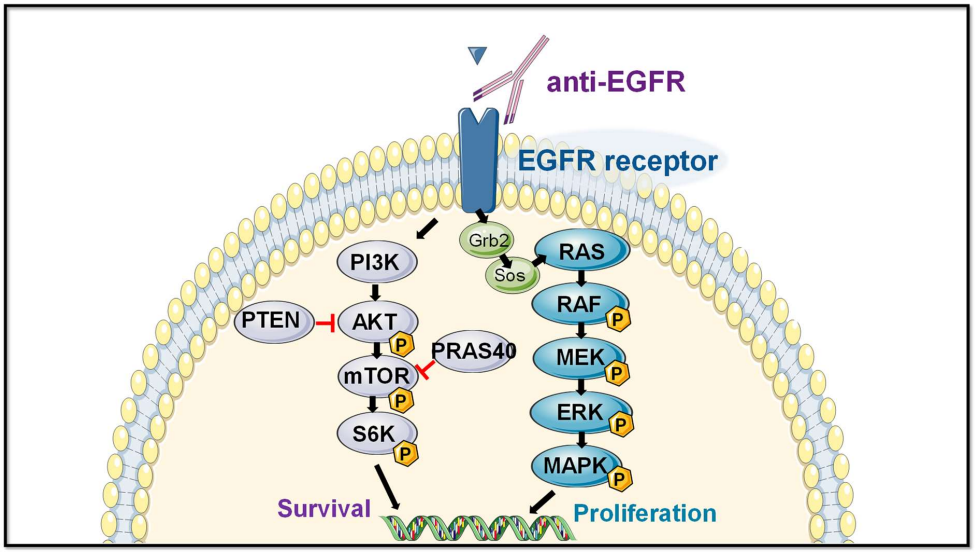


Figure 10. The PI3/AKT and MAPK/ERK pathway (161)*. The figure includes components from smart.servier.com (CCBY3.0).*

KRAS/NRAS mutation status is an established **predictive marker** for the effect of anti-EGFR agents panitumumab and cetuximab (used in mCRC). As *KRAS/NRAS* is downstream from the EGFR receptor, an activating mutation in *KRAS/NRAS* will hinder the pathway blockade initiated by anti-EGFR agents. Treatment with panitumumab and cetuximab is therefore restricted to patients without *KRAS* mutations in exons 2-4 and/or *NRAS* mutations in exons 2-4 (156, 157, 168, 169). The **prognostic** value of *KRAS* mutations is not clear, and the number of studies in localized CRC is limited (158, 170, 171).

BRAF

In 2002, the *BRAF* V600E mutation was reported to be present in a large proportion of malignant melanoma and several other human cancers (154). *BRAF* is downstream from *KRAS* and encodes a serine/threonine protein kinase in the RAS/RAF/MEK/ERK pathway. The *BRAF* V600E mutation leads to phosphorylation of MEK and ERK and activation of MAPK signaling (172). In colorectal cancer, BRAF mutation is associated with female gender, older age, right-sided tumors, high-grade tumors, CIMP, dMMR, and the serrated pathway (60, 92, 173). In dMMR cancers, the presence of a

*BRAF*V600E mutation strongly supports a sporadic origin and excludes Lynch Syndrome (174, 175). *BRAF*V600E and *KRAS/NRAS* mutations are mutually exclusive (158, 176, 177). In patients participating in clinical studies, *BRAF*V600E mutations have been detected in ~8-14% of stage II-III colon cancer patients (94, 158, 160) and ~8% of patients with mCRC (98). A recent publication from a population-based mCRC cohort reports a 20% frequency of *BRAF*V600E mutations (151). *BRAF*V600E mutations are associated with a poor prognosis in both localized and metastatic CRC (177-179), but the negative **prognostic impact** is most prominent in pMMR tumors (164, 180). Although the **predictive impact** of *BRAF*V600E mutation on the effect of EGFR-inhibitors has been the subject of controversy, a majority of studies conclude that this mutation is responsible for conferring resistance to monotherapy with anti-EGFR antibodies in *KRAS* wild-type tumors (53, 181). The BRAF inhibitor vemurafenib is ineffective for treating *BRAF*V600E mutated mCRC (182), despite being efficient in malignant melanoma with *BRAF*V600E mutations (183). However, treatment with encorafenib, a BRAF inhibitor with prolonged pharmacodynamic action, combined with the anti-EGFR antibody cetuximab, improves survival outcomes for previously treated patients with *BRAF*V600E mutated mCRC (60).

1.15.2 Maspin

Maspin (serpin B5) is a member of the clade B of serine protease inhibitor (serpin) superfamily and was first described in 1994 (184). Members of this serpin clade lack a classical secretory signal and are thus localized to the cytoplasm and the nucleus (185). Maspin differs from the other serpins by not going through the stressed-to-relaxed transition needed for having protease inhibitory actions (186). Transfection of mammary carcinoma cells with the maspin gene reduced the tumor-inducing abilities and metastatic potential in nude mice (184). The authors also observed loss of maspin expression in advanced human breast cancer and proposed that maspin might have tumor suppressor functions. Further experiments in mouse models supported that maspin inhibits cell motility, invasion, angiogenesis, and metastasis (187). A role in cell adhesion through binding to collagens, laminin, and β1-integrin has been suggested (188-190).

Maspin is epigenetically regulated (191). Promotor methylation can lead to maspin downregulation and has been demonstrated in breast, thyroid, skin, and colon cancer. Conversely, promoter demethylation leading to overexpression of maspin has been shown in gastric, pancreatic, and ovarian cancer. Histone deacetylation may also lead to maspin downregulation.

Maspin expression has been observed in a wide range of cancer types and may be present in the cytoplasm, membrane, or nucleus. Studies in colon cancer have shown that maspin nuclear expression is a marker of poor prognosis and a possible predictor of response to adjuvant chemotherapy (192). Still, few studies have been performed, and the results are discrepant (193). Maspin expression has also been associated with dMMR (194) and the CIMP pathway (195).

1.15.3 CDX2

The caudal type homeobox transcription factor 2 (CDX2) gene is a homeobox gene that codes for a transcription factor important in intestinal differentiation (196). CDX2 is a human homolog of the Hox gene *caudal* identified in *Drosophila melanogaster* (196-198). Homeobox genes are master regulators of the development of multicellular organisms acting at the top of genetic hierarchies (199). Intact CDX2 expression functions as a biomarker for mature colon epithelial tissue (200). Therefore, it is included the standard repertoire of many pathology departments, identifying gastrointestinal cancers in cancers with unknown primary, together with markers CK20 and CK7. Lack of CDX2 expression is reported in 4-15% of localized colon cancer and is associated with high tumor grade, dMMR, BRAF mutations, and right-sided colon cancer (201-206). Loss of CDX2 expression is uncommon in normal colonic epithelium (207) and in adenomas. The frequency of CDX2 loss increases with more advanced stages of colon cancer (208).

CDX2 exhibits tumor-suppressive functions in pre-clinical studies (209, 210). Mice with homozygous CDX2 deficiency (*Cdx2*$^{-/-}$) die at an early embryonic stage (209, 210). Studies in *Cdx2*$^{+/-}$ mice revealed no spontaneous tumorigenesis, but when treating them with a mutagen, *Cdx2*$^{+/-}$ mice had increased susceptibility to form tumors compared to

wild-type littermates (209). The mechanisms behind the tumor-suppressive functions of CDX2 are not fully established. CDX2 mutations are very rare (211, 212). The main cause of CDX2 downregulation is methylation of the CDX2 gene promotor through the CIMP pathway (206, 213). CDX2 downregulation is associated with colon cancer development through the serrated pathway (214, 215). Several studies have observed that lack of CDX2 expression identifies a subgroup with a poor prognosis (201, 206, 215, 216). A study published in NEJM in 2016 by Dalerba *et al.* proposed that lack of CDX2 expression in colon cancer is **predictive** of the effect of adjuvant 5FU-based chemotherapy (202). This has led to an increased interest in the biomarker potential of CDX2 in recent years.

1.15.4 Consensus molecular subtypes

In 2014, the consensus molecular subtypes consortium classified colorectal cancers based on gene expression patterns (150). Four subtypes were launched: the consensus molecular subtypes (CMS) 1-4, Figure 11.

CMS1 MSI immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
BRAF mutations		*KRAS* mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF-β activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Figure 11. The consensus molecular subtypes. Reproduced with permission from Nature Publishing group (150).

Each subtype is associated with different combinations of histopathological characteristics, immunogenic activation levels, and molecular features. Despite being the most widely recognized way of subtyping CRCs, the CMS classification is not implemented in clinical decision-making.

1.15.5 Sidedness

Colon cancer sidedness, *i.e*., whether the primary tumor invades the right or left side of the colon, is a **prognostic** and **predictive** marker in colon cancer (217). A common definition of right-sided colon cancer is cancers proximal to the splenic flexure, whereas left-sided colon cancer is defined as cancers at and distal to the splenic flexure. Although right- and left-sided colon cancers have the same histology, the molecular biology differs. Right-sided cancers are associated with dMMR, *BRAF* mutations, older age, female gender, increased T stage, the CIMP pathway, CMS1, and CMS3 (97, 217, 218). Left-sided cancers are associated with the CIN pathway, *TP53* mutations, and CMS2 (217, 218). In mCRC *KRAS* wildtype tumors, patients with left-sided primaries have a better prognosis (219) and benefit more from EGFR inhibitors (219, 220) than patients with right-sided primary tumors.

Sidedness is not an acknowledged prognostic or predictive factor in **localized colon cancer**. Sinicrope *et al.* reported that the favorable prognosis for dMMR stage II colon cancer is restricted to right-sided tumors (97), but this has not been confirmed in other studies. In a recent register study including stage II and III colon cancer, left-sided pMMR cancers had a better prognosis than right-sided pMMR cancers (221). Sidedness does not seem to affect the benefit of adjuvant chemotherapy (222).

2. Aims of the study

2.1.1 General aim

The general aim of this study was to investigate markers with potential prognostic and/or predictive value in colorectal cancer.

2.1.2 Specific aims

Paper I: Maspin is a part of the serpin family of protease inhibitors. Several studies imply that maspin expression could predict the benefit of adjuvant chemotherapy in CRC. Paper I aimed to assess the predictive and prognostic value of maspin expression in a randomized cohort of stage II-III CRC patients. We hypothesized that maspin is a prognostic factor for disease control of colon and rectal cancer treated by radical surgery and a predictive factor for the effect of adjuvant chemotherapy.

Paper II: Mismatch repair deficiency (dMMR) is associated with markers that confer a poor prognosis in isolation. Lack of CDX2 expression is associated with dMMR, high tumor grade, a poor prognosis, and a possible benefit of adjuvant chemotherapy. In this study, we aimed to assess the prognostic and predictive impact of CDX2 in colon cancer and investigate if CDX2 expression affected dMMR and pMMR cases differently. We also sought to study the interplay between dMMR, tumor grade, and CDX2. We hypothesized that CDX2 is a marker of poor prognosis in colon cancer but that the prognostic impact might depend on tumor grade and MMR phenotype.

Paper III: Mismatch repair deficiency is a marker of improved prognosis in stage II colon cancer but a marker of poor prognosis in stage IV colon cancer. In this study, we aimed to assess the prognostic impact of dMMR in stage III colon cancer and the prognostic interaction between MMR status, tumor cell PD-L1 expression, and density of CD3+ and CD8+ lymphocytes. We hypothesized that the prognostic impact of MMR depends on stage and that PD-L1 and TIL expression might affect the prognosis of dMMR colon cancer.

3. Material and methodological considerations

The methods utilized in ***paper I-III*** are described in detail in the respective manuscripts, but important methodological aspects and considerations are discussed in the following sections.

3.1 Patient characteristics/study populations

Our study includes patients recruited from two different series (Figure 12).

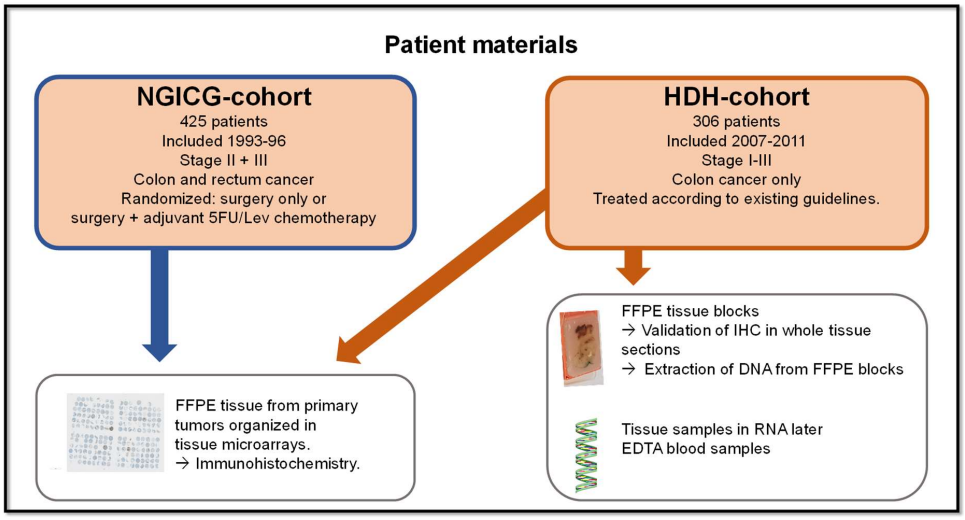


Figure 12. Patient materials in our study.

In the **NGICG series**, tissue from primary tumors was collected from patients participating in a clinical trial published in 2009 (43). This clinical trial was initiated by the Norwegian Gastrointestinal Cancer Group (NGICG) to study the effect of adjuvant chemotherapy in stage II and III colorectal cancer. Between 1993 and 1996, patients between 18 and 75 years of age with radical resection of colon or rectum adenocarcinoma were included. They were randomized to surgery alone or surgery with adjuvant chemotherapy. Surgical radicality was evaluated by pathologists. Preoperative staging included a chest x-ray and ultrasound or CT of the abdomen. Follow-up is described in ***paper I*** and included chest x-ray, abdominal ultrasound, colonoscopies, and

blood tests. None of the patients with rectal cancer received preoperative radiation or preoperative chemotherapy. The adjuvant chemotherapy consisted of 12 months of fluorouracil/levamisole according to the Moertel schedule (41). Fluorouracil was administered for five consecutive days for the first course. From day 28, fluorouracil was administered weekly. Levamisole was administered as 50 mg three times daily every second week. Originally, 425 patients were included, but 13 were later omitted from further evaluation due to reclassification to stage I (9 patients) or discovery of metastasis or previous cancer prior to randomization (4 patients), leaving 412 patients available for evaluation. The plan was to include 1076 patients, but the study was closed prematurely after a joint interim analysis of ongoing Scandinavian studies showed a benefit of receiving adjuvant chemotherapy for stage III colon cancer (42). The benefit for the stage III subgroup was also shown (for DFS and CSS) when analyzing the NGICG trial separately (43). The randomized study design with a control group not receiving chemotherapy offers unique possibilities to study markers predictive of chemotherapy effect. All predictive effects in our study were therefore explored in this cohort only.

The Haraldsplass Deaconal Hospital (**HDH**) series is a population-based prospectively collected single-center cohort (223, 224). In this series, 306 patients with stage I-III colon cancer were included from 2007 to 2011. All patients were operated on with complete mesocolic excision with high vascular tie, and achieving a minimum of 12 excised lymph nodes was a specific surgical goal. The preoperative radiological staging consisted of CT of the thorax and abdomen. None of the patients had preoperative chemotherapy or radiation. The adjuvant Nordic FLOX regimen (fluorouracil and oxaliplatin) was offered to fit patients under 70 years of age, while 70–75-year-old fit patients were offered Nordic FLV (fluorouracil/calciumfolinate). Follow-up was conducted half-yearly and included CT of the thorax and abdomen in addition to relevant blood tests. The first publication from this series evaluated outcomes after implementing CME and compared laparoscopic to open CME (223). Having a population-based series reduces the risk of selection bias and improves the generalizability of the study results.

The selection of patients for the different papers is described in Figure 13. ***Paper I*** included the NGICG cohort only. In ***paper II*** and ***paper III***, patients with colon cancer stage II and III were included from both series.

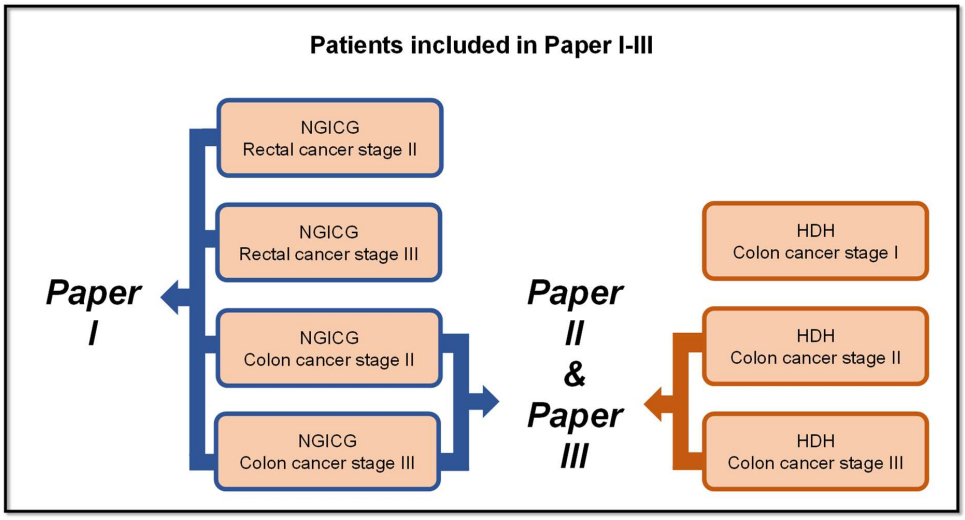


Figure 13. Selection of patients in paper I-III.

3.1.1 Strengths and limitations of our patient materials

The public healthcare system and similar national guidelines in Scandinavian countries lead to limited inter-hospital treatment heterogeneity and facilitate combining patient series from different centers. Detailed clinicopathological data are recorded for all patients in our two study cohorts. Still, in our study, including two separate series increases the heterogeneity of the combined patient material due to differences in treatment. In the period of time between the inclusion of the two different patient series, studies revealed the importance of achieving a high lymph node yield. As a result, the mean number of examined lymph nodes is higher in the HDH series than in the NGICG series. For stage III colon cancer patients, the mean number of examined lymph nodes was 16.7 (SD 5.1) for the HDH cohort and 9.6 (SD 6.6) for the NGICG cohort.

The largest difference between the two series is the selection of patients to receive adjuvant chemotherapy. In the NGICG study, patients in both stage II and III were

randomized to receive adjuvant chemotherapy after surgery or to surgery alone. This means that half the stage III patients would be under-treated by today's standards.

For the prognostic models, we have performed statistical tests and adjustments to minimalize the effect of study group differences on the results in our models. The NGICG and HDH series have similar clinicopathological characteristics and distribution of biomarker expression, as shown in Table 1 of ***paper II*** and Supplementary table 3 of ***paper III***. Our multivariate models statistically adjust for receipt of adjuvant treatment (adjuvant chemotherapy vs. surgery only). In addition, our multivariate Cox regression models in ***papers II*** and ***III*** include the variable cohort to adjust for differences between the two series. Survival is significantly better in the HDH series. Differences in receipt of chemotherapy and lymph node yield are possible explanations for this observation. In addition, the previously mentioned differences in preoperative staging methods between the HDH and NGICG series (chest x-ray + CT/UL abdomen versus CT thorax and abdomen) might contribute to a larger fraction of under-staged patients in the NGICG cohort and a worse recorded outcome for this group of patients.

The material from the NGICG cohort includes TMA blocks only. The original Formalin-fixed paraffin-embedded (FFPE) blocks are no longer available. As a result, the validation of the IHC stains in whole tissue slides was only possible in whole tissue slides from the HDH cohort. The tissue blocks from the NGICG material are older than from HDH — a factor that might influence antibody performance, despite TMAs from both series being stained at the same time using the same protocols. Still, there were no significant differences in variable expression between the HDH and NGICG cohorts, and no apparent differences in antibody performance were observed when scoring. Not having material available from the NGICG cohort for DNA or RNA sequencing also limits possibilities for validation and biomarker exploration.

3.2 Tissue Microarrays

Tissue microarrays (TMAs) are used in ***paper I-III***. The tissue microarray method was developed for high-throughput molecular characterization of large series of tissue

samples (225). Tissue microarrays are commonly used in biomarker studies due to the many advantages associated with this method. Compared to studying biomarker expression in whole tissue slides, using TMAs is less time-consuming and less expensive. As all tumors can (usually) be included in the same staining cycle, batch to batch differences are reduced (226). The main disadvantage associated with TMAs is the reduced ability to reflect tumor heterogeneity which may not be adequately represented in small tumor samples (226).

When making the TMAs for our studies, measures were taken to increase representability. Before making the TMAs, a pathologist reviewed Hematoxylin & Eosin (H&E)-stained slides from each tumor block. Areas with representative tumor tissue suitable for use in the TMAs were marked. Three punches were made from each tumor. A tissue core size of 1.0 mm was chosen instead of smaller core sizes. Tissue cores were mounted into a recipient paraffin block using a custom-made precision instrument (Beecher Instruments, Silver Spring, MD, USA). When planning studies involving TMAs, it is important to acknowledge that some biomarkers are expressed more heterogeneously than others. While using TMAs is a reliable method for evaluating the expression of several biomarkers (227-229), this might not be the case for more heterogeneously expressed biomarkers (230, 231). Therefore, the ability of TMAs to accurately represent tumor biomarker expression should be evaluated for each separate antigen (230).

3.3 Immunohistochemistry

Immunohistochemistry (IHC) is used in ***papers I-III***. IHC is a tissue section-based method that identifies a tissue antigen by exploiting the ability of antigens to bind to specific antibodies (232). The binding between antigen and antibody is visualized with a colored histochemical reaction. IHC allows for determining both biomarker expression and localization as the method preserves tissue histology. It is therefore frequently used for both clinical diagnostics and research purposes.

Several variables can affect the sensitivity and specificity of the IHC staining. Preanalytical variables include tissue fixation time and -method and epitope retrieval approach (233). In addition, antibody type and optimization- and detection methods will influence the IHC results (234). Several different clones of antibodies targeting the same protein are often available, and the antibody performance may vary significantly (233). The Nordic Immunohistochemical Quality Control (NordiQC) (235) is an independent quality assurance organization that can guide antibody- and protocol selection.

Immunohistochemistry for maspin

The anti-maspin antibody in ***paper I*** was selected as it had been used in previous publications (192, 194, 236). Test staining performed in our group resulted in staining that was concordant with the expected staining pattern in colorectal tumors. Cores were scored both for nuclear and cytoplasmic maspin, as previous studies had implied that expression in both locations could contribute to the prognostic value of maspin (236).

Immunohistochemistry for CDX2

CDX2 IHC is well-integrated in the clinical diagnostics of cancer with unknown origin. Several commercially available antibodies exist, and in the recent decade, the NordiQC has run several tests highlighting the performance variation between clones. The EPR2764Y clone has the highest pass rate (235) and is used at our local hospital. Therefore, our TMAs were stained at the Department of Pathology at Haukeland University Hospital using their diagnostic protocol. Details of CDX2 scoring and validation are found in ***paper II*** and ***supplementary material*** of ***paper II.*** Based on the results from our study and other published studies using CDX2 IHC, it is our opinion that CDX2 is a marker very well suited for assessment in TMAs. The majority of patients (78.1%) had a strong CDX2 expression in 95-100% of tumor cells (category D), reflecting the homogeneous nature of the CDX2 staining. Interobserver agreement were high: 89,0% between the four categories (A, B, C, and D) and 98,8 % between CDX2 positive and CDX2 negative cases.

Slik *et al.* observed a high correlation of CDX2 staining between tumor center and tumor front cores (205) which complies with our impression; we also observed little variation

in CDX2 staining between our different tumor cores. The CDX2 staining was validated in 54 whole tissue sections (***supplementary material, paper II***). Discrepancies between TMAs and whole tissue sections were related to cases with scoring just below or just over the cut-off value of 50%. We, therefore, concluded that staining in the TMA was representative of the staining in the whole tissue sections.

Immunohistochemistry for mismatch repair genes

Immunohistochemistry for mismatch repair proteins is used in ***paper II-III.*** IHC and PCR-based methods are used to diagnose defective MMR or microsatellite instability. IHC characterize the expression of mismatch repair proteins, while PCR-based methods diagnose microsatellite instability by studying instabilities in a panel of microsatellite markers. PCR-based molecular testing involves a fast turnaround time and low cost. Limitations of the method include the requirement of ≥20% neoplastic cells in the sample and the risk of false-positive test results due to microsatellite polymorphisms. In addition, MSI-PCR will not produce any information about the affected MMR gene (76). Sensitivity depends on the applied panel; for the Pentaplex panel (using BAT-25, BAT-26, NR-21, NR-24, and NR-27), a sensitivity of 95.8% and specificity of 98.7% is reported (237).

A panel of two or four MMR proteins are used for IHC of MMR proteins. This method will identify the affected deficient gene, which is important when diagnosing Lynch Syndrome. The expression of each MMR protein must be studied separately, which occupies the pathologist's time. The intact expression of a protein on IHC does not always indicate that the homologous gene functions properly. A missense mutation in an MMR gene can lead to MMR proteins being expressed, despite a dysfunctional gene. This can present itself as an intact expression of the MMR protein (238). The reported sensitivity of MMR IHC is 85-100% and specificity 85-92% (237). Several studies report that the discordance rate between the PCR and IHC testing strategies is low (1.0%-2.9%) but varies depending on two versus four analyzed MMR proteins and the choice of MSI panel (237, 239, 240). Others report a higher discordance rate (241, 242) and recommend that both methods should be used when selecting patients with mCRC for immunotherapy (132, 241).

In our work, MMR staining for mismatch repair proteins MLH1, MSH2, MSH6, and PMS2 was performed for all tumors in both study cohorts. Staining for only two MMR proteins is less time-consuming and used in some studies. We chose the recommended four-protein approach to optimize sensitivity and specificity for diagnosing dMMR (132, 237, 243). Co-authors of ***paper II***, NBR and YM both scored all IHC tissue cores. The majority of cases had strong staining in 100% of tumor cells for all four MMR proteins. All cores that either had weak or no staining, cores with positive staining in less than 100% of tumor cells, cases where results between NBR and YM were discrepant or where tissue or staining quality was questioned, were selected for revision. These cases were reviewed and discussed by KEH and MPM. To ensure technical staining validity, the presence of positive, strong staining in control cells on the same tissue core (normal mucosa or stromal cells including stromal lymphocytes) was mandatory for a representative negative tumor stain (132). Cases with negative tumor staining without positive internal control were omitted from analysis ("missing staining result"). As intratumoral lymphocytes may be mistaken for tumor tissue, negative staining was defined as 0-5% stained tumor cells. Whole tissue slides from 47 cases were used for validation. Details about the scoring and validation are found in the supplementary material of ***paper II***. Results from the staining validation in whole tissue slides did not change the MMR phenotype from assessment of TMAs in any of the 47 cases.

The biology of MMR proteins was taken into consideration when scoring. Mutations in *MLH1* and *MSH2* will result in the degradation of the homologous mutated proteins and the proteins of their secondary partners, PMS2 and MSH6, respectively. Conversely, mutations in *PMS2* or *MSH6* may not necessarily result in degradation of their primary partners MLH1 and MSH2, as MLH1 and MSH2 may have other heterodimer partners (PMS1 or MLH3). As a result, a case with negative PMS 2 staining with intact MLH1 staining was regarded as dMMR. A case with negative MLH1 staining with intact negative PMS 2 staining was regarded as equivocal. ESMO guidelines recommend supplementary MSI-PCR for cases with equivocal IHC interpretation. As the material available from the NGICG cohort does not allow for this analysis, cases with equivocal IHC interpretation were omitted from our analysis ("missing staining result").

Although uncommon, heterogeneous staining patterns of MMR proteins have been reported (244, 245). By not assessing all cases in whole tissue sections, we might have missed cases with heterogenous MMR staining. As there is no consensus on how to interpret heterogenous IHC MMR staining and this remains a rare event, we do not think including information about heterogeneity for the whole study cohort would have significantly changed our results.

Immunohistochemistry for CD3 and CD8

Immunohistochemistry for CD3 and CD8 is described in ***paper III.*** There are several ways to assess the degree of immune infiltration in CRC. In addition to the Immunoscore®, a range of different methodologies for quantification of IHC-defined lymphocytic subsets, both manual and digital, have been used in studies. Methods for assessing inflammatory infiltrates in CRC based on H&E-stained slides have also been established and seem to result in similar prognostic information as IHC methods (133, 246). We chose to score the intraepithelial and the stromal lymphocytes separately, as some studies have shown that these subsets have a different prognostic impact (247). The CD8 staining was performed at the Dept. of Pathology, Haukeland University Hospital. The CD3 IHC staining was performed by authors KEH and NBR and scored by KEH and MPM. As CD3+ stromal, CD3+ intraepithelial, CD8+ stromal, and CD8+ intraepithelial lymphocyte density all were prognostic markers in our study, and their expression was strongly related, a combined TIL score was made for prognostic assessment in multivariate models, as explained in ***paper III***.

Immunohistochemistry for PD-L1

Immunohistochemistry for PD-L1 was performed for ***paper III*** by the Dept. of Pathology, Haukeland University Hospital. There are currently two different scoring methods for PD-L1, the Tumor Proportion Score (TPS) and the Combined Positive Score (248). For the TPS score, the percentage of viable tumor cells with partial or complete membrane staining is evaluated. The combined positive score assesses the number of PD-L1 stained cells (tumor cells and immune cells) relative to all viable tumor cells. There was no consensus on what method to use for CRC at the time. We chose the TPS method that was developed for lung cancer. Obtaining representative

biopsies may be challenging in lung cancer, and most patients are never operated on. Therefore, diagnostics are often based on small biopsies. For a TPS score, only ≥100 vital carcinomas cells are needed for evaluation (249). PD-L1 can be heterogeneously expressed (249). As we have chosen a low threshold for positive staining (≥1% of cells) and we chose a scoring method developed for small biopsies, we have aimed to avoid false-negative cases.

3.3.2 Scoring of IHC

Several methods exist for scoring tissue staining (234). Intra- and interobserver variability, reproducibility, biomarker molecular biology, and antibody performance should guide the choice of method. The Histoscore is a well-established, target-agnostic, and semiquantitative scoring method that has been used for several decades (250). Staining intensity is scored from 0-3 and multiplied by the percent of stained tumor cells. This method was used in ***paper I*** to score maspin expression as there was limited clinical experience available for maspin IHC at the time, and methods used in previous studies varied. For some markers, weak staining may result from the staining quality instead of reflecting biological differences in biomarker expression (251). Weak or negative staining in cells expected to express CDX2 may result from insufficient staining and represent a diagnostic pitfall in CDX2 staining interpretation (235). Therefore, in ***paper II***, cases with weak staining in a majority of cells were recorded as CDX2 positive. When scoring CD3+ and CD8+ lymphocyte infiltration in ***paper III***, staining intensity was not recorded, as a weak intensity most likely would reflect technical issues and not biological differences.

3.3.3 Grading of tumors

As mentioned in chapter 1.13.2, the grading of colorectal adenocarcinomas considers the degree of tumor glandular formation: >95% for well-differentiated, 50-95% for moderately differentiated, and <50% for poorly differentiated adenocarcinomas (107). The highest interobserver variability is observed when separating between the categories well- vs. moderately differentiated. A two-tiered grading system is recommended to increase reproducibility and is therefore used in ***paper II*** (107). The two-tiered grading system combines well and moderately differentiated tumors into low grade, and poorly

differentiated tumors are defined as high grade (252). The tumor grading in our study was obtained from the original pathology reports.

3.4 Statistics

The following statistical analyses have been performed in ***paper I-III***. The **t-test** is a parametric test. It was used for testing the distribution of a continuous variable against a categorical variable. The **Mann-Whitney U**-test is a non-parametric that was used for testing categorical variables against a continuous variable that deviated from the normal distribution. The **Chi-square test** was used for testing associations between two categorical variables (253). The **Kaplan-Meier** estimate is a univariate method for survival analysis (254), tested for statistical significance with the log-rank test (255). It is a non-parametric test and can be used for incomplete observations. The **Cox proportional hazard model** is the most used multivariate analysis in cancer research. It can assess the association between a variable and survival rate while adjusting for possible confounding variables (256).

3.4.1 The use of cut-off values.

When analyzing the prognostic and predictive impact of the biomarker variables in statistical models, the biomarker expression can be analyzed as continuous variables or divided into two or more groups by selecting cut-off values. For some markers, *e.g.*, PD-L1 and MMR proteins in ***paper III*** and tumor grading in ***paper II*****,** established scoring systems and cut-off values exist. In ***paper II***, a 50% positive cell cut-off was chosen according to the scoring system made by Dalerba *et al.* (202). By applying an already established cut-off, we have increased the reproducibility of our study. For biomarkers maspin in ***paper I*** and for CD3 and CD8 in ***paper III***, studies use different cut-offs. To avoid statistical overfitting of our models, these markers were treated as semi-continuous variables in the multivariate Cox regression models, and cut-off values were only used for Kaplan Meier curves. Receiver Operating Curves (ROC) are frequently used for selecting an optimal biomarker cut-off (257) but were not used in our papers. Selecting a cut-off point optimized for one particular study could lead to low p-values and high hazard ratios but increases the risk of making type I errors (258).

3.4.2 Multivariate models

The variables entered in our Cox regression multivariate models were chosen based on their clinicopathological relevance. The number of events limited the number of variables in each model: ≤ one variable per ten events. ***Papers I-III*** all have multivariate models with an interaction term that includes two categorical variables. An interaction effect is a situation where the effect of one variable is dependent on the values of other variables in the model. Statistically, this can be modeled by including the product of two or more variables along with corresponding individual variables (259), as we have done in our papers.

3.4.3 The REMARK guidelines

The Reporting Recommendation for Tumor Marker Prognostic Studies (REMARK) guidelines provides a methodological framework for reporting studies involving prognostic markers (260). These have been carefully considered in our studies, and a REMARK checklist is added in the supplementary material for ***paper III***.

3.4.4 Ethics

The Regional Ethics Committee and the Norwegian Data Inspectorate have approved the studies. The patients had signed informed consents before inclusion, which included that the generated data could be used for research purposes. The studies were conducted in agreement with the Declaration of Helsinki. A secure server has been used for data storage, and all analyses have been performed on de-identified data.

3.5 Supplementary methods

These methods are not included in paper I-III but are a part of the discussion of the results. We, therefore, mention these methods briefly.

BRAF immunohistochemistry was performed for the HDH and NGICG cohorts. Whole transcriptome sequencing, DNA extraction, and ddPCR for BRAF V600E was performed in 77 selected patients from the HDH cohort. The number of relapses in the transcriptome group was balanced to match the original cohort. Otherwise, patients were

randomly selected. DNA was extracted from FFPE tissue, and ddPCR was performed for BRAF V600E.

RNA extraction and whole transcriptome sequencing

Members of the research group performed RNA extraction. The RNA extraction has been described previously (261). Immediately after resection, tissue samples were placed in RNAlater™ (Sigma-Aldrich, Merck, Darmstadt, Germany) and stored at -80°C. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia, CA). In addition to the tumor samples, ten samples containing adjacent colon tissues with normal morphology were included. The libraries were prepared using the Illumina TruSeq Stranded Total RNA with Ribo-Zero Gold (PN RS-122-2301) in accordance with the manufacturer's protocol. The RNAs were fragmented and then converted to cDNA by reverse transcriptase. Sequencing was performed on an Illumina HiSeq4000 (2x75bp Paired-End) at the Genomic Core Facility at the University of Bergen.

BRAF Immunohistochemistry.

BRAF IHC was performed at the Department of Pathology, Ålesund Hospital. It was performed on a BenchMark Ultra platform with CC1 buffer (56 minutes at 99°C) for target retrieval followed by incubation for 16 minutes at 36°C with the anti-BRAF V600E mouse monoclonal antibody (Ventana, clone VE1) at 1:100 dilution. Detection was performed using the OptiView DAB Detection Kit Roche (P/N 760-700). BRAF V600E staining was scored by KEH and MPM. The staining was cytoplasmic and scored as 0, 1+, 2+, or 3+. Positive staining results were defined as 2+ or 3+.

DNA extraction

KEH performed DNA extraction. For DNA extraction, archival formalin-fixed paraffin-embedded (FFPE) blocks were obtained. We used the same block that was used for TMA construction, and H&E slides from each block were inspected to locate the optimal place for sampling. Punching was done with sterile disposable 1mm Biopsy Punches, one for each block, and two-four samples were taken from each block. Genomic DNA (gDNA) was extracted using the QIAGEN GeneRead™ DNA FFPE Kit according to the manufacturer's instructions. The samples were dewaxed with Deparaffinization

Solution (Qiagen, Valencia, CA). After heating to reverse formalin cross-links and protein digestion by proteinase K, the samples were subjected to enzymatic removal of cytosine deamination artifacts using the Uracil-N-Glycosylase (UNG)-enzyme. The lysate was added to a spin column, binding DNA to the column. Residual contaminants were washed away using ethanol and supplied buffers. Residual ethanol was removed by additional centrifugation. DNA was eluated using ATE buffer. Total DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific) and samples were stored at -20°C before further processing.

BRAF ddPCR

BRAF ddPCR was performed by KEH and NBR. Samples were subjected to ddPCR using the ddPCR BRAF V600 Screening Kit from BioRad, this is designed for detecting mutations in BRAF V600E (Assay ID dHsaMDV2010027), V600K (dHsaMDV2010035) and V600R (dHsaMDV2010037) in a single well. Samples were thawed, vortexed, and prepared at the concentration of 30-40ng DNA per reaction. The diluted DNA sample, 1.0 µL, was mixed with 10.0 µL ddPCR Supermix for Probes, 1.0 µL BRAF V600 Screening Assay (20 ×), 0.5 µL HINDIII restriction enzyme, and 7.5 µL nuclease-free water. After sealing the 96-well plate, droplets were generated by the Automated Droplet Generator and Droplet Generation Oil for Probes (P/N 1864110, Bio-Rad) before proceeding to thermal cycling using the following conditions: 95°C for 10 min, 40 cycles consisting of 94°C for 30 sec and 55°C for 1 min, 98°C for 10 minutes before cooling to 4°C at 1°C/sec. The Ramp Rate was 2°C/sec. The QX200 Droplet Digital System and the QuantaSoft Analysis Pro software (Bio-Rad) was used for droplet reading and analysis. An amplitude of 4000 was set as the threshold for positive BRAF V600E mutated droplets and 8000 for negative BRAF V600E wt droplets, and duplicate wells were analyzed for all samples.

CMS classification

KEH classified cases with RNA seq data into CMS groups (150). These groups were classified using the R-package CMS classifier (random forest version) and the variable “predicted CMS” (262, 263). Before CMS group classifying, genes with low expression were removed using a row-sum of 200 as the threshold.

4. Summary of the results

4.1 Paper I: Maspin is a part of the serpin family of protease inhibitors and a potential predictive and prognostic marker in colon cancer. In our study, maspin expression was assessed semi-quantitatively by immunohistochemistry. The study cohort was obtained from a trial where 412 patients with stage II and III colorectal cancer were randomized to receive adjuvant chemotherapy with fluorouracil and levamisole (5-FU/Lev) or to surgery without adjuvant chemotherapy. Successful maspin staining results were available for 380 patients. Maspin expression was not associated with any established clinicopathological markers in our cohort. The maspin variable was entered as a continuous variable in our multivariate models. We did not demonstrate any association between maspin expression level and prognosis. In our split multivariate regression model, low expression of nuclear maspin was associated with the effect of adjuvant chemotherapy for colon cancer patients but not for rectal cancer patients. In patients receiving adjuvant chemotherapy, maspin expression level was associated with CSS (HR 1.43 per 50 points increase in maspin score ($p = 0.021$)), but not in patients treated with surgery only. There was a significant interaction between treatment and maspin expression ($p = 0.045$). For illustration in Kaplan-Meier plots, the maspin variable was divided into low, medium, and high expression. Low expression of maspin expressed in the nucleus was associated with the effect of adjuvant chemotherapy, but medium or high maspin expression was not.

4.2 Paper II: Our study investigated the associations between MMR phenotype, CDX2 expression, and tumor grade. The study included 544 patients with colon cancer stage II-III from two different patient materials. We performed immunohistochemistry for MMR proteins and CDX2. We report that pMMR patients with loss of CDX2 expression have a very poor prognosis. Cancer-specific survival for cases with pMMR and CDX2 negativity was 35.8 months (95% CI 23.4–48.3) versus 52.1−53.5 months (95% CI 45.6–58.6, $p = 0.001$) for other cases. Our multivariate regression model showed that CDX2 negativity is an independent negative prognostic marker in pMMR patients (HR 2.93 (95% CI 1.23–6.99, $p = 0.015$)). CDX2 expression did not affect prognosis in dMMR patients. We did not demonstrate a predictive effect of CDX2 expression. High

tumor grade was also a marker of poor prognosis in pMMR patients. When investigating the predictive effect of tumor grade in our randomized cohort, we observed that pMMR cases with high tumor grade respond well to treatment with adjuvant chemotherapy with fluorouracil/levamisole. A statistically significant interaction between tumor grade and treatment ($p = 0.036$) was demonstrated in our multivariate models. High tumor grade conveyed a poor prognosis in the surgery-only group (HR 4.60 (95% CI 1.68−12.61), $p = 0.003$) but not in the group receiving adjuvant chemotherapy (HR 0.66 (95% CI 0.15−3.00), $p = 0.587$).

4.3 Paper III: We studied the clinical significance of dMMR in 544 patients with colon cancer stage II and III. IHC for MMR proteins, CD3, CD8, and PD-L1 was performed. Our multivariate Cox regression models demonstrated a significant interaction between MMR phenotype and stage ($p<0.001$ for DFS). In stage III colon cancer, dMMR was associated with poor prognosis compared to pMMR cases. Mean survival in months was 28.8 (95% CI 18.5-39.1) for dMMR vs. 40.9 (37.2-44.6) for pMMR, $p=0.014$. In our multivariate model, dMMR was a marker of poor DFS and OS in stage III colon cancer: HR 4.17 (95% CI 2.02-8.61), $p<0.001$ for DFS). There was a non-significant trend towards an improved DFS for stage II dMMR patients compared to pMMR patients in our multivariate model HR 0.24 (95% CI 0.06-1.04), $p=0.057$). The prognostic shift demonstrated in the multivariate models was also significant when adjusted for PD-L1 expression, CDX2 expression, chemotherapy, and TIL density. TIL density and PD-L1 expression were not independent prognostic variables in the dMMR subgroup.

5. Discussions of results

This thesis has studied the prognostic and predictive impact of selected biomarkers in stage II-III colon cancer. Our study has confirmed known associations between the biomarkers and clinicopathological features. In addition, we have identified new clinically relevant prognostic subgroups and markers with potential predictive relevance in localized colon cancer. Still, our findings call for validation in larger individual datasets. Here, I will discuss central findings in ***paper I-III*** considering updated knowledge from other studies.

5.1 Maspin

Paper I demonstrates the **predictive** impact of maspin expressed in the nucleus. In this study, patients with a low expression of nuclear maspin benefit from adjuvant chemotherapy with 5FU and levamisole. Patients with high expression of nuclear maspin did not benefit from this treatment. The expression of maspin did not affect the prognosis of patients. As exemplified in the introduction of this thesis, most established biomarkers in colorectal cancer are not strictly predictive or strictly prognostic. A biomarker that affects the chemosensitivity of a tumor without affecting the ability to progress and metastasize is an example of a biomarker whose function is restricted to predicting the chemotherapy effect. The increased benefit of adjuvant chemotherapy in patients with low maspin may be explained by the pro-apoptotic function of maspin (264). One of the mechanisms behind chemotherapy-induced initiation of apoptosis is the release of cytochrome C from the cell mitochondria. A theory behind the anti-apoptotic function of maspin is that maspin can bind to cardiolipin in the mitochondria, thereby competing with cytochrome C and preventing apoptosis (265). The association between low expression of pro-apoptotic proteins and increased effect of chemotherapy has been described in metastatic melanoma (266). Still, to our knowledge, no in vitro or in vivo models assessing the effect of maspin expression on chemosensitivity in colorectal cancer cells have been published.

Few studies have assessed the predictive impact of maspin expression in colon cancer. In the study by Dietmaier *et al*., maspin was a predictor of the effect of fluorouracil, but here a *high* expression was linked to benefit (192). The authors did not provide any supplementing investigations explaining the link between high maspin expression and chemotherapy benefit.

We did not disclose a prognostic impact of either nuclear or cytoplasmic maspin expression. The early enthusiasm for maspin as a prognostic marker has been tempered by the discordant results from assessing the effect of maspin expression in patient cohorts. Most studies performed in colorectal cancer report that maspin expression is increased in colon cancer compared to normal colonic mucosa (192, 267). Other studies describe contrasting findings — a high expression in normal mucosa and loss of maspin expression in cancer (193). Some studies report that the nuclear expression of maspin is a prognostic marker (192). Others report that the prognostic impact of maspin is related to cytoplasmic expression (193, 268, 269). In addition, while most studies associate a *high* maspin expression with poor prognosis (192, 269, 270), others state that a *low* maspin expression is a marker of poor prognosis (193). One study shows that maspin expression indicates a good prognosis in the dMMR subgroup (268), while others show no overall prognostic impact of maspin (267). A recent publication suggests that the function of maspin in colorectal cancer might depend on the subcellular location of maspin expression (236, 264). Still, the discrepant findings in the published studies are difficult to explain from this perspective alone. The discoveries in the published literature diverge, and it is still not clear what the actual contribution of maspin to colon cancer development and progression is.

Maspin expression has been associated with right-sided colon cancer (195, 267), high tumor grade (267, 269), mucinous histology (270), high degree of tumor budding (195, 271), CIMP (195), and microsatellite instability (268). The consequences of these relations are not known, therefore, the prognostic impact demonstrated in some maspin studies might be confounded by these associations.

5.2 CDX2

We did not demonstrate any **predictive** value of CDX2 expression on the effect of adjuvant chemotherapy in ***paper II***. Still, the low prevalence of CDX2 negative patients may have caused our predictive models to be statistically underpowered. The pivotal study by Dalerba *et al.* suggested that patients with CDX2 negative tumors benefit from chemotherapy (202). In their study, the DFS rates were higher for the 23 patients with stage II CDX2 negative colon cancer tumors that had received chemotherapy than for the 25 patients who did not. This was a non-randomized, retrospective multi-center study, and receipt of chemotherapy varied between centers. Therefore, these results must be interpreted with caution. The Dalerba study did not assess whether the assumed increased benefit of chemotherapy in the CDX2 negative stage II subgroup is due to a decreased baseline prognosis compared to CDX2 positive cases or if CDX2 negative tumors are intrinsically more sensitive to chemotherapy than CDX2 positive tumors. However, in vitro studies conducted on colon cancer cell lines suggest that CDX2 positive cancer cells are less sensitive to chemotherapy (272-274).

Paper II demonstrated that the **prognostic** effect of CDX2 expression loss depends on the MMR phenotype, in agreement with other publications (203, 205). Loss of CDX2 expression was not a prognostic factor in dMMR tumors, while pMMR tumors with loss of CDX2 expression had a very poor prognosis.

The reason for the different prognostic effects of CDX2 loss in dMMR versus pMMR tumors is not known. Studies relating CDX2 expression to the different CMS groups may provide an explanation for this phenomenon. Pilati *et al.* report that 94% of tumors with CDX2 negativity belonged either to the CMS1 (MSI/immune) or CMS4 (mesenchymal/stem cell-like) group (203). CDX2 expression loss was a negative prognostic factor in the CMS4 group but not in the CMS1 group (203). There is a large overlap between CMS1 and dMMR (150). Subgroup analysis from the Pilati study showed that CDX2 negativity was not a prognostic factor in dMMR patients, in compliance with the findings of ***paper II***. As mentioned in the supplementary material and methods (section 3.5), we have determined CMS classification in a subgroup of 77

patients from the HDH material. Unfortunately, this subgroup contained only five CDX2 negative cases, all of which belonged to CMS1. Therefore, we cannot assess the validity of the Pilati study in our cohorts.

The CMS4 group has a dismal prognosis and is associated with the upregulation of genes important in epithelial-mesenchymal transition (EMT) (150). In the Pilati study, the CMS4/CDX2 negative cases had a particularly poor prognosis, regardless of BRAF mutational status. Interestingly, intact CDX2 antagonizes epithelial-mesenchymal transition (EMT) activity in several preclinical models (207, 275-277). The poor prognosis of pMMR CDX2 negative cases may be explained by the associations to the aggressive CMS4 phenotype and to increased EMT.

5.3 Tumor grade

In ***paper II***, high tumor grade predicted a good response to chemotherapy in patients with pMMR. Although high tumor grade is an acknowledged prognostic factor in CRC, few studies have explored the **predictive** value of high tumor grade. The recently updated ASCO guidelines (32) include two pooled analyses of studies attempting to determine the benefit of adjuvant chemotherapy for stage II colon cancer with poorly differentiated or undifferentiated tumors. When analyzing studies with OS as the end point (278-280), there was a statistically significant difference in OS favoring adjuvant chemotherapy. None of these studies included the impact of MMR phenotype.

A similar pooled analysis of studies with DFS as endpoint (279, 281, 282) was also performed. There was a non-significant tendency towards a benefit for adjuvant chemotherapy (32). The large heterogeneity in study design and definition of high tumor grade in these studies highlights the need for more research regarding the impact of high tumor grade on chemotherapy effect. The SACURA trial (282) was a randomized controlled study assessing the benefit of the oral-5FU agent tegafur-uracil compared to surgery alone for colon cancer stage II. In this study, tumor grade and tumor histology were not assessed separately. The histology group “poorly” was defined as poor differentiation or mucinous- or signet ring histology and was not a negative prognostic

factor in multivariate analyses. The Liu study (281) did not separate between neoadjuvant and adjuvant chemotherapy, and none of the cohorts were randomized. The Kumar study (279) was a retrospective study of a population-based cohort studying survival in stage II colon cancer among patients who received chemotherapy compared to those who did not. High-risk and low-risk traits were recorded, but the study was not randomized and could not adjust for comorbidities that may have influenced the choice of treatment. In addition to the mentioned study limitations, none of these three studies assessed the effect of MMR phenotype on the results. We, therefore, believe that ***paper II*** represents an important contribution to this field of research.

In ***paper II,*** high tumor grade was a marker of poor **prognosis** in pMMR tumors. Our findings are in line with other studies. High tumor grade is an established negative prognostic marker in CRC (283). Still, the negative impact seems to be restricted to pMMR cancers (284, 285). The ASCO 2021 colon cancer stage II guideline emphasizes that high tumor grade is not a prognostic marker in dMMR tumors, only in pMMR (32).

5.4 MMR and TIL

In ***paper III***, neither MMR phenotype nor TIL density predicted the effect of adjuvant chemotherapy with fluorouracil/levamisole (results not presented). This observation may partly be due to the lack of administered oxaliplatin in our randomized cohort. Oxaliplatin is considered the main driver of immunogenic cell death in tumors treated with FOLFOX (286). Oxaliplatin induces immunogenic apoptosis in colon cancers implanted into mice, but the effect depends on an intact immune system. In addition, as discussed in section 1.14.4, patients with dMMR tumors are believed to have an increased benefit of oxaliplatin-containing chemotherapy compared to receiving fluorouracil only (143-145).

Paper III shows that dMMR patients have a worse survival outcome than pMMR patients in stage III colon cancer. Attempting to explain the negative **prognostic** effect of dMMR in stage III colon cancer, we adjusted for the possible prognostic influence of PD-L1 expression and density of tumor-infiltrating CD3+ and CD8+ lymphocytes. Our

study demonstrated no independent prognostic effect of PD-L1 but known associations between positive PD-L1 expression and right-sided cancer, CDX2 negativity, dMMR, and TIL density were confirmed. dMMR remained a marker of poor prognosis in stage III also when adjusting for these variables.

The beneficial prognostic impact of dMMR on prognosis in stage II colon cancer has been attributed to the immunogenicity of these tumors (287). Still, there is increasing evidence that dMMR cancers are associated with worse survival in metastatic disease (98, 151, 288). The reason for the aggressive biology of dMMR mCRC is not known. Differences in metastatic patterns between dMMR and pMMR mCRC might contribute to the poor prognosis of dMMR mCRC. Liver and lung are the most common metastatic sites in mCRC (53), but dMMR mCRC is associated with a higher frequency of peritoneal metastases and isolated intra-abdominal metastases (101). Peritoneal metastases are associated with a poor prognosis compared to other metastatic sites (289). Differences in metastatic patterns are not likely to contribute to the poor prognosis in dMMR patients in ***paper III*** as dMMR status conveyed both a poor OS and DFS.

Another possible reason for the observed poor prognosis of our stage III dMMR patients could be a higher frequency of *BRAF* mutations in stage III versus stage II. Therefore, immunohistochemistry using the anti-BRAF VE1 antibody was performed on TMAs in the NGICG and HDH cohorts. Several studies report a high concordance between BRAF IHC and PCR-based methods (290), while others report that the results are discordant (291). Based on our IHC results, our study showed 21.5% *BRAF*V600E mutations (25% for stage II and 16% for stage III). 32.5% of the *BRAF* mutated patients were pMMR, 67.5% were dMMR. When *BRAF*V600E mutation status was included in our multivariable models, there was still a significant interaction between dMMR and stage, and dMMR was still an independent marker of poor prognosis in stage III colon cancer. However, when we validated the IHC detection of *BRAF*V600E mutations with *BRAF*V600 ddPCR, we found a 20% discrepancy between the results from BRAF IHC and ddPCR. We decided that this discrepancy was too large to include the BRAF IHC results in our manuscript. Not having other material available than the tissue cores

included in the TMAs, we were not able to extract DNA and perform BRAF PCR for the whole cohort. An example of *BRAF*V600 ddPCR is shown in Figure 14.

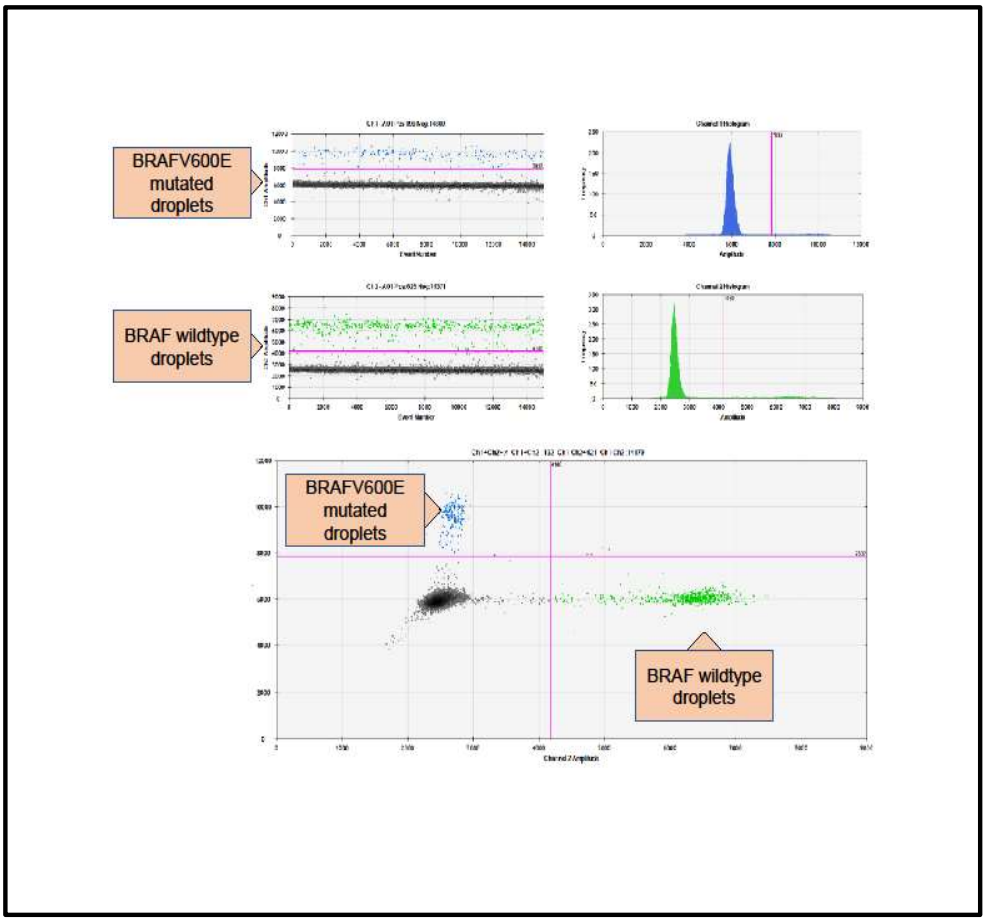


Figure 14: Example of a case with positive ddPCR for BRAFV600E mutation.

Not having *BRAF*V600E-mutation data included in ***paper III*** is an obvious study limitation. Still, we do not expect the survival differences between stage II and III dMMR cases to be explained by *BRAF*V600E mutations. First, based on the IHC results, *BRAF*V600E mutations were more common in stage II colon cancer than stage III. In addition, the prognostic impact of *BRAF*V600E mutation in the published literature seems to be stronger for pMMR cases than for dMMR (180).

In paper III, a high density of CD3+ and CD8+ TILs conveyed an improved prognosis, as expected, and both stage II and III dMMR tumors had a high infiltration of CD3+ and CD8+ TILs. Despite being a **prognostic** marker within dMMR CRC in other studies (290, 292), the density of TILs did not explain the difference in prognosis between stage II and stage III dMMR patients in our study. In a recent publication from a population-based cohort of mCRC patients, dMMR remained a marker of poor prognosis regardless of immune cell density (293). Still, dMMR tumors are associated with up-regulation of several immune checkpoints (294), and advanced-stage cases may represent a selected group of tumors able to escape immune surveillance. The methods in our study only allowed for the assessment of TIL density, and we were not able to assess signs of exhaustion of cytotoxic T cells (295).

6. Conclusions

This study has shown that a low expression of nuclear maspin in colon cancer might predict the effect of adjuvant fluorouracil. Still, maspin expression was not associated with other clinicopathologic markers in our NGICG cohort, and neither nuclear nor cytoplasmic expression of maspin was associated with prognosis. Our data indicated that lack of CDX2 expression in localized colon cancer is associated with dMMR, high tumor grade, and right-sided colon cancer. Patients with pMMR and CDX2 negativity represent a high-risk subgroup within stage II-III colon cancer. In our study, stage II-III colon cancer patients with pMMR and high tumor grade had a poor prognosis when treated with surgery only, but not when receiving fluorouracil/levamisole, indicating that this group of tumors might respond well to adjuvant chemotherapy. Infiltration of CD3+ and CD8+ lymphocytes was associated with improved prognosis in stage II and III colon cancer, both when assessed together and individually. We also demonstrated a prognostic shift in dMMR colon cancer. We propose that dMMR is a marker of improved prognosis in stage II colon cancer but a marker of poor prognosis in stage III colon cancer. The difference in the prognostic impact of dMMR between stage II and III colon cancer was not explained by differences in density of tumor-infiltrating lymphocytes, CDX2 expression, receipt of chemotherapy, or PD-L1 expression.

7. Future perspectives

A substantial scientific effort has been devoted to cancer biomarker discovery for the past decades. However, less than 1% of published cancer biomarkers end up being included in clinical practice (296). Here, I will discuss the potential future implications of our findings.

Despite numerous conducted biomarker studies, most routinely used anti-cancer drugs lack validated predictive biomarkers. In current practice, when conducting clinical studies for cancer types where chemotherapy is the standard treatment, having a randomized group not receiving chemotherapy is not possible. When assessing the predictive value of cancer biomarkers in non-randomized studies, results will be prone to selection bias. Our cohort presents unique opportunities to study biomarkers that may predict the effect of fluorouracil, like the maspin biomarker. The maspin biomarker is not ready to be implemented in clinical practice at this time. The discrepant results in published papers call for consensus on how to interpret the maspin staining. In addition, mechanistic studies should be performed to determine how maspin expression is expected to influence the chemotherapy effect.

In stage II colon cancer, high tumor grade alone does not warrant treatment with adjuvant chemotherapy (15, 32). Current guidelines for colon cancer stage II state that high tumor grade is a *minor* risk factor, and that adjuvant chemotherapy should be offered to pMMR patients with two or more minor risk factors. Still, surprisingly few studies have assessed the prognostic and predictive value of tumor grade. This information is easily obtainable from pathology reports. In addition, the combination of pMMR and CDX2 negativity may identify a group of stage II patients with a high risk of recurrence who may benefit from adjuvant chemotherapy. CDX2 immunohistochemistry is already part of the standard repertoire of many pathology departments. CDX2 staining is easy to interpret and inexpensive. In stage III colon cancer, the duration of chemotherapy is currently based on the TNM-subgroups from the IDEA study. Future studies will likely assess the role of biomarkers in selecting stage

III colon cancer patients for three versus six months of adjuvant chemotherapy, and the effect of CDX2 expression and tumor grade should be explored in this context.

Our study demonstrates a poor prognosis for dMMR stage III colon cancer patients, and the beneficial effect of dMMR seems to be restricted to the stage II subgroup. If validated in subsequent studies, this finding may have important implications for the management of stage III dMMR colon cancer. Treatment with immune checkpoint inhibitors has led to impressive response rates in localized dMMR colon cancer patients receiving neoadjuvant treatment (139, 297) and metastatic dMMR colon cancer (146, 298-300). Ongoing studies are assessing the effect of adjuvant immune checkpoint inhibitors combined with chemotherapy in dMMR colon cancer, *e.g.*, NCT02912559 and NCT03827044 (62). Alternative treatment options for dMMR cancers are also being studied, including the effect of CDK4/6 blockade in mouse models (301).

Our study confirmed the beneficial prognosis of colon cancer patients with increased density of tumor-infiltrating lymphocytes. Still, the clinical implications of TIL density/Immunoscore® need to be established. Due to the improved prognosis of colon cancer stage III patients with a high Immunoscore®, one would expect these patients to not benefit from adjuvant chemotherapy, a hypothesis being tested in the upcoming iMAGINE study (NCT04488159, (62)). Still, surprising results were demonstrated when analyzing the clinical utility of the Immunoscore® for patients from the IDEA study (302). A high Immunoscore® was associated with both an improved prognosis *and* an increased benefit from six months versus three months of adjuvant chemotherapy, both for high-risk and low-risk patients. These counter-intuitive findings might be explained by increased chemosensitivity of high Immunoscore® tumors.

New research methods are likely to impact cancer biomarker studies in the years to come. The Hyperion™ cytometry by time of flight (CyTOF) method (303) and multiplex immunohistochemistry (304) are promising techniques for assessing multiple biomarkers simultaneously in FFPE tissue. In addition, the cost of next-generation sequencing panels has decreased substantially over the last decade, and they are increasingly applied in cancer research. Gene expression signatures assays like the

ColoPrint® might distinguish patient relapse risk (305) and are being validated for clinical utility (NCT00903565). The ongoing Norwegian IMPRESS-N study examines potentially actionable molecular alterations in advanced cancer, including mCRC (NCT04817956) (62).

Novel research approaches in colon cancer may improve the opportunities to personalize treatment. The most encouraging results have been reported in the field of liquid biopsies, especially for the measurement of circulating tumor DNA (ctDNA). In the study by Tie *et al*., postoperative ctDNA was detected in 8% of stage II colon cancers (306). These patients had a 79% risk of recurrence compared to 10% for patients without detected ctDNA. Ongoing studies are evaluating whether adjuvant chemotherapy can lower the risk of relapse in stage II colon cancer patients with detectable ctDNA after surgery, including the COBRA (NCT04068103) and CIRCULATE (NCT04120701) trials (62). In addition, a phase 2 study is assessing the effect of adjuvant pembrolizumab in solid dMMR tumors with detectable ctDNA after resection (NCT03832569).

Colon cancer is a biologically diverse type of cancer, and disease outcomes can be difficult to predict. Further studies of colon cancer biomarkers are highly warranted to improve patient survival, avoid over-treatment, and ensure cost-efficacy for emerging expensive therapeutics.

8. References

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