Presence and effect of tumour biomarkers in a population-based series of metastatic colorectal cancer patients

Kristine Øvreås Aasebø

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2021

UNIVERSITY OF BERGEN

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Date of defense: 22.10.2021

Year: 2021

Title: Presence and effect of tumour biomarkers in a population-based series of metastatic colorectal cancer patients

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Print: Skipnes Kommunikasjon / University of Bergen

Scientific environment

This work was conducted at the Department of Clinical Science, Faculty of Medicine, University of Bergen and the PhD grant was funded by the University. Professor Halfdan Sørbye has been my main supervisor, with co-supervisors Professor Olav Dahl and Professor Ragnhild A. Lothe.

The studied patient cohort was enabled by a Scandinavian collaboration with Professor Bengt Glimelius at the Department of Immunology, Genetics and Pathology at Uppsala University in Sweden and professor Per Pfeiffer at Department of Oncology at Odense University Hospital in Denmark. Laboratory work was conducted by Professor Fredrik Pontens research group at Science of Life laboratory, Uppsala University, Sweden, and Ragnhild Lothes research group at the Department of molecular oncology, Institute for Cancer Research, Oslo University Hospital, Norway.

Acknowledgements

I am very grateful for the University of Bergen granting the PhD position and giving me the opportunity to start a scientific career. First of all, I would like to thank my main supervisor Professor Halfdan Sørbye for letting me take part in his research project on colorectal cancer patients. You have been a great supporter throughout these years, sharing your tremendous knowledge in the field and innovative ideas. Thank you for always being available, interested and engaged in all parts of my PhD work. Our meetings always made me feel motivated and encouraged me to continue. Thank you to my co-supervisor, Professor Olav Dahl, for introducing me to his research group and sharing his knowledge obtained after a life-long career as a clinician and scientific researcher. My co-supervisor, Professor Ragnhild A. Lothe, for taking part in our project and sharing her research facilities and deep knowledge. I really appreciated the warm welcome to your research group and the time you attributed to me in your busy schedule.

My heartfelt thanks are directed to Mette Myklebust, Benedicte Rosenlund and Kjersti Hestetun at Olav Dahl's research group for discussions and advice during our group meeting. A special thanks to Mette for always being available for questions, guiding me throughout this project with your cancer research expertise. Kjersti, my college and now a close friend. Thank you for all our scientific and non-scientific discussions during our long lunches and coffee breaks. Thank you for being there through this PhD roller-coaster adventure, always supporting me and cheering me on. Thank you for all our memorable travels together. I am so grateful that we were in this together. I would also like to thank the members of the prominent research group “Ladies who Lunch” for making my days so much more enjoyable. A special thanks to Hanna Dillekås and Hilde Smeland, for sharing ups and downs in our PhD journey, for always listening and for your good advices.

I would like to thank all the excellent co-authors of my papers; this thesis would not have been possible without your contributions. The study and patient inclusion were conducted through a Scandinavian collaboration with Professor Per Pfeiffer at Odense

University Hospital in Denmark and Professor Bengt Glimelius at Uppsala University in Sweden. Thank you for giving me the opportunity to do research on this cohort. A sincere thanks to Professor Bengt Glimelius for your tremendous support throughout the project. Thank you for your constructive comments and ideas for the manuscripts and for all the time you spent reading my drafts. Thanks to the thorough work of Randi Eikeland at the Clinical Cancer Research office at Haukeland University Hospital for data management and establishment of the clinical database. Geir Egil Eide at Centre for Clinical Research, Haukeland University Hospital and Department of Global Public Health and Primary Care, Lifestyle Epidemiology Group, University of Bergen, thank you for your excellent statistical advices.

All the patients included in the study also deserves my sincere thanks.

I am very grateful for the outstanding laboratory work conducted by Professor Fredrik Pontens research group at Uppsala University Hospital and Jarle Bruun and Christian Bergsland at Lothes research group at Oslo University Hospital. Anca Dragomir and Arthur Mezheyeuski, thanks for all your help interpreting the results and the great cooperation. A special thanks to Jarle Bruun for your thorough and invaluable attributions to my last publication, with your impressive scientific knowledge and research skills, introducing me to the laboratory world of cancer research. Thanks for the great discussions, sharing your opinions and ideas for future cancer biomarker research, travels and bicycling.

Last but not least, thanks to all my family and friends for your great support and encouraging words. My warmest thanks to my husband Olof Nilsson for all your love and support, for believing in me and pushing me to reach my goals. Your positive spirit always comforts me in difficult times. And our beloved children Alva and Arthur, you mean the world to me. Thank you for bringing so much joy into my life, constantly reminding me what's really important.

List of abbreviations

5-FU	Fluorouracil
AJCC	American Joint Committee on Cancer
APC	Adenomatous polyposis coli
BRAF	B-raf proto-oncogene
*BRAF*mut	*BRAF* mutation
*BRAF*wt	*BRAF* wildtype
CAPOX	Capecitabine and oxaliplatin
CDX2	Caudal-type homeobox 2
CEA	Carcinoembryonic antigen
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subgroup
CRC	Colorectal cancer
CRS	Cytoredctive surgery
CT	Computer tomography
CTLA-4	Cytotoxic T-lymphocyte protein 4
CTLs	Cytotoxic T-cells
DFS	Disease-free survival
dMMR	Defective DNA mismatch repair
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EMT	Epithelial-mesenchymal transition
FAP	Familial adenomatous polyposis
FDA	Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FOLFIRI	5-FU, folinic acid and irinotecan
FOLFOX	5-FU, folinic acid and oxaliplatin
FOLFOXIRI	5-FU, folinic acid, oxaliplatin and irinotecan
HER2	Human epidermal growth factor 2
HIPEC	Hyperthermal intraperitoneal chemotherapy
HNPCC	Hereditary nonpolyposis colorectal cancer
HRAS	Harvey rat sarcoma viral oncogene homolog
ICIs	Immune checkpoint inhibitors
IHC	Immunohistochemistry
KRAS	Kirsten rat sarcoma viral oncogene homolog
*KRAS*mut	*KRAS* mutation
MAPK	Mitogen-activated protein kinase
mCRC	Metastatic colorectal cancer
MHC	Major histocompatibility complex
MMR	DNA Mismatch Repair
MSI	Microsatellite instable

MSS	Microsatellite stable
NK	Natural killer
NRAS	Neuroblastoma rat sarcoma viral oncogene homolog
*NRAS*mut	*NRAS* mutation
ORR	Overall response rate
OS	Overall survival
PD-1	Programmed death 1
PD-L1	Programmed death ligand 1
PFS	Progression-free survival
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alfa
POLE	DNA polymerase epsilon, catalytic subunit
RAS	Rat sarcoma viral oncogene homolog
*RAS*mut	*RAS* mutated
RR	Response rate
TAMS	Tumour associated macrophages
TCR	T cell receptor
TILs	Tumour infiltrating lymphocytes
TMA	Tissue microarray
TP53	Tumour protein 53
Tregs	T regulatory cells
TSG	Tumour suppressor gene
UICC	Union of international cancer control
VEGFR	Vascular endothelial growth factor

Abstract

Background: Colorectal cancer (CRC) is one of the most common malignancies worldwide, and a substantial group of patients will develop metastases. Survival for metastatic CRC (mCRC) has improved, but mainly for trial patients. Precision medicine is essential to improve survival and avoid overtreatment. Studies of prognostic and predictive markers for mCRC patients are mainly based on highly selected patients in clinical trial cohorts.

Objective: We aimed to report real-world data on the incidence and impact of predictive and prognostic tumour biomarkers in a prospectively collected Scandinavian population-based cohort of mCRC patients.

Methods: Immunohistochemistry and DNA sequencing of tumour biomarkers was performed.

Results: The incidence of tumour microsatellite instability (MSI) and *BRAF* mutation (*BRAF*mut) was 7 % and 20 %, and both markers were associated with poor patient outcome. MSI was associated with *BRAF*mut and patient age, indicated poor response to 1st-line chemotherapy, and few patients received 2nd-line treatment.

Loss of CDX2 expression was identified in a subgroup of tumours, defining patients with poor prognosis and indicated inferior chemotherapy benefit. CDX2 loss defined new prognostic subgroups in *BRAF*mut and *KRAS* mutated cases, respectively.

In chemotherapy-treated patients, a high density of tumour infiltrating CD3 lymphocytes and CD68 macrophages were independent good prognostic markers for overall survival. MSI was an independent poor prognostic marker despite high immunogenicity.

Conclusions and consequences: We found a higher frequency of MSI and *BRAF*mut in this population-based mCRC cohort than previously reported. Patients with MSI tumours were much older and most harboured tumour *BRAF*mut, in strong contrast to patients in recent clinical trial cohorts. Furthermore, CDX2 status and immune markers beyond T-cell markers are emerging risk assessment biomarkers for mCRC.

List of publications

I. Aasebø K., Dragomir A., Sundstrøm M., Mezheyeuski A., Edqvist P.H., Eide G. E., Ponten F., Pfeiffer P., Glimelius B., Sorbye H.: Consequences of a high incidence of microsatellite instability and *BRAF*-mutated tumors: A population-based cohort of metastatic colorectal cancer patients. Cancer Med. (2019) 8:3623–35.

II. Aasebø K., Dragomir A., Sundstrøm M., Mezheyeuski A., Edqvist P. H., Eide G. E., Ponten F., Pfeiffer P., Glimelius B., Sorbye H.: CDX2: A Prognostic Marker in Metastatic Colorectal Cancer Defining a Better *BRAF* Mutated and a Worse *KRAS* Mutated Subgroup. Front Oncol. (2020) 10:8.

III. Aasebø K., Bruun J., Bergsland C., Nunes L., Eide G. E., Pfeiffer P., Dahl O., Glimelius B., Lothe R., Sorbye H.: Prognostic role of tumour infiltrating lymphocytes and macrophages in relation to MSI, CDX2 and *BRAF* status: A population-based study of metastatic colorectal cancer patients. *Manuscript*

Contents

1. Introduction

1.1 epidemiology and aetiology

Colorectal cancer (CRC) is ranked as the fourth leading cancer disease worldwide and the third leading cause of cancer-related death, with the highest incidence among Western and high-income countries. Globally there were 1.9 million estimated new cases and 0.9 million deaths in 2020 (1)

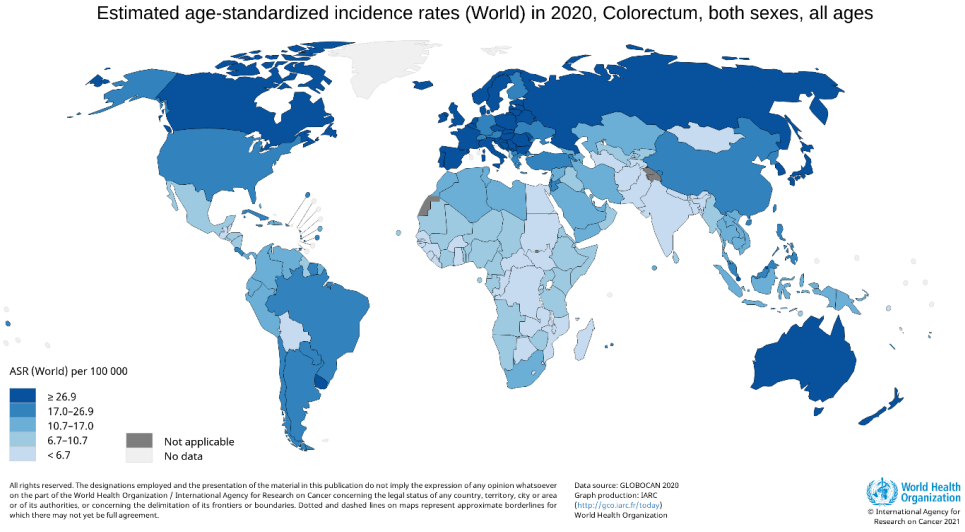

Figure 1 Estimated age-standardised incidence of colorectal cancer worldwide in 2020. Available from: https://gco.iarc.fr/today, accessed [13 03 2021](2).

In Scandinavia, CRC is the second most common cancer disease in men and women (3). The age-standardised incidence rate for men in 2018 was 82.5 in Norway and 76.2 in Denmark, and higher than what is observed in Sweden (56.2) (Figure 2) (3). CRC is generally a disease of the elderly population with a median age of around 70 years (4). Due to increased life expectancy and change in lifestyle, CRC incidence is still rising in many countries. In Norway, the incidence rate has nearly doubled since the 1970s, is steadily increasing, and now displays the highest incidence in Scandinavia. A less steep increase is seen in Denmark, and a relatively flat incidence curve is observed in Sweden during the same period (Figure 2) (3). The reason for the more pronounced

increase in Norway is not known, but obesity, dietary factors and gene pool vulnerability to environmental changes and lifestyle factors have been suggested. A substantial increase in the incidence among younger patients (<50 years) was recently reported in a study of seven high-income countries (5). A significant proportion of CRC patients have metastatic disease, approximately 25% of patients present with metastatic CRC (mCRC) at diagnosis, and another 20% will eventually develop metastasis (4).

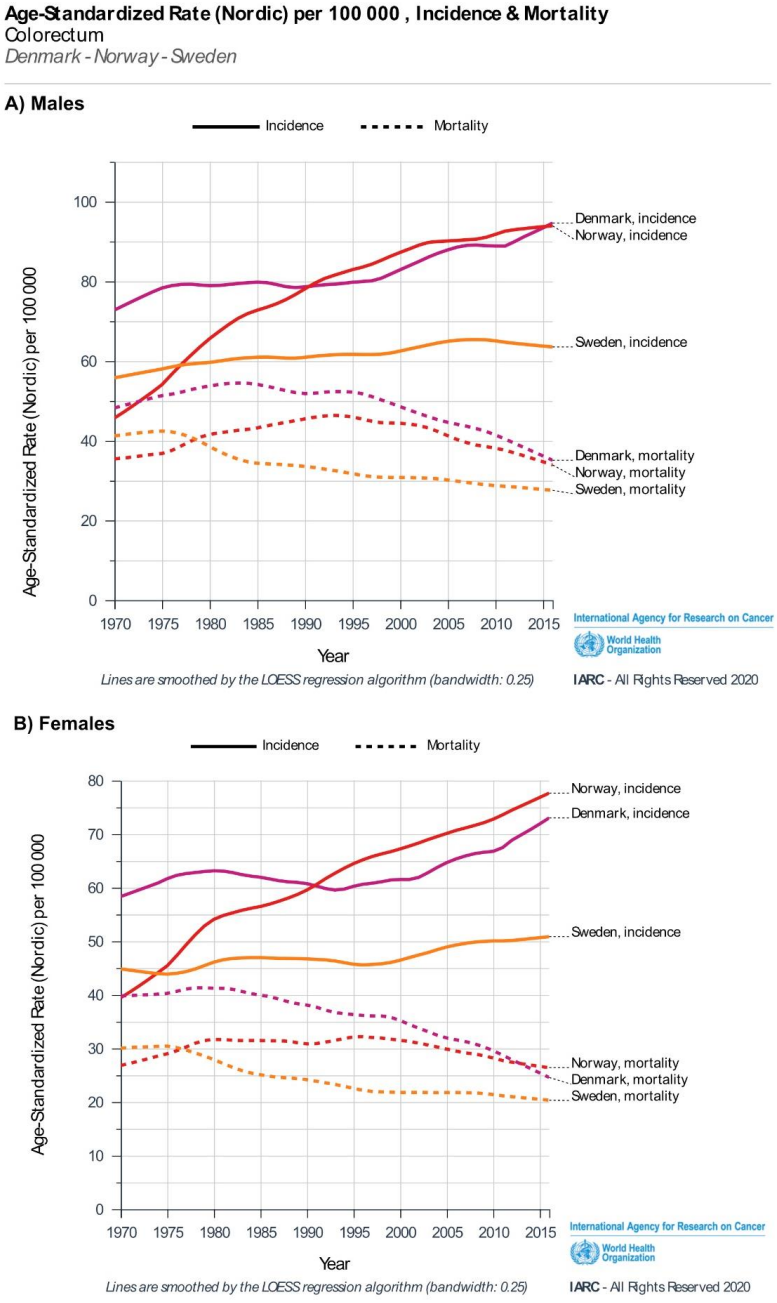

Figure 2 Trends in age-standardised incidence and mortality rates for colorectal cancer patients in Scandinavia. Available from: https://nordcan.iarc.fr/, accessed [13 03 2021] (3).

Several risk factors have been identified, causing epigenetic and genetic changes in colorectal epithelial cells, eventually developing into cancer. The epithelium displays the highest cell division (mitotic turnover) in the human body. Due to age-related changes such as stem cell senescence, genome instability, accumulation of mutations, telomere attrition and epigenetic alterations, age is considered the leading risk factor for epithelial cancers (6). Different lifestyle characteristics have also been linked to increased risk, such as a diet rich in red or processed meat, obesity, excessive alcohol consumption, smoking and reduced physical activity (7, 8). Changes in these lifestyle factors have been estimated as the most important preventive measures for CRC development (8, 9). Long-term use of aspirin (acetylsalicylic acid), a non-steroidal anti-inflammatory drug, has also been associated with reduced CRC incidence (10, 11). Several studies suggest that the gut microbiome plays a role in CRC development (12, 13) as intestinal dysbiosis and increased colonisation of specific microbes in intestinal mucosa and tumour tissue of CRC patients has been observed. However, factors that predispose to CRC, such as diet, physical activity and obesity, could alter the gut microbiome, and it is not clarified if the altered microbiome is a cause or a consequence of CRC development (14). Chronic inflammation is a well-established risk factor for cancer development, and an increased risk for CRC is observed in patients with chronic mucosal inflammation conditions such as ulcerative colitis (15). Around 20-30% of CRC occur in first- or second-degree relatives, indicating a hereditary component. However, only around 5% of cases are identified as hereditary cancer syndromes with known germline mutations (16). The two main cancer syndromes are hereditary nonpolyposis colorectal cancer (HNPCC), also termed Lynch Syndrome, and familial adenomatous polyposis (FAP). Most HNPPC cases present heterozygous genetic mutation in one of the DNA mismatch repair (MMR) genes, and in FAP, heterozygous mutation is found in the tumour suppressor gene (TSG) adenomatous polyposis coli (*APC*).

1.2 Diagnosis

1.2.1 Diagnosis and staging

The most commonly reported symptoms at diagnosis of CRC is faecal bleeding, abdominal pain and change in bowel habits (17). However, many patients report no symptoms, and if present, it often occurs late in the disease course. The transformation of precancerous lesions to CRC takes many years, and early detection is essential for curation. For these reasons, different screening programs, such as faecal blood tests, sigmoidoscopy and colonoscopy, of the healthy population above 50 years has been implicated in many countries. In European countries with long-standing screening programs, CRC incidence decreased over time, and these countries obtained the most significant decrease in CRC mortality (18). These findings support the initiation of screening programs, particularly in high-incidence countries such as Norway. A CRC screening pilot in Norway was recently published (19), and the health authorities have decided to initiate a national screening program for people above 55 years in 2021. In Denmark, a CRC national screening program was initiated in 2014.

Colonoscopy and proctoscopy with tumour biopsy are standard diagnostic procedures for colorectal cancer. Other imaging methods such as computer tomography (CT) and Magnetic resonance imaging (MRI) are also implemented for preoperative evaluation/investigation, staging the disease, and identifying metastatic spread. Histopathological staging of the tumour is performed after surgical resection. The most commonly used system for staging CRC is the TNM system for solid tumours, regularly updated by the Union for International Cancer Control (UICC) (Table 1) (20). According to the American Joint Committee on Cancer (AJCC) staging system, the TNM classification is further used to categorise CRC into four stages. Stage I-III represent localised tumour according to T and N status, and stage IV is the presence of distant metastases (Table 2). Prognosis is strongly associated with this staging system, with a poorer prognosis with increasing stage due to a higher risk of metastases. Estimated 5-year relative survival for male colon and rectal cancer patients in Norway diagnosed in 2015-2019 is 98.3 for stage I-II, 84.4 % for stage III and 15.5 % for stage IV disease (4). Localised disease is most often curable with surgery, while stage IV is

usually fatal. Staging is therefore essential for prognostic assessment and is used to guide treatment strategies for each patient.

Table 1 The Union for International Cancer Control (UICC) TNM classification of malignant tumours, colorectal cancer, eight edition (20)

T – Primary Tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ: invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades subserosa or into non-peritonealised pericolic or perirectal tissues
T4a	Tumour perforates visceral peritoneum
T4b	Tumour directly invades other organs or structures
N – Regional Lymph Nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2 to 3 regional lymph nodes
N1c	Tumour deposit(s), i.e. satellites, in the subserosa, or in non-peritonealised pericolic or perirectal soft tissue without regional lymph node metastasis
N2a	Metastasis in 4–6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
M – 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
M1c	Metastasis to the peritoneum with or without other organ involvement

Table 2 American Joint Committee on Cancer staging of colorectal cancer according to the UICC TNM classification of malignant tumours, eight edition (20)

AJCC staging	**TNM**
Stage I	T1, T2 N0 M0
Stage II	T3, T4 N0 M0
Stage III	Any T N1, N2 M0
Stage IV	Any T Any N M1

1.2.2 Histopathological evaluation

Adenocarcinoma is the most commonly identified phenotype in colorectal cancer. Adenocarcinomas are further graded according to differentiation from grade I-III. Grade I refer to highly differentiated tumours, and grade III poorly differentiated tumours. A high tumour grade is associated with a poor prognosis. Some of these tumours produce mucin, and others are composed of signet ring cells. Tumour budding, extramural venous invasion (EMVI) and perineural infiltration has also been

associated with poor prognosis and is recommended to be included in the pathology report (21).

1.3 Prognosis

The prognosis for CRC patients has greatly improved in the last decades, and 5-year overall survival (OS) is around 66% in Scandinavia (Figure 3). The observed improved prognosis is probably due to earlier and enhanced detection methods, improved surgical treatment, chemoradiation for localised rectal cancer and adjuvant chemotherapy for locoregional disease. Improved prognosis is also observed for mCRC patients due to development in systemic treatment options, personalised treatment approach, and a more aggressive surgical approach if resectable metastases. In 2010-2015 the 5-year OS for mCRC patients in Norway was 16% and 20% for colon and rectal cancer, respectively, compared to 5% and 4% in 1980-1984 (4). The large majority of patients with metastatic disease cannot be cured, illustrated by a median survival of 20-30 months and five-year survival of 9-19% in study patients (22, 23). However, median OS is only 15 months for chemotherapy-treated patients in population-based cohorts (24, 25), and an even more grim prognosis is reported in population-based registries with median survival of 5-10 months and five-year survival 0-9% (26).

Figure 3 Trends in 5-year age-standardised relative survival for colorectal cancer patients in Scandinavia. Available from: https://nordcan.iarc.fr/, accessed [13 03 2021]

1.4 Treatment

1.4.1 Localised CRC

In localised CRC without metastases, surgical resection of the primary tumour with sufficient margins is mandatory for curating the disease. The addition of chemo- or radiotherapy before (neoadjuvant) or following (adjuvant) surgery is based on specific risk factors for recurrence and survival (27). In colon cancer with lymph node metastases (stage III) or high-risk stage II, adjuvant chemotherapy with fluorouracil (5-FU)/folinic acid (FA) or capecitabine with oxaliplatin (FOLFOX or CAPOX) for 3-6 months is recommended to improve disease-free survival (DFS) and OS for these patients. For rectal cancer, neoadjuvant radiotherapy (5Gy x 5) followed by chemotherapy (FOLFOX or CAPOX) is recommended if the tumour is classified as T4 or N2, presence of extramural venous invasion, tumour or pathological lymph node is located close to (< 1mm) or outside the mesorectal fascia. However, preoperative long-term chemoradiation is recommended for very locally advanced tumours. In the case of tumour perforation or insufficient resection margins obtained during surgery, adjuvant chemoradiotherapy is recommended for previously untreated patients. The use of adjuvant chemotherapy for rectal cancer is debated internationally and generally not recommended in Norway but might be considered if risk factors are identified after surgery (27).

1.4.2 Metastatic colorectal cancer

The most frequent location of synchronous mCRC is liver (74%) or peritoneum (23%) (28). In a metachronous setting, liver (60%), lung (39%), lymph nodes (22%) or peritoneum (19%) are the most commonly affected sites (29).

Metastatic surgery

The main treatment option for most mCRC patients is systemic treatment with palliative intent, although some patients have a possible curative option with metastasectomy if resectable single organ metastasis to liver, lung or peritoneum. Metastasectomy is performed either primarily or after downsizing with chemotherapy.

The effect of perioperative or adjuvant chemotherapy after metastasectomy is not clarified. In Norway, around 20% of mCRC patients with liver metastases undergo liver resection, with a 4-year OS of 55 % compared to 9 % in the unresected group (30), but unfortunately, the majority relapse (31, 32). Pulmonary resection is less frequent and, in a Danish study, only performed in 28 of 736 cases with solitary pulmonary metastases (33). If the patient is considered inoperable due to poor performance status or technical reasons, radiofrequency ablation or stereotactic radiation could be an option in the case of minor and limited lung or liver metastases. Maximal cytoreductive surgery followed by hyperthermal intraperitoneal chemotherapy (CRS HIPEC) is recommended for selected patients with resectable peritoneal metastases (34). This treatment method has shown 40 % 5-year OS in this poor prognostic group (35). The method consists of surgical removal of all macroscopic tumours, followed by intraperitoneal heated chemotherapy administration, attempted to kill remaining cancer cells.

Systemic treatment

Chemotherapy is the major backbone of systemic treatment (Figure 4), although the response and survival benefits are limited. The two main chemotherapy treatment options are combination treatment of 5-FU/FA with oxaliplatin (FOLFOX) or irinotecan (FOLFIRI). The response and survival benefit of these regimens are considered equally efficient, with a 20-30 % increased overall response rate (ORR) and 2-3 months increased progression-free survival (PFS) and OS compared to 5-FU/FA alone (36, 37). A higher RR and survival are obtained with combined targeted antibody treatment; epidermal growth factor receptor (EGFR) inhibitor (cetuximab or panitumumab), or vascular endothelial growth factor (VEGFR) inhibitor (bevacizumab). EGFR inhibitors bind to the EGFR receptor on the surface of tumour cells and other human body cells, blocking the signalling cascade of the RAS-RAF-MAPK pathway, otherwise leading to cell growth and proliferation. Patients with tumour Rat Sarcoma viral oncogene homologue mutations (*RAS*mut) have no survival benefit from EGFR-inhibitors due to constant signalling through the RAS-RAF-MAPK pathway. For *RAS* wildtype patients, the addition of EGFR inhibitors has

shown 10-20 % increased RR, one-two months increased PFS and around five months increase in OS compared to FOLFOX or FOLFIRI alone (38, 39). VEGFR inhibitor binds to VEGF, secreted by several cells of the human body, including endothelial cells and cancer cells. VEGF induces the development of blood vessels (angiogenesis), important for tumour development and progression. The addition of the VEGFR-inhibitor bevacizumab has shown around 10 % improvement in RR and 1-4 months improved PFS and OS compared to combination chemotherapy alone (40, 41).

Studies of oral fluoropyrimidine TAS-102 (Lonsurf) and a multikinase inhibitor regorafenib have shown survival benefit in 3rd line setting. Due to marginal survival benefit and a high toxicity profile, it is considered the last treatment option for selected patients with good performance status.

Patients with tumour B-raf proto-oncogene mutation (*BRAF*mut) have a poor prognosis with often rapidly progressive disease. These patients are often recommended intensified 1st-line chemotherapy with triplet chemotherapy regimen FOLFOXIRI plus bevacizumab if considered eligible for this intensive treatment with a high toxicity profile (42, 43). Recently 2nd-line treatment with BRAF inhibitor (encorafenib) combined with EGFR inhibitors has shown promising results in patients with *BRAF*mut tumours (44). This treatment is now approved as 2nd-line treatment by the USA Food and Drug Administration (FDA) and European Medicines Agency (EMA). Reimbursement is approved by the health authorities in Denmark but currently under consideration at New Health Technologies (Nye Metoder) in Norway (45).

A major breakthrough in cancer treatment was achieved by developing immune checkpoint inhibitors (ICIs), inhibiting the negative regulation of immune cells. For mCRC patients, durable response and survival benefit of programmed death 1 (PD-1) ICIs (pembrolizumab and nivolumab) have been obtained in patients with tumour microsatellite instability (MSI) status (46, 47). This was recently validated in a randomised 1st-line study of pembrolizumab vs investigators choice of standard chemotherapy, with median PFS 16.5 vs 8.2 months and ORR 44 % vs 33 % (48). Pembrolizumab is approved by both FDA and recently EMA as 1st-line treatment for

mCRC patients with MSI tumours, and reimbursement is under consideration at New Health Technologies in Norway.

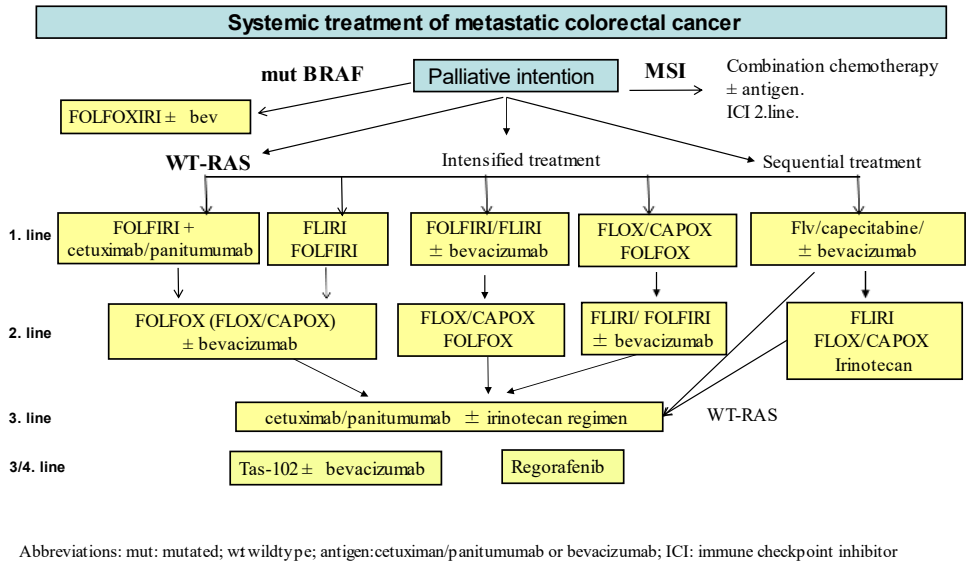

Figure 4 Recommended treatment algorithm for metastatic colorectal cancer patients. Figure from the Norwegian national guidelines for the treatment of colon and rectal cancer, modified with permission from the author (27)

1.5 Population-based cohort studies

Studies on population-based cohorts are essential to understand the biology and prognosis of the patients that we meet in the clinic. However, most biomarker studies are based on trial patient cohorts, and these patients are highly selected compared to the general mCRC patients. In our Scandinavian population-based cohort, patients included in trials were significantly younger, had better performance status, less often peritoneal metastases and abnormal blood tests (haemoglobin, white blood cell count, ALP and LD) (49). As the majority of mCRC patients are not represented in clinical trials, there is a risk that the trial results will not replicate in the clinical practice. In recent mCRC trials, median survival has reached up to 30 months, compared to 10-15 months in population-based cohorts (24, 26). Previous biomarker studies have revealed

few cases with specific tumour molecular alterations. But due to the inferior survival in population-based cohorts, there is reason to believe that poor prognostic markers would be more frequent in unselected cohorts. We have previously demonstrated a higher frequency of *BRAF*mut in our cohort (25), and we hypothesize that other poor prognostic markers would also be more frequent. There is a need for predictive and prognostic markers validated in population-based cohorts to guide treatment selection and improve mCRC patients' survival.

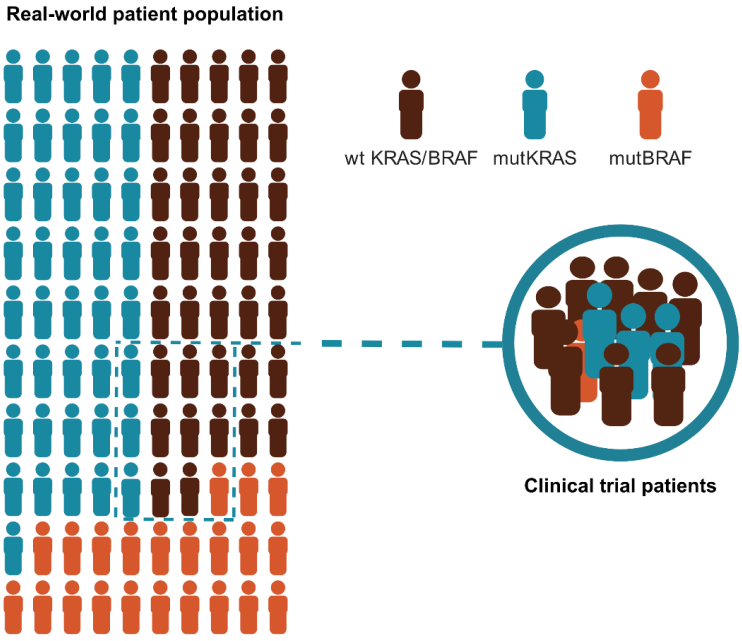

Figure 5 Most biomarker studies are based on trial patient cohorts, but the selected patients in clinical trials might not represent the general patient population.

With a median age of 70 years at diagnosis, mCRC is generally a disease of the elderly. However, little is known about the optimal treatment of elderly patients, as they are usually not included in clinical trials. Elderly patients have more comorbidities and could have functional and organ decline with increased risk of side effects. They have an inferior outcome than younger patients, and chemotherapy

receipt is inversely associated with age (26). However, elderly patients today are more fit and eager to receive chemotherapy (50). The selection of elderly patients that may profit from treatment without risking too much toxicity and which treatment to choose for each patient is an everyday challenge for clinicians. Population-based cohorts are therefore ideal for studying this large subgroup of mCRC patients.

1.6 Molecular characteristics of CRC

Both gene defects and epigenetic changes are involved in tumour development. Gene defects have been identified as causes of inherited CRC syndromes, including *APC* gene alterations in FAP and defects in the DNA MMR system in HNPCC. In sporadic CRC, alterations in these and other genes accumulate in a somatic lineage during life. Such alterations include point mutations, amplifications, insertions or deletions of DNA sequence stretches. Three classes of genes are affected in the malignant cells; tumor suppressor genes and repair genes often need to be in a loss of function state to exert a selection advantage to the tumor cells, whereas protooncogenes will be become active oncogenes through increased expression, typically by mutation, gain/amplification or chromosomal translocation.

Epigenetics has a vital role in preserving genomic instability, embryonic development and tissue differentiation. The epigenetic machinery controls gene expression by attaching or removing chemical groups (mainly methyl, phosphor and acetyl-groups) to DNA, chromatin and histones, causing modification and accessibility of DNA structure. Factors influencing epigenetic regulation are diet, chemicals, age, bacteria, although some traits are inheritable (6).

The Adenoma-Carcinoma sequence

Adenomatous polyps (adenomas) are identified as precursor lesions to CRC that develop from glandular epithelium. They display dysplastic morphology and altered differentiation compared to polyps or normal colonic epithelium. However, only a fraction of adenomas eventually develop into adenocarcinoma, and the progression is thought to take many years (51). Accumulation of multiple genetic and epigenetic events is required for CRC development by an evolutionary process termed clonal

selection. This multistep genetic model of colorectal carcinogenesis is proposed with mutation of the TSG *APC* occurring as an early event, followed by Kirsten Rat Sarcoma viral oncogene homologue (*KRAS)* mutation (*KRAS*mut) and subsequent inactivation of the TSG Tumour Protein 53 (*TP53) (52)*. Genes that control cell survival, cell fate and genome stability are referred to as driver genes. At least 2-3 driver gene mutations are needed for a normal cell to differentiate into a cancer cell (6). Each individual tumour contains numerous mutations, with only a handful of driver gene mutations, and the majority classified as passenger mutations (53). The most common driver gene mutations in CRC are *APC*, *KRAS*, *BRAF*, *PIK3CA*, *SMAD4* and *TP53*.

It was later identified that not only adenomas but also serrated polyps could develop into adenocarcinoma through an alternative pathway (CIMP). In general, three distinct pathways for CRC development have been identified: 1) mutations in DNA mismatch repair leading to microsatellite instable (MSI) phenotype, 2) mutations in *APC*/Wnt pathway characterised by Chromosomal instability (CIN) phenotype and 3) genome hypermethylation in CpG island methylator (CIMP) phenotype (figure 6). These pathways are not mutually exclusive, and a tumour can evolve through multiple pathways (54).

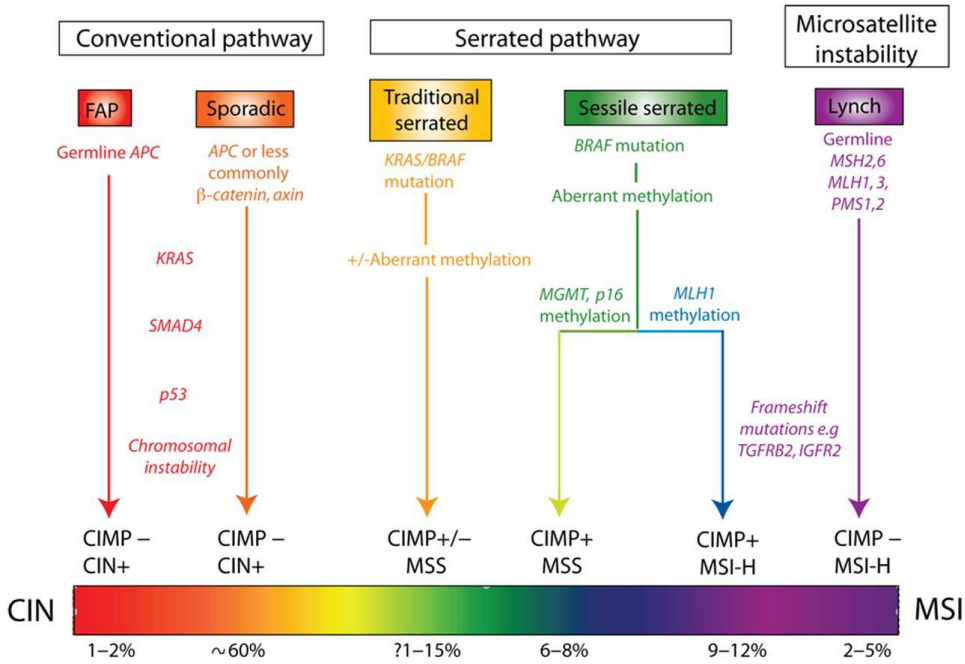

Figure 6 Pathways leading to colorectal cancer development, reprint from East 2017 (55) with permission.

1.6.1 Microsatellite instability

Microsatellites are short repetitive DNA sequences flanked by unique sequences, scattered throughout the genome, and prone to high mutation rates when replicated. The DNA mismatch repair (MMR) complex's role is to identify and repair DNA replication errors. This complex consists of several genes, the essential being: *MLH1*, *MSH2*, *MSH6* and *PMS1/2*. The MMR system is well conserved from the bacterial MutS,L,H system to humans. Among the human homologs of MutS, a heterodimer of MSH2 with MSH6 or MSH3 recognizes mismatches and small insertions or deletions. The homologs of MutL make a heterodimer of MLH1 with PMS2, PMS1 or MLH3, acting as endonucleases after complexing with MutS. Genetic or epigenetic inactivation of MMR genes, termed deficient MMR (dMMR), results in microsatellite instability (MSI) (56). A tumour demonstrating MSI in ≥ 30 % mononucleotide or dinucleotide repeats are termed MSI high (57). CRCs with dMMR/MSI are hypermutated with >10 mutations/Mb (58). They typically acquire somatic mutations

in short repetitive sequences, frequently found in TSG. Protooncogenes and TSG with repetitive sequences are the major somatic mutations in these tumours (54). MSI-induced frameshift mutations lead to a significant amount of neoantigens that make MSI tumours more immunogenic with increased amounts of tumour infiltrating lymphocytes (TILs) compared to Microsatellite stable (MSS) tumours. MSI tumours are more often located in the proximal colon, poorly differentiated, with mucinous or signet ring histological type (56).

HNPCC is identified as the cause of 2-4 % of localised CRC. These patients present an autosomal dominant inheritance of CRC caused by germline mutations in one of the four MMR genes or the epithelial adhesion molecule (EPCAM) gene, leading to transcriptional inactivation of MSH2. Some families have also been identified with heritable MLH1 or MSH2 promoter methylation. Patients with HNPCC often develop several tumours at an early age, including colorectal, ovarian, endometrium and stomach cancer, amongst others (56). Alterations of the microsatellite sequence are seen at many loci across the genome in all tumours. Sporadic MSI tumours occur in around 15 % of localised CRC (54, 59) and are generally caused by epigenetic loss of MLH1 gene expression by promoter hypermethylation through the CIMP pathway (discussed later). It often coexists with the gain of *BRAF*mut, and the presence of *BRAF*mut generally rules out an inherited cause. Patients diagnosed with MSI and *BRAF* wildtype (*BRAF*wt) CRC are recommended to proceed with genetic screening with blood-based germline mutation analysis for HNPCC (60).

1.6.2 Chromosomal instability

Chromosomal instability (CIN) tumours develop through the classical adenoma-carcinoma sequence and are present in 70-85 % of CRC (61, 62). They are characterised by an imbalance in chromosome number and loss of heterozygosity. Mutations in several genes that drive chromosome alterations have been proposed as potential mechanisms of CIN (62). The resulting alterations in chromosome segregation, telomere dysfunction and DNA damage response leads to mutations in specific oncogenes and TSG such as *APC*, *KRAS*, *PIK3CA* and *TP53* (61). *APC* inactivation is thought to be a key initial mutation in CIN, followed by *KRAS*mut.

1.6.3 CpG island methylator phenotype, the serrated pathway

CpG-islands are regions in the genome rich in CpG dinucleotides often seen in promoter regions of genes. More than 50 % of human genes are epigenetically regulated by methylation at CpG-islands, leading to silencing of gene expression. CpG island methylator phenotype (CIMP) tumours are hypermethylated at many different CpG-islands resulting in epigenetic instability and inactivation of TSG. They are believed to represent around 20 % of all CRC (62) and present a distinct histological phenotype, apparent already in early lesions, termed Sessile serrated adenomas (55). They are more often observed in patients with right-sided primary tumour location, more advanced tumour stage, female gender and older age and has also been associated with a poor prognosis (63). There is considerable overlap with the MSI pathway as they often display methylated promoter CpG island of MLH1, which accounts for most sporadic MSI (62, 64). They often have co-occurring *BRAF*mut and promoter methylation induced loss of the TSG caudal-type homeobox 2 (CDX2) (55, 65).

1.6.4 Oncogenes and tumour suppressor genes

The RAS proteins control several signalling pathways within the cell (66). In response to extracellular growth factors binding to endothelial growth factor receptors (EGFR), they regulate multiple cellular functions, such as proliferation, apoptosis and angiogenesis (Figure 7). RAS proteins belong to the family of small GTP binding proteins (GTPases). In the inactive state they bind GDP. Upon activation, GDP is phosphorylated to GTP, changing the conformation of the RAS protein. Mutated *RAS* remains active, leading to continuous signalling in downstream pathways (Ras/Raf/MAP/MEK/ERK and PI3K) (Figure 6). The subgroups of the RAS protein family consist of KRAS, Neuroblastoma rat sarcoma viral oncogene homolog (NRAS) and Harvey rat sarcoma viral oncogene homolog (HRAS). *KRAS*mut is the most common *RAS* mutation and has been identified as an early event in CRC development and is found in 40-50 % in mCRC (25, 38, 67). The most frequent location of *KRAS*mut is point substitutions in exon two codons 12 and 13 and less common at

exon three and four. The frequency of *NRAS* mutation (*NRAS*mut) in mCRC is 3-7 % (38, 68, 69).

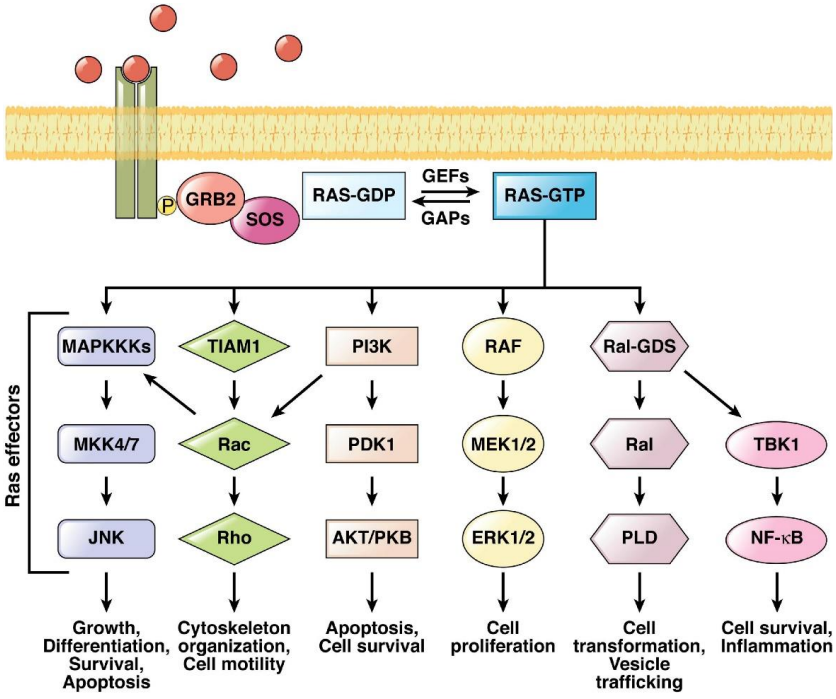

Figure 7 The RAS signalling pathway is activated by growth factors binding to endothelial growth factor receptors on the cell surface, leading to GTP binding and conformation of RAS to its active state and subsequent signalling in downstream pathways. Reprint from (61) with permission

The *BRAF* proto-oncogene belongs to the RAF family of serine/threonine protein kinases. BRAF proteins are activated by RAS in the EGFR-mediated RAS pathway. They regulate the Mitogen-activated protein kinase (MAPK) pathway, activating MAPK effectors MEK and ERK, leading to cell growth, proliferation, differentiation, migration, apoptosis and survival (70). *BRAF*mut is frequently found in human cancers, especially malignant melanoma, thyroid, ovarian and colorectal cancer. The most common *BRAF*mut is V600E *BRAF* with T>A transversion at position 1799 of exon 15, resulting in the substitution of Valine by Glutamate at position 600 (70, 71). This resembles the phosphorylation needed for BRAF activity, leading to continuous activation. *BRAF*^{V600E}mut occurs in 10-15 % of mCRC (72) and has been associated

with right-sided tumours, female sex, older age, and sporadic MSI. *BRAF*^{V600E}mut and *KRAS*mut are mutually exclusive. Non-V600E mutations have been identified in approximately 2 % of mCRC (73, 74). These have a distinct clinical subtype associated with left-sided primary tumour location and fewer peritoneal metastases and are not associated with MSI, as compared to *BRAF*^{V600E}mut (73, 75).

Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alfa (*PIK3CA)* protooncogene is a kinase in the PI3K/AKT1/MTOR pathway, located downstream of the RAS signalling pathway, regulating cell proliferation, survival and motility(6). Mutations lead to continuous activation and signalling and occur in approximately 15-20 % of CRC (76).

TP53 TSG is the main cell cycle checkpoint, halting the cell cycle in response to DNA damage and initiate repair or apoptosis (77). Loss of function allows excessive proliferation and mutations. *TP53* mutation is common in many cancers and occurs in nearly 60 % of CRC (78) and > 70 % in liver metastases from CRC (79) and plays an essential role in the classical adenoma to carcinoma sequence.

The adenomatous polyposis coli (*APC*) gene is a TSG and is the most frequently mutated gene in sporadic CRC. It is considered a gatekeeping mutation of CRC, present in 70-80 % of carcinomas (6). It is found in small benign adenomas and dysplastic epithelium, suggesting an early event in the development of most adenomas. Germ-line mutations in *APC* is also identified in some hereditary syndromes (FAP, Turcot and Gardner) (54). APC is thought to regulate cell-cell adhesion, migration, chromosomal segregation and apoptosis in the colonic crypt. It binds and regulates β-catenin, inhibiting the β-catenin-dependent Wnt signalling pathway. If APC is inactivated, β-catenin accumulates in the cytoplasm and translocate to the nucleus, activating many different genes leading to proliferation, differentiation, migration and adhesion of colorectal cells (54). Together with TGF-β, β-catenin induces epithelial-mesenchymal transition (EMT) (6), which is important for cancer progression and metastasis.

The *POLE* gene encodes the catalytic subunit of DNA polymerase epsilon, one of the main DNA replication enzymes. Germline mutation is identified in polymerase proofreading associated polyposis. Somatic *POLE* mutations give rise to an ultramutable tumour (62). *POLE* mutation is an uncommon event in CRC, identified only in 1-2 % of cases (80). As for MSI/Lynch syndrome CRC patients, tumour *POLE* mutation is associated with younger age at diagnosis, right-sided primary tumour and increased TILs, and improved prognosis in early-stage (80). Despite similar clinicopathological characteristics to MSI tumours, they are generally identified as MSS (62).

Human epidermal growth factor 2 *(HER2)* oncogene, also known as *ERBB2*, encodes a transmembrane glycoprotein receptor and is a member of the EGFR family. Upon activation, it stimulates signal transduction through the RAS mediated pathway(81). In CRC, the prevalence of overexpression is only around 2 %, and the prognostic effect is not clarified (82, 83). Mutations in *HER2* occurs in 2-6 % (84, 85) in CRC. These mutations are believed to be activating mutations, further stimulating the RAS signalling pathway.

Caudal-type homeobox 2 (*CDX2*) is an intestine-specific transcription factor and function as a master gene regulator of gene expression in intestinal epithelial cells, regulating cell differentiation, proliferation, adhesion, and migration. It is essential for intestinal embryonal development and the homeostasis of the continuously renewing mature intestinal epithelium. The protein expression of CDX2 is considered one of the most sensitive and specific intestinal differentiation markers (86). Loss of CDX2 expression causes changes in the mucosal architecture leading to developmental disorders and is believed to be involved in intestinal inflammation such as inflammatory bowel disease (87). There is also increasing evidence that *CDX2* play a tumour suppressive role in carcinogenesis. Studies of heterozygous *CDX2* mice developed multiple colonic polyps with an increased risk of tumour development (88). A recent study demonstrated that CDX2 inhibited EMT and the development of liver metastases of CRC (89). Loss of CDX2 expression is identified in a subset of CRC, but the incidence in the general population of mCRC patients is not clarified. Loss is

rarely caused by a mutation in the *CDX2* gene but rather as a consequence of promoter methylation. Together with *BRAF*mut, it is often associated with the serrated (CIMP) pathway (65). It has also been associated with MSI, right-sided primary tumour location, poor tumour differentiation and advanced tumour stage (90). CDX2 loss has been found enriched in consensus molecular subgroup (CMS) 1 and CMS 4 subgroup (91, 92).

1.6.5 Consensus molecular subgroup (CMS) classification

In 2015 a consensus of molecular subgroups based on gene expression data from the primary tumour of 4151 CRC patients was published (93). Four different clusters were identified, termed consensus molecular subgroup (CMS) 1-4. However, 13 % of patients were defined as mixed or unclassified. The CMS1 (MSI) subgroup was found in 14 % of patients and was associated with MSI, CIMP high, hypermutation, *BRAF*mut, immune infiltration, and worse survival after relapse. The CMS2 (epithelial) subgroup was found in 37 % of patients and was associated with the Wnt pathway and MYC activation. The CMS3 (epithelial) subgroup was found in 13 % of patients and was associated with mixed MSI status, CIMP low, *KRAS*mut and metabolic deregulation. The CMS4 (mesenchymal) subgroup was found in 23 % of patients. It was associated with stromal infiltration, TGF-β activation, angiogenesis, metastatic disease and worse OS and recurrence-free survival. The development of CMS classification was mainly based on analyses of localised CRC patients but has later been reported as a potential prognostic classifier in mCRC trial cohorts (94, 95). It has also been suggested to stratify treatment response or resistance, but standardization of methods on FFPE tumour tissue and prospective clinical trials are needed (96, 97). At present, CMS classification has no clinical implication.

1.7 Cancer development and the immune system

For centuries, it has been acknowledged that the immune system plays an essential role in cancer development and progression in conflicting ways. Tumour-promoting inflammation and evasion of immune destruction have been recognised as common traits for the cancerous transformation of normal cells and are proposed as important

contributors to the “Hallmarks of cancer” (98) (Figure 8). It is well known that some tumours are highly infiltrated by immune cells, and some chronic inflammatory conditions and viral infections eventually develop into cancer. Inflammation and tumour infiltrating immune cells secrete growth factors and other signalling molecules, stimulating tumour proliferation, survival, angiogenesis, invasion and metastasis (99, 100). Although driver gene mutations and genomic- and epigenomic instability are essential for the initial tumour formation, microenvironmental stimuli are needed to evolve metastasis (99, 101). The immune system is therefore believed to play an essential role in tumour development.

On the contrary, the immune system can recognise and evade tumour cells at an early stage (100, 102), a process called immune surveillance. Immune surveillance is believed to be the reason for the increased risk of cancer developement in immunocompromised individuals and the improved prognosis in patients with highly inflamed tumours (103). In the last two decades, the discovery of ICIs that inhibits T cells' negative regulation has led to a major breakthrough in cancer treatment and was awarded the Nobel Prize in Physiology or Medicine in 2018.

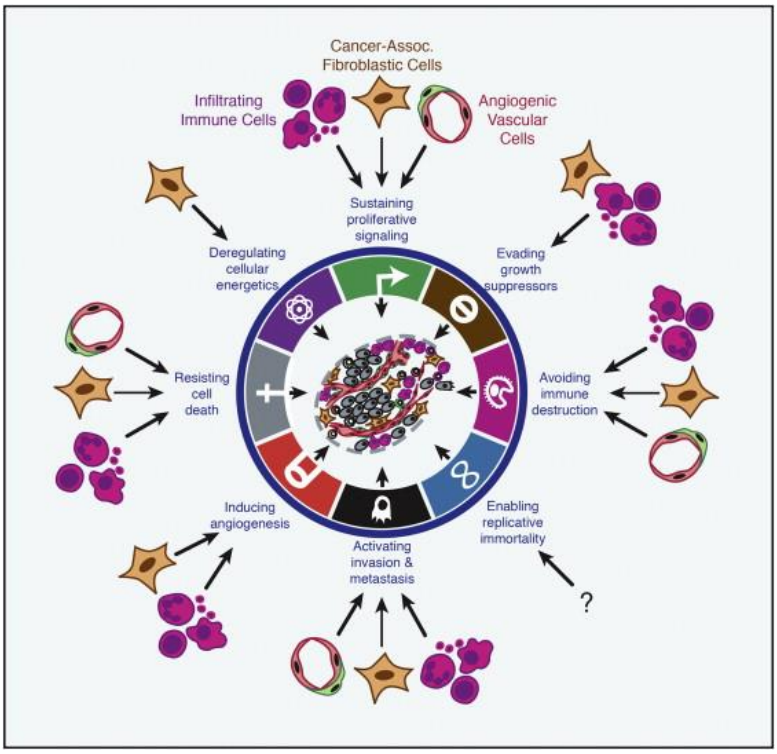

Figure 8 The hallmarks of cancer by Hanahan and Weinberg. Avoiding immune destruction is one of the recognised hallmarks, and six of eight hallmarks involve contributions by infiltrating immune cells. Reprinted from (104) with permission

1.7.1 Immune cells of the tumour microenvironment

The human body's immune system consists of different cells that recognize, memorize or eradicate foreign cells. Although cancer cells are developed from the host's cells, mutations can create neoantigens that are detected by the immune system. TILs consists of subsets of T lymphocytes. They are important mediators of the adaptive immune system, inducing cell-mediated immunity. The three major groups are CD4 helper T cells, CD8 cytotoxic T cells (CTLs) and CD4 regulatory T cells (Tregs). Activation is dependent on antigen-presenting cells (such as macrophages), presenting tumour antigen on major histocompatibility complex (MHC) class II on their cell surface, which binds to the T cell receptor (TCR). The TCR is then rearranged to

recognize this specific antigen upon binding to MHC class I on tumour cells. All nucleated cells express MHC class I on their surface, presenting fragments of any protein component within the cell. Upon tumour antigen exposure, CD4 helper T cells secrete cytokines that activate other immune cells (lymphocytes and macrophages), while CTLs can directly recognize and kill cancer cells.

On the other hand, Tregs function mainly as inhibitors of the immune response, suppressing T cell proliferation and activation. They play an important role in preventing the immune system's overactivation and autoimmunity. Other minor subgroups of T cells have also been identified, such as Natural Killer (NK) cells. Co-inhibitory cell surface receptors, such as PD-1 and cytotoxic T-lymphocyte protein 4 (CTLA-4), are often up-regulated on T-cells after activation (105) (Figure 9). These immune checkpoints function as “immunological brakes” by reducing T cell proliferation and cytokine production upon stimulation and is essential to prevent autoimmunity and retain immune homeostasis. This is also seen with chronic antigen exposure, such as cancer, a process called T cell exhaustion. ICIs blocks co-inhibitory receptors (e.g. PD-1, CTLA-4) on T cells and other immune cells or their ligands (e.g. PD-L1) on tumour cells or various immune cells, thereby enhancing the anti-tumour effect of the immune system. This paradigm shift in cancer treatment has led to an impressive survival advantage for many different cancers, but unfortunately, most patients have no clinical benefit from this treatment (105).

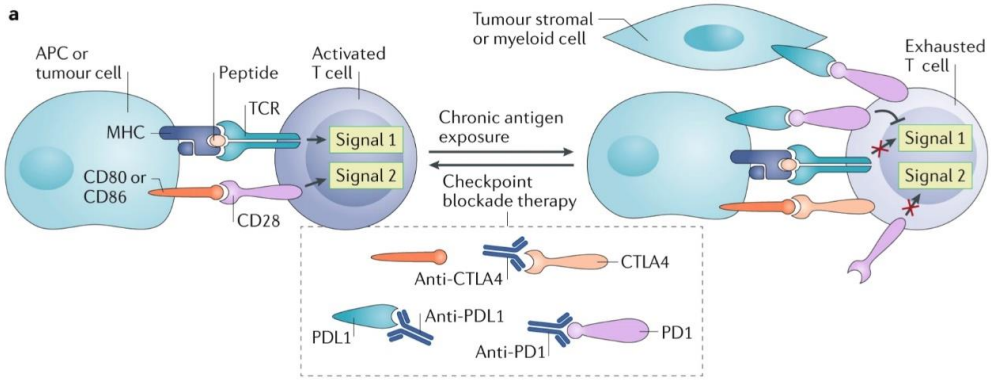

Figure 9 Activation of T cells requires two signals; binding of TCR to MHC and stimulation of co-stimulatory cell surface receptors. Upon activation and chronic antigen exposure, co-inhibitory cell surface receptors (CTLA-4 and PD-1) are up-regulated. Blocking these co-inhibitory signals with immune checkpoint blockade (Anti-CTLA4, Anti-PDL-1 or anti-PD-1) enhances the activity of T cells and reactivate exhausted T cells. Reprinted from (105) with permission.

Macrophages have a diversity of functions in normal and tumour tissue (106). As part of the innate immune system, they recognise and phagocytose pathogens and function as antigen-presenting cells for the adaptive immune system. They also secrete cytokines and chemokines affecting other immune cells and are essential in regulating the immune response and wound healing. A spectrum of macrophages has been identified, and the phenotype is determined by stimulation from the surrounding microenvironment. The two major groups have antagonising effects; the classically activated M1 phenotype with anti-tumour/pro-inflammatory effect and the alternatively activated M2 phenotype with pro-tumour/anti-inflammatory effect. It is believed that M1 macrophages are the most prominent in the early stage of tumour development. However, with tumour progression, M2 activation is induced by the secretion of cytokines from other immune cells, stromal cells, and tumour cells (107). Many studies have revealed that tumour-associated macrophages (TAMs) resemble the M2 phenotype, producing growth factors that stimulate proliferation, angiogenesis and survival of cancer cells and suppress infiltration and cytolytic activity of CTLs (107,

108) (Figure 10). They also play important parts in the metastatic process by remodelling the extracellular matrix components, facilitating tissue invasion (101, 104).

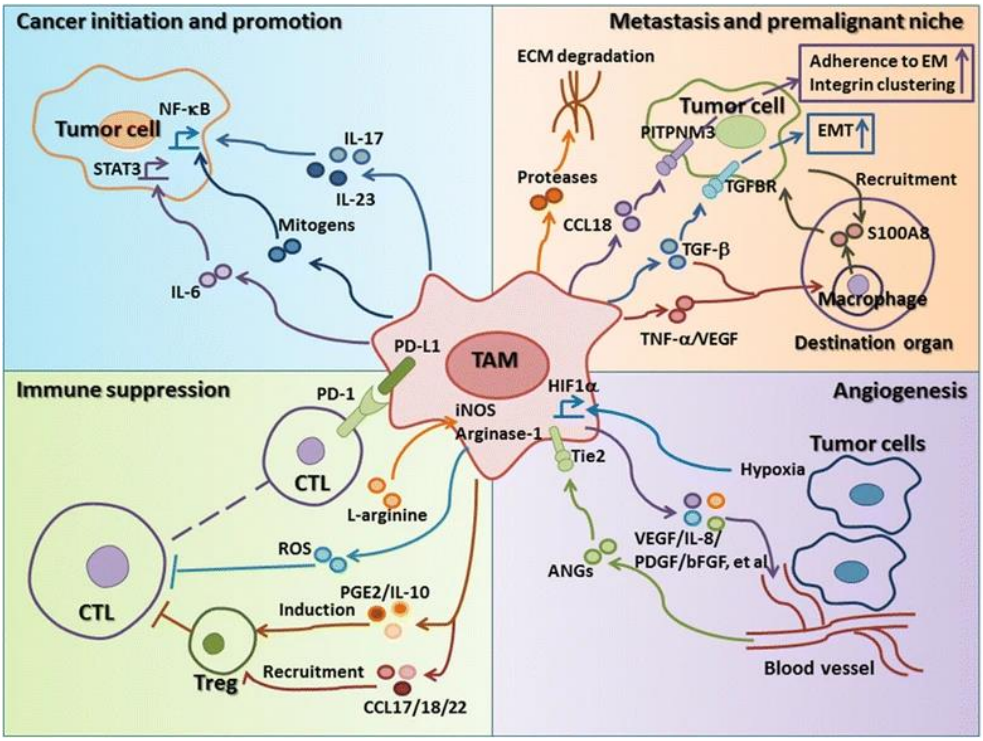

Figure 10 The effects of tumour-associated macrophages. Reprinted from (108) with permission.

1.7.2 Immune escape

Due to immune surveillance, cancer cells must evade the host's immune system to develop into tumours. The ability of cancer cells to avoid immune destruction is recognised as one of the hallmarks of cancer (98). Immune escape by tumour cells, a process termed immunoediting, can be achieved by lack of recognition and sensitivity to immune effector mechanisms and induction of immune suppression (100).

In 2001 it was discovered that apart from preventing tumour formation, the presence of an active immune system also shapes tumour immunogenicity (102). Proliferation and mutation of tumour cells with tumour progression also reduce tumour immunogenicity and leads to the escape of the immune response (100). In a CRC study, active

immunoediting resulted in a decrease of neoantigens from early to advanced stage, eliminating immunoreactive subclones and selecting immune-privileged subclones (109). Several studies have also shown defects in the antigen-presenting machinery and antigen processing of cancer cells, such as loss or downregulation of MHC class I (100). Lack of sensitivity to the immune system's cytotoxic effect can also be achieved by resistance to apoptosis, either by overexpression of anti-apoptotic receptors (BCL-2) or activation of pro-oncogenic transcription factors (STAT3) (100).

Cancer cells induce immune suppression by active secretion of immunosuppressive cytokines, paralysing tumour infiltrating CTLs and NK cells and recruiting immunosuppressive inflammatory cells (Tregs, macrophages and myeloid-derived suppressor cells). These immunosuppressive cytokines also upregulate co-inhibitory receptors on T cells, suppressing T cell activity. Ligands of co-inhibitory receptors, such as PD-L1, is also expressed on tumour cells in many types of cancers (98, 110, 111).

1.8 Prognostic and predictive tumour markers

In the era of precision oncology, specific tumour gene or protein information is analysed to tailor the patient's treatment based on their predicted response or disease risk. This enables clinicians to give each patient the most optimal and effective treatment regimen to improve survival and avoid non-beneficial overtreatment and toxicity for all non-responders. A key concept of precision medicine is the use of biomarkers, defined by the National Cancer Institute as “a biological molecule found in blood, other body fluids or tissues that is a sign of a normal or abnormal process, or of a condition or disease” (112). These biomarkers are categorised as prognostic or predictive, or both. Predictive markers identify patients that most likely benefit from a particular treatment. On the other hand, a prognostic marker gives information on patient outcome/disease course and categorises patients into different risk groups. This could also influence treatment choice. A patient with a poor prognosis might benefit from a more intensified chemotherapy regimen upfront as few make it to second and third-line treatment. Carcinoembryonic antigen (CEA), a blood-based biomarker, was

the first biomarker implemented for CRC patients. It is recommended to be measured preoperatively for prognostic assessment and monitoring of disease recurrence. If initially elevated, CEA is also recommended to be monitored at response evaluation during palliative chemotherapy for mCRC patients (113), as increasing levels could be an indication of treatment resistance. However, a surge in CEA level is observed in certain responding patients after initiating chemotherapy treatment (114), and about one-third of patients have no elevation of CEA at diagnosis.

In the era of precision medicine, a major focus has been on developing targeted treatment by identifying tumour specific targets or affecting critical tumour signalling pathways, thereby avoiding or minimizing the effect on normal cells. The development of new cancer treatment is expensive and often benefit a smaller subgroup of patients. Therefore, predictive biomarkers are also considered cost-effective. Much effort has been made to find prognostic and predictive markers to optimize cancer treatment, but so far, remarkably few have entered the clinic. In fact, precision oncology guided by cancer genome profiling has so far provided only a modest increase in treatment benefit. The estimated proportion of patients who showed benefit from genome-informed treatment in the US in 2018 was less than 7 % (115). And CRC lags behind: systemic treatment options are few compared to other major cancer types (116), and each of the “actionable” molecular markers is found in only 1-5 % of the cancers (117).

In this thesis, we have focused on tumour molecular biomarkers. Of note, certain clinicopathological factors, such as performance status, age, comorbidity, blood test (e.g. CEA, leukocytosis, ALP, CRP), sidedness of primary tumour, site and number of metastasis, histopathological grading and other features remain important prognostic factors. They should be considered in combination and relation to tumour molecular markers.

1.8.1 *BRAF/KRAS/NRAS*

Tumour *BRAF*^{V600E}mut status is a well-validated poor prognostic marker in localised (118) as well as mCRC (72) and is recommended to be assessed in the clinical management of mCRC patients (119). However, in the clinic, we observe that some

mCRC patients have an unexpectedly prolonged survival despite tumour *BRAF* mutational status. This was recently addressed in a study of 395 *BRAF*mut patients, where clinical factors defined three vastly different prognostic subgroups (120). Tumour *BRAF*mut is also associated with MSI status, and some studies have revealed that the poor prognosis of *BRAF*mut is limited to MSS tumours. However, results are conflicting and inconclusive due to the limited number of patients in these subgroup analyses (118, 121, 122). Future studies are needed to explore the reason for the survival heterogeneity in the $BRAF^{V600E}$mut subgroup. Studies of rare non-V600 *BRAF*mut have revealed distinct clinical subtype with a good prognosis, even exceeding the prognosis of *BRAF*wt patients (73, 75). However, insufficient data is currently available to determine the prognostic value of non-V600E *BRAF*mut due to the limited number of patients in these studies. Since $BRAF^{V600E}$mut generally predicts a poor prognosis, patients with a good performance status are recommended an intensified chemotherapy regimen in 1st-line palliative treatment (FOLFOXIRI + bevacizumab). *BRAF*mut is considered a predictive marker for BRAF-inhibitors (vemurafenib or dabrafenib) in metastatic malignant melanoma patients (123, 124). However, results on mCRC patients have been disappointing (125). Recently, encouraging data has been published on BRAF-inhibitor treatment combined with EGFR-inhibitors for these patients (44). At least a dual-targeted inhibition of the MAPK pathway seems needed for the obliteration of the signalling cascade. BRAF-inhibitor combination treatment is now recommended as 2nd-line treatment for *BRAF*mut cases according to NCCN and Danish national guidelines.

*KRAS*mut is a common event in CRC. This might explain why it has not been a promising marker to identify prognostic subgroups. However, survival rates after radical metastatic surgery for mCRC varies according to mutation status. *KRAS*mut and especially *BRAF*mut has been identified as adverse prognostic markers after liver surgery (126-128). Following hepatectomy for mCRC patients with available *BRAF* status, 5-year survival was 37 % in *BRAF*mut vs 67 % for *BRAF*wt (129). Another study reported inferior median survival in *BRAF*mut (23 months) compared to 42 months in *RAS*mut and 63 months in double wildtype (130). Five-year survival after

lung surgery in mCRC patients was 0 % for *BRAF*mut, 44 % for *KRAS*mut and 100 % for double wildtype, with corresponding median survival rates of 15 months, 55 months and 98 months, respectively (131). *RAS*mut and *BRAF*mut also impair survival after cytoreductive surgery with hyperthermic intraperitoneal chemotherapy(132).

Randomised trials have shown that EGFR inhibitors adds no survival benefit in patients with tumour *KRAS*mut status and later *NRAS*mut (133, 134), as these mutations occur downstream of the EGFR signalling pathway. The primary tumour site might also implement the treatment effect, as studies have shown improved benefit in patients with left-sided *RAS* wildtype tumour (135). There is reason to believe that other mutations in the RAS-signalling pathway also negatively predict the EGFR inhibitors effect. Recent studies have reported *BRAF*mut as a potential predictive marker in this setting (136, 137). Nevertheless, since *BRAF*mut is a less frequent mutation, associated with poor prognosis and inadequate chemotherapy response, predictive assessment is challenging (138, 139). Randomised trials are lacking and will probably not be conducted in the future due to new upcoming treatment options for these patients (BRAF inhibitors and ICIs for MSI). Taken together, *RAS* and *BRAF* mutation accounts for >50 % of mCRC patients who then are not eligible for EGFR inhibitor treatment. Due to RAS proteins' central role in intracellular signalling and the high frequency of *RAS*mut in CRC and other cancers, targeted treatment of RAS or RAS effector pathway seems promising. The development of RAS inhibitors has been challenging and ongoing for more than 40 years. It also needs to be constructed specifically for the mutated protein of interest to minimise the effect on normal RAS signalling. A specific inhibitor of KRAS G12C (sotorasib) has recently shown promising results in a phase II trial of previously treated metastatic non-small cell lung cancer with this specific mutation (140). The potential effect of KRAS G12C inhibitor for mCRC patients is currently under investigation. Still, this specific *KRAS*mut is a rare event in mCRC, and a phase I trial showed inadequate response compared to NSCLC (141).

1.8.2 MSI

The good prognostic effect of tumour MSI status in localised CRC has been thoroughly demonstrated in several studies, including randomised trials and meta-analyses (142, 143). In mCRC, on the contrary, MSI has been associated with an adverse prognosis in most studies (121, 122, 144). MSI leads to the accumulation of mutation-associated neoantigens on tumour cells, registered as foreign by the immune system, thereby activating the immune cascade resulting in increased TILs. This is believed to explain why MSI is recognised as a good prognostic marker in localised CRC (145). The cause of poor prognosis of MSI in mCRC is not known, and the association to and prognostic role of TILs in mCRC MSI tumours is not established. Previous studies have found MSI CRC enriched in mutations of critical immune-modulating pathways and antigen-presenting machinery, including MHC class I and B2M, as well as upregulation of checkpoint inhibitors (146-149). This has also been demonstrated in studies of Lynch syndrome patients (150). This immunoediting process allows immune escape despite the highly immune infiltrated tumour environment and is believed to be one of the reasons why MSI tumours are not eliminated by the activated immune system itself. Transcriptome analysis revealed that MSI CRC tumours display upregulation of checkpoint inhibitors that exhausts cytotoxic T cells and predicts a poor outcome, independent of tumour stage (148). They also reported an adverse prognosis of PDL-1 expression and immune checkpoint metagenes in an independent cohort of 28 MSI mCRC patients. MSI tumours have also been enriched in resistance mechanism to interferons secreted by activated T cells, such as Jak and Stat mutations, and could be a potential immune escape mechanism (151). Other factors have been proposed to explain the stage-dependent heterogeneous prognosis of MSI in CRC, such as the observed lower frequency of liver metastases and higher frequency of peritoneal metastases (122, 152) and right-sided tumour (153). Tumour MSI status is frequently cooccurring with *BRAF*mut (121, 122) in sporadic CRC, and the independent prognostic effect of these tumour molecular markers are not clarified.

MSI is recognised as a predictive marker for immunotherapy with ICIs in mCRC (46). The treatment effect is thought to be due to increased infiltration of immune cells in these tumours. However, not all patients respond clinically, as around 30 % of patients had direct progression on pembrolizumab in the recent 1st-line randomised trial (48). Assessment of TILs has been suggested as a potential predictive marker for these patients (154), but so far, studies are lacking. A higher ORR was observed in a small study of 85 MSI patients with high TILs (155). No studies have yet explored if TILs are predictive in the MSS subgroup of mCRC. A minor study of localised CRC that underwent neoadjuvant ICIs demonstrated a significantly higher tumour density of CD8/PD-L1 lymphocytes in responders of the MSS subgroup.

1.8.3 *HER2*

HER2 overexpression is a rare event in CRC, and the prognostic effect is not clarified (82, 83), although the presence of high-level amplifications of at least one gene (including *HER2*) was associated with poor survival among stage I-III MSS tumours (156). Studies suggest that HER2 overexpression is predictive of resistance to EGFR inhibitors (117). In recent phase II trials, encouraging data on HER2 targeted treatment has been published for HER2 positive *KRAS* wildtype mCRC patients. In heavily pretreated patients in the HERACLES II trial, 30 % ORR was observed with dual HER2/EGFR inhibitor (lapatinib) in combination with HER2 antibody (trastuzumab) (157). In the basket study Mypathway, 40 % ORR was observed with dual anti-HER2 antibody treatment (trastuzumab + pertuzumab) (158).

As for HER2 overexpression, the prognostic effect of *HER2* mutation in CRC is uncertain (85). Preclinical studies suggest resistance to EGFR inhibitors with *HER2* mutations(117). Monotherapy with anti-HER2 antibodies have so far not proven effective, but dual inhibition of *HER2* signalling might be promising (117). Studies of HER2 inhibitors (neratinib) in mCRC patients with tumour *HER2* mutations or amplifications are ongoing.

1.8.4 CDX2

Tumour CDX2 expression is downregulated in a subset of CRC patients, and loss of CDX2 expression has been associated with poor prognosis (91, 159-161). It has also been associated with other poor prognostic markers in mCRC, such as poor tumour differentiation, right-sided primary tumour, *BRAF*mut and MSI (90, 91, 162). No studies have fully explored if these associations confound the poor prognosis. It has also been associated with the poor prognostic subgroups CMS 1 and CMS 4 (91, 92, 163). CDX2 loss was reported as a negative prognostic marker for mCRC patients undergoing curative liver metastasectomy, indicating CDX2 as a potential biomarker to be assessed before surgery to identify patients with limited benefit (164). However, this study did not assess *KRAS* or *BRAF* mutation status, identified as negative predictive markers for liver metastasectomy patients (126, 129, 165).

In contrast to MSI, CDX2 loss has been reported as a potential predictive marker for adjuvant chemotherapy benefit in stage II and III CRC (91, 159). These data are limited to retrospective analyses and small sample sizes. In vitro drug screening and gene expression analyses has shown higher responses in CDX2 negative cell lines (91). However, the difference observed with 5-FU and oxaliplatin, used for adjuvant treatment, did not reach significance. Retrospective studies of smaller mCRC cohorts have not found tumour CDX2 assessment predictive for chemotherapy effect (91, 161, 164). Due to the potential predictive and prognostic value of CDX2 loss in CRC, determining the prevalence and survival effect in unselected population-based cohorts of mCRC patients is warranted.

1.8.5 Tumor infiltrating lymphocytes and macrophages

Dense infiltration of TILs is associated with a favourable prognosis in several types of cancer (103). But consensus on methods and threshold to define high tumour immunogenicity is lacking. In localised colorectal cancer (CRC), the Immunoscore® has been developed to standardise tumour immune infiltration analyses for clinical implementation (166). This is a combined score of CD3 and CD8 positive T-lymphocytes at the tumour centre and invasive margin, evaluated on whole tissue sections. It has been internationally validated as a prognostic marker in localised

colorectal cancer, even superior to the TNM staging system in such cancers (145). The prognostic effect of TILs has also been reported in studies of CRC tumour centre/tissue microarray (167-169). In a recent metanalysis, combined effect models were similar regardless of whether immune cells assessed intratumorally or at the invasive margin (170). In the clinic, whole-tissue sections are not always available. A substantial group of mCRC patients only have small biopsies taken for diagnostic purposes and is not emitted for primary tumour surgery if no clinical benefit of this procedure is expected. Prognostic assessment of TILs in studies of central tumour/TMA is therefore of clinical importance. Despite several studies in localised CRC, the prognostic impact of TILs in the primary tumour of patients with metastatic CRC (mCRC) is not established. Most previous reports have been performed on metastases of small and highly selected patient cohorts after secondary metastatic surgery (171-173), and validation in population-based mCRC cohorts are warranted.

The observed response of ICIs in MSI tumours is believed to be due to higher immunogenicity in these tumours. However, far from all patients with MSI CRC respond to ICIs. An intensive effort is currently being made to find a more specific and sensitive predive marker for ICIs response. It is believed that the antitumour effect of ICIs is achieved by removing the negative regulation of the pre-existing tumour immune microenvironment, and assessment of TILs has therefore been suggested as a potential predictive marker (105, 174), although studies are lacking. Studies of lung and melanoma cancer patients have shown higher tumour density of CD8+PD-1+ lymphocytes in responding patients (175, 176). In a small study of MSI mCRC, high TILs predicted better response, OS and PFS to ICIs (155). Future studies are needed to determine the predictive effect of TILs on ICIs treatment with consensus on threshold and methods to determine high and low TILs. The predictive effect of TILs in the generally non-responsive MSS mCRC patients has so far not been reported. Interestingly, a small study of neoadjuvant ICIs in localised CRC demonstrated a significantly higher tumour density of CD8+ PD-1+ lymphocytes in responding MSS cases (177).

TAMs have been associated with poor prognosis in several types of cancer (103). Studies of CRC patients have shown contradictory results (178-181), and recent meta-analyses conclude with a favourable prognosis of tumour infiltrating CD68 macrophages (103, 170, 182). However, studies on mCRC cohorts are lacking. The contradictory results could be due to the contrasting function of the different phenotypes. The location of TAMs in tumour tissue could also be essential as studies (180, 181) and recent metaanalyses concluded with improved survival with higher infiltration at the invasive margin. In contrast, studies of tumour centre infiltration did not reach significance (170, 182). Due to the evidence of tumour promoting effects of TAMs and macrophages plasticity, the potential of targeting TAMs as an anticancer treatment seems promising, and several studies are ongoing (183).

2. Aims of the study

The overall aim of this PhD project was to find new and validate known prognostic and predictive tumour molecular markers in a population-based cohort of mCRC patients.

Specific aims of the three papers included in the thesis were:

I. To determine the frequency and evaluate the prognostic effect of MSI in a population-based cohort of mCRC, taking into account known prognostic clinicopathological variables, including tumour molecular markers.
II. To determine the prognostic and predictive effect of CDX2 loss in a population-based cohort of mCRC related to clinicopathological variables, including tumour differentiation and tumour molecular markers.
III. To determine the prognostic effect of tumour infiltrating lymphocytes and macrophages in a population-based cohort of mCRC and explore the impact of immune cell density on prognosis for patients with MSI and *BRAF* mutated tumours.

3. Materials and methods

3.1 Patients

In this project, we analysed tumour and clinical data from a Scandinavian population-based cohort of mCRC patients (SPCRC). This cohort was collected by prospective registration of all patients with non-resectable mCRC adenocarcinoma referred to the oncology units of three university hospitals in Scandinavia from 2003-2006. These hospitals received all oncology department referrals of their administrative area. Uppsala University Hospital in Sweden serves 280 000 inhabitants, Odense University Hospital in Denmark 475 000 inhabitants and Haukeland University Hospital in Norway 450 000 inhabitants. Patients not referred to the oncology department was later identified via national (Norway and Sweden) and regional (Denmark) cancer registries (n=49). The cohort consists of 796 patients and represents a truly unselected population-based cohort of all non-resectable mCRC patients in a defined area (Figure 11). Clinical characteristics, pathological evaluation, blood tests and treatment regimens were retrieved from CRF. According to Response Evaluation Criteria in Solid Tumours (RECIST), treatment response was evaluated on CT scans after 1st, 2nd, 3rd and 4th-line chemotherapy. It needs to be mentioned that our cohort initially consisted of 798 patients. After publishing our first paper, we discovered that two patients included did not receive any systemic treatment, underwent curative surgery for metastatic disease and should not have met the inclusion criteria's for our study. Removal of these patients had no impact on our published results.

Figure 11 Patient selection and tissue microarray generation in a Scandinavian prospective mCRC cohort (SPCRC)

3.2 Methods

3.2.1 Tissue microarray

Tissue microarray (TMA) of tumour specimens enables high-throughput analysis of hundreds of specimens on one glass slide. This method facilitates large-scale studies and is both time and cost-effective compared to whole-section tumour analysis. Furthermore, TMA enables many tumour spots to be stained simultaneously, under the same conditions, time interval and method. In our study cohort, formalin-fixed paraffin-embedded (FFPE) tissue blocks were retrieved from primary tumour specimen in most cases or from a metastatic lesion (6 cases), and corresponding haematoxylin-eosin stained glass slides were examined. TMA generation was performed in 460 cases according to the standards used in the Human Protein Atlas program (184), with two 1-mm diameter tumour cores extracted per patient. The lack of inclusion was generally due to small biopsies or necrotic tissue (n = 239), and a proportion was displaced in the archive or had no cancer tissue (n = 97). For patients without enough tumour material to generate TMA, we collected sufficient tumour material for immunohistochemistry (IHC) analyses for MMR and $BRAF^{V600E}$ in 167 cases.

3.2.2 Immunohistochemistry

IHC is a low cost and well-established method for biomarker research and applied in routine clinical diagnostics for most cancer types worldwide. The major advantage of IHC is the visualization of the biomarker expression in a morphological and subcellular context. In papers I and II, we used IHC to detect MMR and BRAF status, using methods and image analysis according to standards within the Human Protein Atlas (185). Automated staining was performed, reducing operator variability and increasing reproducibility. Visual scoring was assessed by two independent pathologists without knowledge of clinicopathological data, and annotation discrepancies were re-evaluated to reach a consensus. In paper I, a consensus was made between MMR staining interpretation (n = 581) and previous results of MSI DNA sequencing in *BRAF*mut cases (n = 91) (25), further referred to as MSI or MSS. Furthermore, a consensus was made between $BRAF^{V600E}$ IHC staining and previous

BRAF V600E DNA pyrosequencing results (25), available for 446/591 stained cases. In paper III, we were able to add results on MSI DNA sequencing for additional 66 patients (69), and a final MSI consensus was made for 591 patients. Next-generation DNA sequencing of a customised Ampliseq hotspot targeted panel (69) was also considered for the final *BRAF* ^{V600E} conclusion in paper III (n = 595).

3.2.3 Multiplex IHC

In paper III, we used multiplex IHC to identify tumour infiltrating lymphocytes and macrophages with The Vectra 3 intelligent slide analysis system (PerkinElmer/Akoya). A multispectral camera obtains images at every ten nanometres of the visible spectrum, enabling unmixing of multiplexed staining with up to 7 colours. Both chromogenic and fluorescent stains can be analysed with the advantage of fluorochromes having a much higher dynamic range. The system is based on automated imaging and digital image analysis. After imaging, high-resolution cell segmentation is performed with a trainable software algorithm for pattern recognition. This automation of image analysis increases throughput and is more objective and quantitative compared to visual analysis (186). Furthermore, multiplexing enables the study of biological networks and facilitates a deeper analysis of heterogeneity and spatial relationships between different proteins in situ. Hence, this method is well suited to study the complex network of the immune cells in the tumour microenvironment.

3.2.4 Statistical methods

To evaluate correlations between the studied biomarkers and clinicopathological characteristics, exact chi-square test and multiple binary logistic regression were used for group comparisons between dichotomous variables, such as *BRAF*, MSI and CDX2 status. Immune cell markers were analysed as a continuous variable, with non-parametric Mann-Whitney U-test and Spearman's rank-order correlation. In multivariate analysis, linear regression was used with square root-transformed density of each immune cell to resemble a normal distribution.

OS was calculated from the date of radiologically confirmed unresectable metastatic disease to the date of death and censored if the patient was alive on 4 February 2014. PFS was the interval from the date of the first administration of chemotherapy to the date of progression (on CT scan) or death and censored if the patient was alive without progression on 4 February 2014. The Kaplan-Meier method was used to generate survival curves, and the log-rank test was used to compare the curves. Multivariable models were developed according to the Cox proportional hazards method, and OS and PFS were used as clinical endpoints. Formal interaction tests were integrated into the Cox models to assess whether effects were different between subgroups. However, results must be interpreted carefully due to the low power of such tests. In the fully adjusted Cox multiple regression analyses, we included variables statistically significant for survival in our cohort and available prognostic variables recommended by *Goey et al*. (187). All analyses were performed using the statistical software program IBM SPSS v25.

3.3 Ethical considerations

The prospective collection of a Scandinavian population-based cohort of mCRC (SPCRC) was approved by the regional committee for Medical and Health Research Ethics – REC West (Norway) (114 03), Regional Ethical Committee Uppsala (Sweden) and the Regional Scientific Ethical Committees of Southern Denmark. The study was conducted according to the declarations of Helsinki. Patients at the oncology department signed a written informed consent to participate in the study. Patients with mCRC not referred to the oncology department who died of the disease during the inclusion period were later identified through national (Norway and Sweden) and regional (Denmark) cancer registries, approved by REC West (2009/2052). General biobank approval was granted by REC West (2018/2111). REC West also approved the current PhD project on the general biobank (2019/30).

3.4 Methodological considerations

3.4.1 Tissue microarray

A major concern with the TMA method is the risk of not detecting possible heterogenous expression of tumour biomarkers. Tumour formation and progression is an evolutionary process, and subclones with survival advantage will be selected for further growth and division (188). Therefore, a small histospot from one particular part of a tumour might not represent the majority of tumour tissue. However, large-scale studies generally compensate for sampling errors. In the generation of TMA in our cohort, two tumour spots were taken from each patient to reduce the potential bias of tumour heterogeneity. Although rare, previous studies have raised concern about tumour heterogeneity of MMR staining (189, 190). However, we recently reported compliance between TMA and whole-tissue section staining of MMR and CDX2 in a cohort of early-stage CRC (191).

Another concern might be that the metastatic site has a different molecular profile than the primary tumour. In the generation of TMA, most spots were taken from the primary tumour and not from the metastases. However, for CRC, there seems to be a good correlation between driver gene mutations in the primary and the metastatic site (127). Studies have shown that the dominant clone of metastatic cells can change genotype/phenotype from the start of 1st-line chemotherapy to the last line of treatment, especially for *RAS* mutation status. The emergence of treatment-induced *KRAS*mut with acquired resistance to anti-EGFR treatment has been demonstrated in several studies of mCRC (192, 193). Heterogeneity between primary tumour and metastases is difficult to assess. It is generally not feasible to biopsy all metastatic lesions of a patient, as many patients harbour multiple metastases in multiple organs.

Another problem with the TMA method is the selection bias of omitting cases without sufficient tumour tissue. This could be due to small biopsies or rectal cases with massive necrosis after preoperative chemoradiation. This bias is, however, present for all studies of tumour tissue biomarkers. Our research group have previously shown that patients without sufficient tumour tissue to generate TMA were significantly

older, had worse performance status, more metastatic sites, primary tumour less often resected, more often abnormal baseline prognostic blood tests, received less chemotherapy and had an inferior prognosis compared to patients with TMA available (25). In paper I, we included additional patients outside the TMA cohort for IHC analyses of MMR proteins and $BRAF^{V600E}$ mutation. However, results of CDX2 staining and tumour immune markers studied in paper II and III are only based on patients with TMA generated.

The failure to detect tumour heterogeneity and tissue selection bias might be overcome by the use of liquid biopsies. This is a steadily evolving, minimally invasive method, detecting circulating tumour DNA (ctDNA) shed into the bloodstream by tumour cells. Recent studies report good concordance between driver gene mutations detected in tissue and blood (193, 194). Detection of mutations from ctDNA could also enable monitoring tumour evolution over time (195, 196). There is a risk of false-negative results with non-shedding tumours or low tumour burden, and standardisation of methods and large prospective studies are needed (195, 196). This method will only apply to mutations detectable by DNA-based methods, and therefore, for instance, not applicable for studies of immune cells in the tumour microenvironment.

Despite heterogeneity concerns, TMA enables large scale studies with high throughput analyses that reduce sampling bias and increase statistical power. For these reasons, TMA is widely used and considered a highly efficient method to discover and validate prognostic and predictive biomarkers.

3.4.2 Immunohistochemistry

The IHC method contains various steps, and many factors could impact the results. Even preanalytical factors, such as neoadjuvant radiotherapy for rectal cancer (197), the time spent from tissue resection to formalin fixation and length of formalin fixation (198), could give rise to different degrees of epitope degradation. The use of archived tissue specimens could also affect the staining, as some epitopes might be less stable than others. Still, FFPE tumour tissue is believed to retain its antigenicity for several

decades in proper storage conditions. With the use of high-quality antibodies, the staining is retained for most markers over time.

Antigen retrieval after formalin-fixation can be applied by many different methods, a potential cause of variable results between labs. Furthermore, antibody specificity and sensitivity may vary from different vendors. Commercial antibodies are often poorly validated (199), although some companies provide higher quality antibodies with more data on validation (e.g. XP range from Cell Signaling Technology). It is important to optimise antibody concentration for each particular antibody of interest. A short dynamic range will not differentiate between weakly stained tumours in low antibody concentration, and a high antibody concentration will lead to overstaining of the highly stained tumours. The method of detection and signal amplification is another crucial step. For detection, we used chromogen diaminobenzidine (DAB), which is well validated and most frequently used. Chromogen detection is based on light absorption, leading to a limited dynamic range, which means that there is no linear relationship between staining and intensity and the amount of biomarker detected (Beer lambert law). In comparison, emission-based fluorochrome detection, utilised for immune cell detection in paper III, has the advantage of a higher dynamic range.

3.4.3 Staining interpretation and threshold determination for biomarkers

Visual semi-quantitative assessment is the established standard in IHC but is prone to bias of interobserver variability. Firstly, the localization of the antigen of interest needs to be determined, as proteins have different functions in different subcellular localization. Staining is then scored according to levels of intensity and fraction of positive cells or reported as multiplication (H-score) (200) or summation (Allred score) (201) between the two, making it difficult to compare results from different labs. Furthermore, different thresholds are used to identify positive and negative staining, and in the early stage of biomarker research, there is generally no consensus on how to interpret the staining results. Staining intensity is especially prone to interpretation bias by using different fixatives, storage time of unstained sections and variations in IHC protocols and antibody concentration (202).

For this reason, we chose only to evaluate the fraction of CDX2 stained cells when interpreting CDX2 staining in paper II. Furthermore, no consensus on the staining interpretation of CDX2 as a prognostic marker in CRC has been established. The threshold for negative staining was set to nuclear fraction < 10 % based on the distribution of expression across our cohort, with most patients expressing a high nuclear fraction.

Determination of dMMR by IHC is a widely used method implemented in the clinical diagnostics of CRC. However, no well-studied, evidence-based threshold for normal MMR expression has been established (197). We defined the threshold for loss of MMR proteins as complete loss of nuclear staining, as applied by the College of American Pathologists POET report (203). Patients were further categorised as dMMR if staining was absent for MLH1 + PMS2, PMS2 alone, MSH2 + MSH6 or MSH6 alone. For $BRAF^{V600E}$, the cytoplasmic staining was qualitatively scored as positive (mutated) or negative (wildtype) as applied by previous CRC studies (204, 205). With the applied fluorescent multiplex IHC method for staining tumour immune cells in paper III, the scoring was much more objective and quantitative due to automated image analysis. This method is more standardised and robust (186), with higher resolution than IHC with chromogen staining. Evaluating the staining results as a continuous density variable could also enable the detection of additional clinical subgroups. Nevertheless, to be implemented as a prognostic or predictive marker, it is advantageous to apply a threshold to divide patients into different risk groups. There is no consensus on a threshold for high and low tumour immune cell infiltration in central tumour/TMA studies. Several studies have shown that the median value is associated with prognosis in CRC (169, 173, 206, 207). This value will, however, differ from each patient cohort studied and is not an objective measure. Hence, we chose to report survival prediction as both continuous variable and median value.

3.4.4 Validation of IHC results

Several studies have shown similar sensitivity and specificity of MMR detection by IHC and MSI detection by DNA based assays utilizing polymerase chain reaction (PCR). Both tests are readily used in clinical diagnostics (197). However, around 5 %

of cases with MSI have normal staining of MMR proteins (203). In paper I, we compared the staining results of MMR and previously sequenced MSI available for most cases with *BRAF*mut. As most of our dMMR/MSI cases also harboured *BRAF*mut (87 %), we were able to validate the IHC results for the majority of dMMR cases. In paper III, we were able to add MSI PCR results on additional patients, enabling validation of IHC results for 157/581 cases with final staining interpretation. Only one patient had inconsistent results between these two methods, supporting that the methods are comparable and sufficient to detect dMMR/MSI. In clinical practice, tumour *BRAF* mutation status has routinely been analysed by DNA-based PCR methods. However, these tests are not always available as they require molecular pathology expertise in routine labs. Previous studies utilising VE1 antibody for detection of $BRAF^{V600E}$ have shown variable sensitivity and specificity (204, 205, 208). In paper I, we compared our IHC $BRAF^{V600E}$ interpretation with previous DNA pyrosequencing for *BRAF* ^{V600} on the TMA cohort, with results available for 446/591 stained cases. This resulted in six inconsistent results. In paper III, we had the opportunity to include results on *BRAF* mutation from targeted sequencing of a customised Ampliseq hotspot panel on the TMA cohort, with results available for 447 patients. A consensus of all three methodological results was made, further securing correct interpretation of *BRAF* status.

4. Summary of results

4.1 Paper I

Recent encouraging treatment options for patients with mCRC include ICIs and BRAF inhibitor combination treatment provided for MSI and *BRAF*mut cases, respectively. Assessment of prevalence and prognostic impact of these biomarkers in a general population of mCRC is therefore warranted. Our cohort of mCRC patients identified 7 % MSI and 20 % *BRAF*mut cases, around twice as high as previous trial publications. MSI and *BRAF*mut were highly correlated. Most of our MSI cases were *BRAF*mut (87 %) with a median age of 75 years, in contrast to patients included in recent ICIs trials, with 0-25 % *BRAF*mut cases and a median age of 46-63 years. MSI was also associated with right-sided primary tumour, female sex and poor tumour differentiation.

MSI indicated poor survival in mCRC, with median OS of 6 vs 11 months for MSI vs MSS cases (p=0.004). We confirmed the negative prognosis of tumour *BRAF*mut status with 7 vs 12 months median OS in *BRAF*mut vs *BRAF*wt cases, respectively ($p < 0.001$). In patients treated with chemotherapy, both MSI and *BRAF*mut were independent poor predictors for OS and PFS in multivariate regression analyses. MSI cases had limited benefit of 1st-line chemotherapy, with an ORR of 5 % compared to 40 % in the MSS group, and few made it to 2nd-line treatment. Subgroup survival analyses revealed that the prognostic effect of MSI only reached significance in the *BRAF*wt subgroup, and the poor prognosis of *BRAF*mut was only observed in MSS cases.

4.2 Paper II

CDX2 is an important intestine-specific transcription factor and TSG, and recent studies indicate tumour CDX2 staining as a new potential predictive marker for adjuvant chemotherapy in CRC. The prognostic and predictive effect in mCRC cohorts has not been thoroughly investigated. In our unselected population-based cohort of mCRC patients, CDX2 loss was identified in 19 % of patients and indicated a poor prognosis. For patients given 1st-line combination chemotherapy, median OS was 10

vs 24 months in cases with tumour CDX2 loss vs expressed ($p < 0.001$). CDX2 loss was associated with other poor prognostic markers such as MSI, *BRAF*mut and poor tumour differentiation.

CDX2 loss was also confirmed as an independent negative prognostic marker for OS and PFS in multivariate regression analyses. Expression of CDX2 identified subgroups of *BRAF*mut and *KRAS*mut cases with a much better prognosis (median OS 21 months), comparable to wildtype patients (27 months). Loss of CDX2 expression identified subgroups of *BRAF*mut and *KRAS*mut cases with poor prognosis (median OS 8 and 11 months, respectively).

Immediate progression on 1st-line combination chemotherapy was seen in 35 % of patients with tumour CDX2 loss, compared to 10 % with tumour CDX2 expressed ($p = 0.003$). After 1st-line combination chemotherapy, median PFS was four vs nine months in cases with CDX2 loss vs expressed ($p = 0.001$). Furthermore, patients with tumour CDX2 loss received less 2nd-line treatment (23 % vs 39 %, p=0.006) and secondary surgery (1 % vs 9 %, $p = 0.019$) compared to patients with tumour CDX2 expression. Patients with tumour CDX2 loss had no survival benefit of doublet chemotherapy vs monotherapy in 1st-line treatment.

4.3 Paper III

Due to lack of knowledge of the impact of tumour immunogenicity in mCRC, we explored the prognostic effect of immune cell infiltration in the primary tumour of metastatic colorectal cancer patients using fluorescent multiplexing IHC panel including antibodies detecting CD3 and CD8 lymphocytes and CD68 macrophages. We found that tumour immune cell infiltration was associated with MSI status. However, the distribution was heterogenous, with two-thirds of MSI and one-fourth of MSS cases displaying the highest quartile infiltration of CD8 lymphocytes.

In patients treated with chemotherapy, high tumour infiltration of CD3 lymphocytes was associated with a favourable prognosis, with a median OS of 20 vs 16 months (HR: 0.76, p=0.025). This was of particular importance on long-term survival with a three-year OS of 27 % vs 13 %, respectively. Tumour infiltrating CD3 lymphocytes

was an independent positive prognostic marker for OS, corrected for other important prognostic markers such as *BRAF*mut, MSI and CDX2 status. The poor prognosis of MSI, *BRAF*mut and CDX2 loss was independent of tumour immune infiltration. Subgroup analysis revealed that CD3 TILs was only prognostic in the major groups of patients with tumour MSS, *BRAF*wt and CDX2 expression.

For CD68 high vs low cases, median OS was 23 vs 15 months (HR: 0.69, p=0.003) with a three-year OS of 28 % vs 12 %. Tumour infiltration of CD68 macrophages was an independent good prognostic marker when dichotomised by the median value but did not reach significance when assessed as a continuous density variable. Patients with a high combined tumour density of CD3 and CD68 cells had a median OS of 25 months compared to 15 months in patients with low infiltration of CD3 and CD68 cells (p=0.002).

Patients with low infiltration of CD3 lymphocytes had no survival benefit of oxaliplatin-based combination chemotherapy compared to 5-FU monotherapy in 1st-line treatment.

5. Discussion

5.1 Discussion of results

5.1.1 Prevalence of adverse prognostic biomarkers in population-based cohorts

Most previous studies of prognostic and predictive biomarkers in mCRC are based on selected and better prognostic subgroups of patients included in clinical trials or referral hospital cohorts (49). We, therefore, hypothesize that the incidence of poor prognostic markers in population-based cohorts will be higher. Indeed, this is what we showed in paper I, with around twice as high incidence of both MSI and *BRAF*mut cases as compared to previous studies (121, 209, 210). We believe this is due to the unselected nature of our cohort, with many elderly patients and patients with poor performance status generally excluded from clinical trials. In fact, the same frequency of *BRAF*mut was reported in a recent Nordic phase II trial of elderly vulnerable patients with mCRC (211). Due to the poor prognosis related to these biomarkers with new upcoming encouraging treatment options, our study emphasises the importance of assessing these tumour biomarkers for all mCRC patients.

5.1.2 MSI

In our population-based mCRC cohort, MSI is a poor prognostic marker, in accordance with other previous publications (121, 209, 210). Our results were later validated in a large cohort of 281 dMMR cases (144). In early-stage CRC, MSI is associated with a good prognosis, and we attempted to explore potential reasons for this heterogeneous prognosis across tumour stage. We found that MSI was highly associated with *BRAF*mut status and CDX2 loss. This could not solely explain the poor prognosis, as MSI was an independent poor prognostic marker for survival. MSI was also a poor prognostic marker in *BRAF*wt cases, although we had few patients in this subgroup analysis. In paper III, we demonstrated that, as for early-stage, MSI is highly associated with TILs. In studies of early-stage CRC, the good prognosis of MSI has been addressed to this fact (145). But although CD3 TILs is an independent good prognostic marker for survival in our chemotherapy-treated patients, MSI is still an important independent negative prognostic marker. We hypothesize that this could be

due to accumulating immune escape mechanism with tumour progression. Previous studies of MSI CRC tumours have revealed evidence of tumour immune evasion, such as enrichment in mutations of immune-modulating pathways and antigen-presenting machinery and upregulation of checkpoint inhibitors compared to MSS tumours(146-150). Future studies should evaluate if accumulating immune evasion mechanisms with tumour progression could explain the heterogeneous prognosis of MSI across tumour stage.

MSI is recently implemented as a predictive marker of ICIs effect, as FDA and recently EMA have approved ICIs as 1st-line treatment in MSI mCRC patients. In our population-based cohort, MSI patients have a particularly poor prognosis with an inferior response to standard chemotherapy compared to MSS patients. The obtained 5 % ORR after 1st-line chemotherapy in our cohort is in great contrast to 44 % ORR obtained in recent 1st-line ICIs trials (48), with 83 % ongoing responses at 24 months. This could be particularly important for MSI patients with potentially resectable disease, needing the best response, which our study shows are not achieved by regular chemotherapy. We also observed that a substantial amount of MSI patients never made it to 2nd-line treatment. This further supports the use of ICIs in early lines, preferably in 1-st line, to ensure that most patients with MSI will have the opportunity to receive ICIs.

The marked difference between our MSI patients and patients included in recent ICIs trials should be considered when transferring trial results to the general population (46-48, 212). Most of our MSI cases were *BRAF*mut (87 %), in strong contrast to patients included in recent ICIs trials (0-25 %). MSI was more often identified in elderly patients, with a median age of 75 years, compared to 46-63 years in ICIs trials. Although no difference in response and survival has been observed according to *BRAF* status in these trials, additional data on ICIs in the more prevalent *BRAF*mut MSI cases in the general population is needed. Elderly patients are generally not included in clinical trials, and studies on efficacy and side-effects of ICIs in elderly mCRC are clearly warranted.

Despite the impressive response of ICIs in the poor prognostic subgroup of MSI mCRC patients, around 30 % of these patients have immediate disease progression on this treatment, and assessment of TILs have been proposed as a potential predictive marker in this setting (105, 174). Our study demonstrates that although TILs are highly associated with MSI status, the distribution is somewhat heterogeneous between the groups. Two-thirds of MSI cases and one-fourth of MSS cases displayed the highest percentile group of CD8 lymphocyte infiltration and might be potential subgroups of ICIs effect. This finding further stresses the need for future ICIs studies exploring the predictive effect of TILs in both MSI and MSS subgroups.

5.1.3 BRAF mutations

Tumour *BRAF*mut is a well-validated and widely used poor prognostic biomarker in the clinical assessment of mCRC patients (72, 119). However, in the clinical setting, we observe a heterogenous prognosis of these patients despite detected mutation. *BRAF*mut is an independent poor prognostic marker for OS in our population-based cohort and significantly predicts poor prognosis in MSS patients. *BRAF*mut and MSI status is highly correlated, and the poor prognostic effect of MSI did not reach significance in *BRAF*mut cases, although the limited number of cases affects statistical power. CDX2 expression, on the other hand, identified a subgroup among *BRAF*mut cases with a good prognosis, with survival comparable to *BRAF*wt. *BRAF*mut and promoter methylation induced CDX2 loss is often observed in serrated tumours developed within the CIMP pathway (65), and a synergistic oncogenic activity between CDX2 loss and *BRAF*mut has been observed in these tumours (213, 214). Serrated tumours have been associated with poor prognosis and can be used to stratify patients with *BRAF*mut tumours (63, 215). We believe that CDX2 status, at least in part, could explain the observed heterogeneous prognosis of *BRAF*mut patients, and our findings are supported by a study of 155 *BRAF*mut mCRC (216). Furthermore, CDX2 loss has also been shown to identify a particular poor subgroup among *BRAF*mut in stage I-III CRC (91). As BRAF inhibitor combination treatment is introduced for *BRAF*mut patients, it would be relevant to study if CDX2 status could affect response.

5.1.4 CDX2

Loss of tumour CDX2 expression has been consistently observed in a subset of CRC with poor prognosis and is proposed as an emerging prognostic and predictive biomarker in CRC. Nevertheless, studies of consequences in mCRC cohorts are warranted. Loss of CDX2 expression has also been associated with other poor prognostic markers in mCRC, such as *BRAF*mut, MSI, right-sided primary tumour and poor tumour differentiation. To our knowledge, no previous studies have fully explored if these associations could confound the negative prognostic effect of CDX2 loss in mCRC. In paper II, we report CDX2 loss as an important independent negative prognostic marker in mCRC, corrected for these associated prognostic markers. With the feasibility and low cost of this method, already implemented in the clinical diagnostics for other purposes, we believe its potential as a new prognostic biomarker for mCRC patients should be further investigated in larger cohorts.

As for *BRAF*mut, we also demonstrate that CDX2 loss defines a small subgroup of *KRAS*mut patients with a poor prognosis. Although, few cases in our analysis preclude a firm conclusion. Both *BRAF*mut and *KRAS*mut have been associated with poor prognosis after metastasectomy (126, 131) and may be factors to consider before possible metastatic surgery in patients with very advanced disease. We believe that CDX2 status also could add important prognostic information prior to surgery when considering treatment strategies for these patients. A recent study reported CDX2 loss as a poor prognostic marker after liver metastasectomy, but this study did not correct for *KRAS* or *BRAF* status (164). Our study indicates that CDX2 loss is a negative prognostic marker for these patients regardless of mutational status. However, as CDX2 loss affects a smaller group of mCRC patients, few cases were available in our subgroup analyses, particularly in cases with *KRAS*mut and CDX2 loss, and larger studies are needed to verify our results.

Recent retrospective cohort studies suggest CDX2 loss as a potential predictive marker for adjuvant chemotherapy benefit in stage II-III CRC (91, 159). However, no predictive value for chemotherapy treatment response was observed in a series of mCRC patients (91) or in a cohort of patients that underwent liver metastasectomy

(164). In our population-based cohort, patients with tumour CDX2 loss had worse PFS and response to 1st-line chemotherapy, and our results are supported by a previous study (161). Furthermore, few patients made it to 2nd-line treatment and secondary metastasis surgery. This might indicate that these patients need a different treatment regimen than given today. Due to the poor prognosis of tumour *BRAF*mut status, these patients are generally recommended intensified chemotherapy upfront. For patients with CDX2 loss, this might not be the best treatment option, as our study indicates that these patients have no survival benefit from receiving doublet chemotherapy instead of monotherapy. However, few patients were included in these subgroup analyses, and more extensive randomised studies are warranted to determine the predictive effect for all stage CRC.

5.1.5 Tumour immune microenvironment

Prognostic evaluation of TILs and macrophages in the primary tumour of population-based mCRC cohort adds warranted information to this field, as previous studies are mainly based on early-stage or metastases of selected mCRC patients that underwent curative surgery. In our chemotherapy-treated series, tumour infiltrating CD3 lymphocytes was an independent good prognostic marker for OS, with apparently greater influence on long-term than median survival. Our finding is supported by a previous study of mCRC patients that underwent surgery for primary tumour and metastases (179). We could not confirm any prognostic impact of tumour infiltrating CD8 in our cohort, in line with two previous studies (179, 217). Prognostic studies assessing a combined score of CD3 and CD8 TILs in selected mCRC trial patients are conflicting (167, 218). Our study found no significant association with TILs and PFS, this has also been demonstrated in a small study of 68 mCRC patients (219).

Although tumour infiltration of CD3 lymphocytes was associated with OS in our cohort, it only reached significance in chemotherapy-treated patients. The effect might seem more pronounced in studies of early-stage CRC. Our study also illustrates that the prognostic effect might be restricted to the major groups of patients with tumour MSS, *BRAF*wt and CDX2 expression status. However, we had limited cases within the poor prognostic subgroups to conclude. With tumour progression, proliferation and

mutation of tumour cells reduce tumour immunogenicity and escape from the immune response; a process termed immunoediting. Previous studies have shown evidence of several immune escape mechanisms during tumour progression, such as upregulation of checkpoint inhibitions, tumour-secreted immune-suppressive cytokines and exhaustion of cytotoxic T-cells (98, 111).

As recent studies have suggested that a high Immunoscore® could predict benefit from adjuvant chemotherapy in stage III CRC (220, 221), we wanted to investigate the survival effect of oxaliplatin-based chemotherapy according to TILs. We found that patients with low infiltration of CD3 TILs had no survival benefit of oxaliplatin-based chemotherapy compared to 5-FU monotherapy, in contrast to patients with high infiltration of CD3 TILs. These results need to be evaluated with caution due to a limited number of patients. Oxaliplatin induces immunogenic cell death, which could be the reason for the observed inferior response in tumours with low immunogenicity (222). Patients with high TILs seem to have a generally better prognosis with improved benefit of both oxaliplatin-based chemotherapy and ICIs treatment.

In our chemotherapy-treated patients, CD68 TAMs was associated with improved prognosis when stratified by the median value but did not reach significance when analysed as a continuous variable, which might suggest that the prognostic effect is less robust. TAMs have generally been associated with poor prognosis in various malignancies. In contrast, most studies of CRC report a favourable prognosis. A spectrum of different TAMs has been identified, with diverse functions in the tumour microenvironment. Two major phenotypes have antagonizing functions on tumour cells; M1 with anti-tumour features and M2 with cancer progressive effects, and our study could not differentiate between these phenotypes. Future studies should include markers to differentiate between these phenotypes to further understand the prognostic effect of TAMs in mCRC patients.

5.2 Strengths

An important strength of our cohort is the prospective design and the effort to make the cohort truly population-based. Due to the health care system in Scandinavia with

public health insurance, all patients with mCRC are referred to a regional cancer clinic if considered eligible for oncological treatment. In our study, the three attending hospitals covered all oncological treatment in their region, and patients not referred were later identified via cancer registries, making our cohort truly population-based. Other factors that underline the value of our cohort are the detailed and quality controlled clinical annotations and long-term survival observation.

Most studies and current knowledge on tumour biomarkers are based on selected patients included in clinical trials or referral hospital cohorts. Our population-based study adds important knowledge on tumour biomarkers in the general population of mCRC, including the poorly studied subgroups of elderly patients, patients with poor performance status and rapidly progressive disease.

Last but not least, we should mention the strength of the Scandinavian collaborative network behind this study. Our study enables the assembly of different research- and clinical expertise across the borders and collecting more extensive patient series, facilitating cancer research of high standard. Similarities among the countries demographics, health care system, cancer incidence and treatment recommendations for mCRC patients makes Scandinavian studies reasonable.

5.3 Limitations

Population-based studies are important to validate prognostic markers for the general cancer patient. However, patients in such cohorts are very heterogeneous regarding treatment regimens, comorbidities and other clinical characteristics. This leads to many potential confounding factors and smaller subgroups of patients with reduced statistical power to detect significant differences. In this non-randomised study, patients not given chemotherapy are negatively selected with poor performance status, rapidly progressing disease, older age and/or other treatment-limiting comorbidities. These patients die rapidly with a median OS of three months and cannot be directly compared to the treated group. Therefore, the effect assessment of predictive markers is challenging and needs to be confirmed in randomised cohorts. Another challenge with our cohort is the inclusion period, as these patients were treated more than ten

years ago. Although treatment options for mCRC patients have not changed much in the past decade, intensified treatment regimens and metastatic surgery are used more often today, leading to an increase in survival for these patients.

Although the attempt to make this cohort truly population-based, a significant number of patients in our cohort did not have enough archived tumour tissue to proceed with biomarker analyses. However, this selection bias is present for all studies of tumour tissue biomarkers, and these patients belong to the worse prognostic group of mCRC (25).

In biomarker studies, subgroup analyses of patients according to different clinicopathological variables are of interest, as biomarkers could have diverse prognostic effect in different subgroups. Such analyses are especially prone to bias, as various variables could influence the results, and smaller group analyses affect statistical power. When exploring the prognostic effect of different biomarkers in subgroups of tumour molecular alterations or certain treatment regimens, we had too few patients in some groups to draw firm conclusions, and these results need to be interpreted with caution.

6. Conclusions

This study has shown that in the population-based tumour series of mCRC, the presence, effect and clinicopathological associations of known tumour biomarkers (here MSI and *BRAF*) differ from results obtained in clinical trials. For CRC, this discrepancy is mainly caused by the inclusion of younger and better fit patients in clinical trials than in the general cancer population. Our data show that each of the three tumour biomarkers studied, MSI, *BRAF* and CDX2, carry independent prognostic information for mCRC. Patients with tumour MSI status or loss of CDX2 have little effect of chemotherapy in end-stage disease, supporting an earlier and different treatment strategy for these subgroups. Tumour CDX2 status identified prognostic subgroups of *BRAF*mut cases that might explain the observed heterogeneous prognosis of these patients in the clinical practice. High expression levels of immune markers of the tumour microenvironment (CD3 and CD68) are prognostic biomarkers for chemotherapy-treated patients with mCRC, with particular importance for long-term survival. Despite the high immunogenicity of MSI tumours, MSI was still an independent negative prognostic marker.

7. Future perspectives

Studies of prognostic biomarkers in population-based cohorts are needed to better understand the tumour biology and prognosis of the patients we meet in the daily clinic. With new targeted treatments steadily emerging, it is important to know the prevalence of these targets and predictive markers in population-based cohorts. We experience an increasing demand for real-world data from the regulatory authorities when new targeted treatment options are being evaluated for reimbursement by the Scandinavian health authorities. Our study on real-world data reports a doubling in frequency of MSI and *BRAF*mut compared to the current estimate. These are predictive markers for ICIs and BRAF inhibitor combination treatment, and our research is currently being referred to in the ongoing reimbursement evaluation by the Norwegian health authorities.

Due to new encouraging targeted treatment options for these poor prognostic groups of patients, our finding underlines the importance of tumour biomarker assessment for all mCRC patients. However, since selected patients included in clinical trials are vastly different from the general mCRC population, there is a risk that the trial effect will not replicate in the clinical practice. In our study, most MSI patients harboured *BRAF*mut and were elderly, in stark contrast to recent ICIs trials. Future studies of ICIs effect in the major group of sporadic and elderly MSI patients are clearly warranted as ICIs treatment is implemented for the general MSI population. We also report that CDX2 stratify prognosis in *BRAF*mut cases, and future BRAF inhibitor combination treatment studies should evaluate if CDX2 loss affects response to this treatment.

Due to our findings and the following verification in a large MSI mCRC cohort, we now state that MSI should be acknowledged as a poor prognostic marker in general mCRC patients. This knowledge should be adapted in the clinical practice and future studies of new prognostic biomarkers in mCRC patients. The short survival and poor response to standard chemotherapy in the MSI group indicate that ICIs treatment should be given up front, as many patients never reach secondary treatment. This could be of particular importance in cases with potentially resectable disease. As these

patients are treated with curative intent, this could have major implications on survival for future patients.

CDX2 is an emerging prognostic and predictive biomarker in CRC, and our study supports this observation and particularly generates new evidence of the independent prognostic effect in mCRC. The feasibility, availability and low cost of this method, already implemented in the clinical diagnostics for other purposes, further supports its potential as a new biomarker for mCRC. However, verification in larger prospective and randomised trials is demanded before clinical application. As CDX2 loss defined new prognostic subgroups of *BRAF*mut and *KRAS*mut cases, this is of particular interest with potential treatment implications for patients assessed prior to metastasectomy and validation of our results in larger cohorts is demanded.

By combining the predictive biomarker strategy with future pharmacological profiling of the patients own tumour cells, grown as 3D patient-derived organoids and screened for drug sensitivities (223, 224), we may better identify the patients who will benefit targeted treatments.

Studies of the function and presence of tumour immune cells have led to a broader understanding of the impact of the immune system in cancer and the development of targeted immunotherapy. Our study provides novel evidence for a prognostic effect of tumour immune cell infiltration in mCRC patients. However, standardisation of methods and threshold for the identification of high tumour immunogenicity is needed. Moreover, the prognostic effect in localised CRC might seem more convincing. Future studies should attempt to reveal tumour immune escape mechanisms that could also identify new potential targets of immunotherapy in mCRC patients. In future studies of our cohort, we plan to evaluate the presence and prognostic effect of different checkpoint inhibitors and immunosuppressive Tregs. The observed independent poor prognosis of MSI despite high immunogenicity, in contrast to studies of early-stage CRC, provides important information in the attempt to reveal the heterogeneous prognosis of MSI across tumour stage. To gain a deeper understanding, studies

exploring evidence of accumulating immune escape mechanisms with tumour progression should be conducted in larger MSI cohorts.

Continued research on population-based cohorts of mCRC is clearly indicated. Patients without enough tumour tissue for analyses have a particularly poor prognosis, and new cohorts should aim to include all patients within a defined period for tumour biomarker analyses. Therefore, our Scandinavian research group has recently initiated a prospective collection of mCRC patients (NewSPCRC) with blood samples to assess ctDNA in an assumed tumour heterogeneity dependent manner. Using this strategy, we believe we will contribute with new knowledge for this poorly studied patient group and a better understanding of the effect of tumour biomarkers for all mCRC patients.

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Received: 31 January 2019 | Revised: 8 April 2019 | Accepted: 14 April 2019

DOI: 10.1002/cam4.2205

ORIGINAL RESEARCH

Cancer Medicine Open Access WILEY

Consequences of a high incidence of microsatellite instability and *BRAF*-mutated tumors: A population-based cohort of metastatic colorectal cancer patients

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Funding information
AD received funding from Uppsala University Hospital ALF-fund, Sweden. MS received funding from Selanders Stiftelse and Lions Cancer Foundation, Uppsala, Sweden. FP and BG received funding from the Swedish Cancer Society. HS received funding from the Norwegian Cancer Society. The funding was used for collection and preparation of tissue and analysis.

Abstract

Background: Immunotherapy for patients with microsatellite-instable (MSI-H) tumors or *BRAF*-inhibitors combination treatment for *BRAF*-mutated (mut*BRAF)* tumors in metastatic colorectal cancer (mCRC) is promising, but the frequency of these molecular changes in trial patients are low. Unselected population-based studies of these molecular changes are warranted.

Methods: A population-based cohort of 798 mCRC patients in Scandinavia was studied. Patient and molecular tumor characteristics, overall survival (OS) and progression-free survival (PFS) were estimated.

Results: Here, 40/583 (7%) tumor samples were MSI-H and 120/591 (20%) were mut*BRAF*; 87% of MSI-H tumors were mut*BRAF (non-Lynch)*. Elderly (>75 years) had more often MSI-H (10% vs 6%) and MSI-H/mut*BRAF* (9% vs 4%) tumors. Response rate (5% vs 44%), PFS (4 vs 8 months), and OS (9 vs 18 months) after first-line chemotherapy was all significantly lower in patients with MSI-H compared to patients with microsatellite stable tumors. MSI-H and mut*BRAF* were both independent poor prognostic predictors for OS ($P = 0.049$, $P < 0.001$) and PFS ($P = 0.045$, $P = 0.005$) after first-line chemotherapy. Patients with MSI-H tumors received less second-line chemotherapy (15% vs 37%, $P = 0.005$).

Conclusions: In unselected mCRC patients, MSI-H and mut*BRAF* cases were more common than previously reported. Patients with MSI-H tumors had worse survival, less benefit from chemotherapy, and they differed considerably from recent third-line immunotherapy trial patients as they were older and most had mut*BRAF* tumor (non-Lynch).

KEYWORDS

colorectal neoplasm, microsatellite instability, proto-oncogene proteins, B-raf, prognosis, neoplasm metastasis, KRAS protein

1 | BACKGROUND

Colorectal cancer (CRC) is a heterogeneous group of tumors with a wide range of genetic changes. Microsatellite instability (MSI) is caused by mutations in DNA mismatch repair (MMR) genes, which leads to failure to repair errors that occur in DNA replication in repetitive sequences (microsatellites). This leads to accumulation of frameshift mutations in genes with microsatellites, also called MSI-high (MSI-H). Most MSI-H tumors result from accumulated mutations during life but can also occur due to inherited MMR deficiency (Lynch syndrome). Most previous studies on metastatic CRC (mCRC) have reported that around 4% of the tumors are MSI-H[1-5] and 5%-12% *BRAF* mutated (mut*BRAF*).[3,4,6,7] Most previous reports are based on patients included in clinical trials, and these patients are highly selected with both younger age and better performance status (PS) compared to patients in the general clinical practice.[8,9] Sporadic MSI-H CRC is associated with a *BRAF* mutation in about 40%-60% of the cases, whereas Lynch syndrome tumors are essentially *BRAF* wild-type (wt*BRAF*).[10,11] In nonmetastatic CRC, MSI-H is associated with less risk of recurrence and improved survival compared to microsatellite stable (MSS) tumors.[12,13] In mCRC, MSI-H tumors appear to have poor prognosis, but the number of patients in these studies are limited.[2-6,14] However, mut*BRAF* has a strong negative prognostic impact in mCRC, but the possible relevance of MSI status for poor prognosis is not clarified.[3-6,15-17]

Recent studies have shown that mCRC patients with MSI-H tumors respond to immunotherapy given mainly as third-line treatment.[18-20] The recently updated National Comprehensive Cancer Network guidelines recommend second-line treatment with a PD-1 inhibitor in patients with MSI-H tumor and addition of BRAF-inhibitors to standard treatment in patients with mutBRAF tumors.[21] For these reasons, it is important to know the proper frequency, clinical characteristics, prognosis and treatment response in patients with MSI-H and mut*BRAF* tumors in population-based cohorts. The aim of this study was to analyze MSI-status in relation to clinical and pathological variables, mut*BRAF* status and survival in a population-based cohort of mCRC.

2 | MATERIALS AND METHODS

2.1 | Patient cohort

The study cohort is a prospective registration of non-resectable mCRC patients referred to the oncology units of three university hospitals in Scandinavia (Odense University Hospital in Denmark, Uppsala University Hospital in Sweden and Haukeland University Hospital in Norway) between October 2003 and August 2006. Cases not referred (n = 49) were identified via the regional cancer registries. This cohort therefore includes all patients diagnosed with nonresectable mCRC in these three Nordic geographical regions. A total of 798 patients were included.[7] The clinical data is from date of inclusion and was obtained from case report forms filled in by clinicians.

2.2 | Tissue retrieval and tissue microarray generation

Paraffin-embedded tissue blocks of the primary tumor or from a metastatic lesion were retrieved and corresponding hematoxylin-eosin stained glass slides were examined. Tumor tissue from 462 cases (58%) was available for initial tissue microarray (TMA cohort) construction as described previously[7] according to standards used in the Human Protein Atlas.[22] DNA was extracted from the tissue cores using Recoverall Total Nucleic Acid Isolation (Ambion, Austin, TX). In the present study we supply additional analyses from patients without enough tumor material for TMA/DNA analysis (167 patients), called the immunohistochemistry (IHC) cohort. Totally 604 cases had tumor tissue available for analysis, as 25 cases failed due to technical reasons (Supplementary Figure S1).

2.3 | Tumor analyses

BRAF and *KRAS* analyses of the TMA cohort had been done previously by pyrosequencing mutational analysis with 5% mutation signal as cut off, and the use of PCR primers for *KRAS* codon 12/13 and *BRAF* codon 600.[7] MSI status for *BRAF*-mutated patients in the TMA cohort had previously been

obtained by DNA analysis using MSI Analysis System, version 1.2 (Promega, Madison, WI) with 6 ng genomic DNA.[7]

Immunohistochemistry (IHC) and image acquisition was performed according to standards used within the Human Protein Atlas.[23] TMA sections 4 mm thick were subjected to heat-induced antigen retrieval using PT module buffer 1 (pH 6, ThermoScientific) in a Decloaking Chamber (Biocare Medical), except for *BRAF* stained with special protocol HIER with TRIS-EDTA at pH8. Automated IHC was performed using a LabVision Autostainer 480S (Thermo Fisher Scientific, Runcorn, UK). BRAF mutation was assessed with mouse antibody from Spring Bioscience, E19292, Clone VE1, diluted 1:50. MSH-2 and MLH-1 with mouse antibody from Becton Dickinson and Company (formerly PharMingen), Clone = G219-1129 and G168-15, diluted 1:200 and 1:100, respectively. PMS-2 and MSH-6 with rabbit antibody from Abcam plc, ab110638 clone = EPR3947 and ab92471 clone = EPR3945, diluted 1:75 and 1:125 respectively. IHC for V600E *BRAF* mutation was analyzed in both TMA and IHC cohorts. Cytoplasmic staining for *BRAF* mutation was qualitatively scored as positive (mutated) or negative (wildtype) in tumor cells. The results from the IHC and DNA analysis were compared and found inconsistent in seven cases. One of them had a V600R mutation discovered by sequencing and obviously not detected by the V600E IHC analysis. This patient was defined as mut*BRAF*. One patient turned out to be mut*BRAF* according to pyrosequencing, but wt*BRAF* according to IHC evaluation. This case had low amount of mutated DNA (8%), was mut*KRAS* and was therefore considered wt*BRAF*. The other five patients with inconsistent results were excluded from further analysis. A final *BRAF* status conclusion was made in 591 patients (Supplementary Figure S1 and Figure S2).

IHC for expression of MLH1, PMS2, MSH2, and MSH6 was performed for all patients included in the TMA cohort. Only MSH6 and PMS2 was analyzed in the additional IHC cohort due to limited amount of material in most cases. Nuclear fraction (NF) of the four MMR proteins were estimated. The samples were denoted as deficient DNA mismatch repair (dMMR) if complete loss of PMS2 or MSH6 staining. One patient had complete loss of MSH2 staining, but clearly positive staining for PMS2. This is an unexpected finding and the patient was therefore excluded from the final analysis. Results from IHC and DNA analysis were compared and merged, further referred to as MSI-H or MSS, in 583 patients (Supplementary Figure S1).

2.4 | Statistics

Group comparisons were performed using the exact chi-square test for dichotomous or nominal variables and the log-rank test for survival times. Multiple binary logistic regression was used for dichotomous outcome variables. Results are reported as odds ratios (ORs) and 95% confidence intervals (CIs). Overall survival time (OS) was the interval from the date of metastatic disease to the date of death or censored if the patient was alive on February 4, 2014. Progression-free survival (PFS) was the interval from the date of first administration of chemotherapy to the date of progression (on CT scan) or death or censored if the patient was alive without progression on February 4, 2014. OS and PFS were analyzed using the Kaplan-Meier method and Cox multiple regression. For the multivariate survival analyses we used Cox regression and backward stepwise selection of covariates to the final model. At the first step, we included all relevant covariates. These were prognostic variables for mCRC patients as recommended by Goey et al,[24] only excluding the volume of liver involvement as this was not available. In addition, we included tumor grade, female sex and high alkaline phosphatase in blood samples as these variables are prognostic markers for survival. CEA >4 μg/L and high LDH were statistically significant when included in the multiple regression model, but were excluded from the analysis due to many missing values. From this model, we removed the variable with the largest *P*-value >0.05. In the second step, we removed the covariate with the largest $P > 0.05$ among the remaining variables from the first step. The process continued until all the remaining variables were significant at level 0.05 and a final model was obtained. Results are reported as hazard ratios (HRs) and 95% CIs. All analyses were performed with the statistical program SPSS v22. All statistical tests were two-tailed using significance level 5%.

3 | RESULTS

3.1 | Study population

In the 604 patients with sufficient morphological material of invasive adenocarcinoma for analyses (Supplementary Figure S1), the median age was 70 years and 209 patients (35%) were >75 years. In total 215 patients (36%) had PS >1. First-line chemotherapy was given to 377 patients (62%, 75% below 75 years, and 26% above). Of those, 287 patients (76%) received combination chemotherapy, 28 patients (7%) received bevacizumab, and 27 patients (7%) received an EGFR-inhibitor. Supplementary Table S1 illustrates follow-up data on frequency of second- and third-line treatment according to the different first-line treatment given. Median OS (95% CI) was 11 months (9.6-12.3) for all patients. For patients treated with first-line chemotherapy, median OS and PFS were 17 months (15.0-19.0) and 8 months (7.2, 8.4), respectively. At last follow-up, 24 patients (3%) were alive.

3.2 | MSI, *BRAF* status, and patient characteristics

Totally 40 (7%) of 583 evaluable tumors were MSI-H. Tumors with MSI-H status had more often mut*BRAF* compared to MSS tumors (87% vs 16%, $P < 0.001$), and consequently less often *KRAS* mutations (mut*KRAS*) (6% vs 44%, $P < 0.001$). Figure 1A illustrates *KRAS*, *BRAF*, and MSI status in the TMA cohort (n = 428). MSI-H patients had less often liver and lung metastases, but more often lymph node metastases (Table 1). Female sex, right-sided primaries, elderly patients, and grade 3-4 tumors were more common in the MSI-H group. Patients with MSI-H tumors given first-line palliative chemotherapy received less often second- and third-line chemotherapy compared to MSS (30% vs 58%, $P = 0.019$ and 5% vs 27%, $P = 0.033$, respectively). In fully adjusted multiple logistic regression, right-sided primaries, mut*BRAF*, and no lung metastases were significantly associated with MSI-H status (Supplementary Table S2).

When analyzing all 591 patients with *BRAF* status available, the frequency of mut*BRAF* was 20% (120 of 591 patients) and MSI-H was 7%. Tumors with mut*BRAF* were more often MSI-H compared to wt*BRAF* (28% vs 1%, $P < 0.001$). We divided the patients into four groups according to MSI/*BRAF* status (Figure 1B, Supplementary Table S3). Elderly patients (>75 years) had more often MSI-H/mut*BRAF* tumors compared to patients <75 years (9% vs 4%, $P = 0.012$). Patients with MSI-H/mutBRAF tumors had also more often lymph node metastases and tumor grade 3, but less often liver metastases compared to the other groups. Patients with MSI-H/wtBRAF tumors had more often liver metastasis as well as liver-only metastasis compared to the other groups.

3.3 | Overall and progression-free survival

Both median OS and PFS were shorter in patients with MSI-H tumors (Figure 2, Table 2, Figure 3). Median OS was 6 months for patients with MSI-H compared to 11 months for patients with MSS tumors ($P = 0.004$). Patients with mut*BRAF* tumors had a median OS of 7 months compared to 12 months with wt*BRAF* tumors ($P < 0.001$). Median OS in elderly patients was 4 versus 5 months for MSI-H vs MSS cases ($P = 0.024$) and 3 versus 6 months ($P < 0.001$) for mut*BRAF* versus wt*BRAF* cases, respectively. For patients <75 years, median OS was 8 versus 15 months ($P = 0.012$) for MSI-H vs MSS cases and 11 versus 16 months ($P < 0.001$) for mut*BRAF* vs wt*BRAF* patients, respectively. In the best supportive care group, median OS was 2 versus 3 months for MSI-H versus MSS patients, respectively ($P = 0.025$). Among patients given first-line chemotherapy, median OS was 9 versus 18 months for MSI-H versus MSS cases ($P = 0.010$), and 13 versus 18 months for mut*BRAF* vs wt*BRAF* cases ($P = 0.005$). Median PFS after 1st-line chemotherapy was 4 versus 8 months for MSI-H versus MSS cases ($P = 0.101$) and 7 versus 8 months for mut*BRAF* versus wt*BRAF* cases ($P = 0.125$). For patients with response registered after first-line chemotherapy with the MSI status (n = 321) and BRAF status (n = 328) available, the objective response rate (ORR) was 5% versus 44% for MSI-H versus MSS cases ($P = 0.002$), and 37% versus 43% for mut*BRAF* versus wt*BRAF* cases ($P = 0.609$).

In log-rank survival analyses, the negative prognostic potential of MSI-H was only statistically significant in wt*BRAF* tumors and the negative prognostic potential of mut*BRAF* was only seen in MSS tumors (Figure 4A-D, supplementary Figure S3). The test of interaction between these two variables was significant ($P = 0.010$), also when

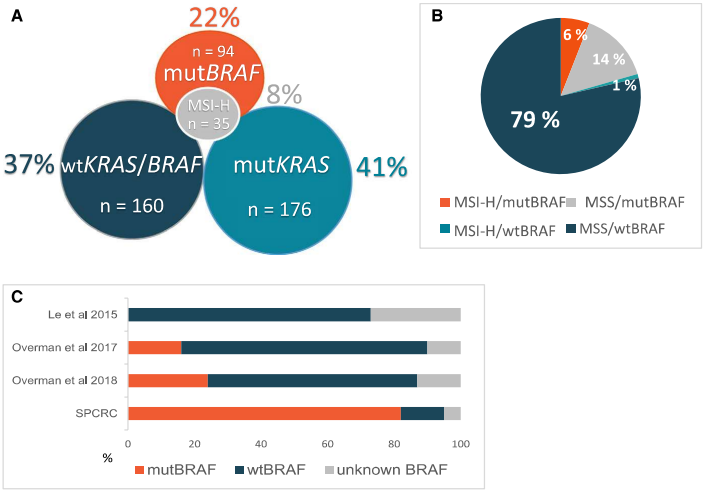

FIGURE 1 Mutation status in a population-based Scandinavian cohort of metastatic colorectal cancer: (A) Venn diagram illustrating *KRAS*, *BRAF* and MSI status in primary tumors of patients in the TMA cohort with analysis available (n = 428), (B) Distribution of *BRAF*/MSI subgroups in patients with sufficient material for these analyses (n = 569), (C) Incidence (%) of *BRAF* mutations in MSI-H tumors in the Scandinavian prospective colorectal cancer cohort (SPCRC) (n = 40) compared to recently published immunotherapy trials by Le et al 2015 (n = 21), Overman et al 2017 (n = 74) and Overman et al 2018 (n = 119)

TABLE 1 Characteristics of a population-based Scandinavian cohort of metastatic colorectal cancer patients with MSI status available (n = 583)

Characteristic n (%[a])	All patients n = 583 (100%)	Missing *n*	MSI-H n = 40 (7%)	MSS n = 543 (93%)	MSI-H vs MSS *P*-value
Age in years, *median*	70		75	70	0.157
Age >75 y, n (%)	200 (34)		19 (48)	181 (33)	0.084
Female, n (%)	281 (48)		28 (70)	253 (47)	0.005
PS ECOG >1, n (%)	207 (36)	1	18 (45)	189 (35)	0.231
ECOG 0	206 (35)		10 (25)	196 (36)	0.464
ECOG 1	169 (29)		12 (30)	157 (29)	
ECOG 2	117 (20)		11 (28)	106 (20)	
ECOG 3	90 (15)		7 (18)	83 (15)	
Right-sided, n (%)	202 (35)	11	33 (85)	169 (32)	<0.001
Liver metastases, n (%)	378 (65)		13 (33)	365 (67)	<0.001
Liver only, n (%)	120 (21)		5 (13)	115 (21)	0.228
Lung metastases, n (%)	148 (27)		3 (8)	145 (27)	0.007
Lymph node metastases, n (%)	156 (27)		20 (50)	136 (25)	0.001
Peritoneal metastases, n (%)	108 (19)		6 (15)	102 (19)	0.676
> 1 metastatic site, n (%)	357 (61)		20(50)	337 (62)	0.178
Synchronous metastases, n (%)	332 (57)		23 (58)	309 (57)	1.000
Local relapse, n (%)	37 (6)		6 (15)	31 (6)	0.033
Comorbidity, n (%)	320 (56)	6	21 (54)	299 (56)	0.868
Weight loss >10%, n (%)	239 (45)	50	22 (60)	217 (44)	0.086
CEA >4 μg/L, n (%)	235 (78)	280	11 (69)	224 (78)	0.538
ALP high, n (%)	297 (57)	63	17 (57)	290 (57)	1.000
LDH high, n (%)	227 (48)	107	11 (37)	216 (48)	0.258
Primary tumor resected, n (%)	474 (81)		36 (90)	437 (81)	0.205
Tumor grade					<0.001
1-2, n (%)	339 (79)	152	15 (43)	324 (82)	
3, n (%)	92 (21)		20 (57)	72 (18)	
KRAS					<0.001
Mutation, n (%)	177 (41)	151	2 (6)	175 (44)	
Wildtype, n (%)	255 (59)		34 (94)	221 (56)	
BRAF					<0.001

(Continues)

TABLE 1 (Continued)

Characteristic n (%[a])	All patients n = 583 (100%)	Missing *n*	MSI-H n = 40 (7%)	MSS n = 543 (93%)	MSI-H vs MSS *P*-value
Mutation, n (%)	117 (21)	14	33 (87)	84 (16)	
Wildtype, n (%)	452 (79)		5 (13)	447 (84)	
Double wildtype, n (%)	160 (48)	249	3(60)	157 (48)	0.673
Curative surgery for metastases, n (%)	40 (7)	1	1 (3)	39 (7)	0.349
First-line chemotherapy, n (%)	364 (62)		20 (50)	344 (63)	0.127
Combination chemotherapy, n (%)	278 (48)		14 (35)	264 (49)	0.103
Second-line chemotherapy, n (%)	206 (36)	1	6 (15)	200 (37)	0.005
Third-line chemotherapy, n (%)	92 (16)	1	1 (3)	91 (17)	0.022
Trial treatment, n (%)	131 (23)	1	7 (18)	124 (23)	0.448
BSC only, n (%)	216 (37)		19 (48)	197 (36)	0.176
Reason BSC, n (%)		36			0.049
Reduced general health	89 (50)		13 (87)	76 (46)	
Old age	27 (15)		0 (0)	27 (16)	
Comorbidity	16 (9)		2 (13)	14 (9)	
Patient declining treatment	26 (15)		0 (0)	26(16)	
Reduced liver function	4 (2)		0 (0)	4 (2)	
Other	18 (10)		0 (0)	18 (11)	

Abbreviations: ALP high, alkaline Phosphatase >105 U/L; BSC, best supportive care; CEA, carcinoembryonic antigen; Curative surgery, for metastatic disease; Double wildtype, both *BRAF* and *KRAS* wildtype; LDH high, Lactate Dehydrogenase above normal level according to age; Left sided, Site of colon cancer in descending colon, sigmoid and rectum; Metastases, at time of diagnosis of metastatic disease; MSI-H, microsatellite instable high; MSS, microsatellite stable; PS ECOG, performance status score developed by Eastern Cooperative Oncology Group; *P*-value, chi-square test except for age (*t* test); Right sided, Site of colon cancer in ascending colon and transversum; Synchronous metastases, within 6 months after initial diagnose.

[a]Due to rounding not all percentages are 100 in total.

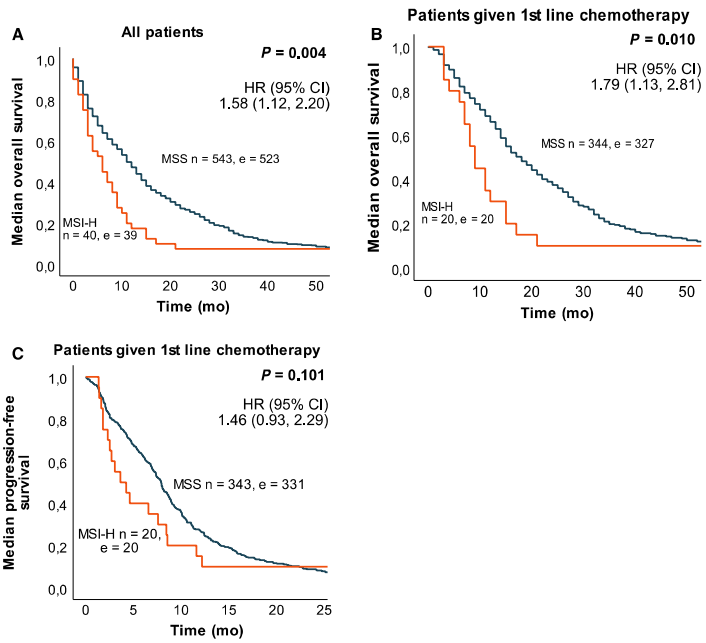

FIGURE 2 Survival in a population-based Scandinavian cohort of patients with metastatic colorectal cancer according to MSI status. Kaplan-Meier curves was calculated with log-rank test for p-value and univariate Cox regression for HR and 95% CI. A, Median overall survival for all patients with MSI status was 6 mo for patients with MSI-H tumors and 11 mo for patients with MSS tumors. B, Median overall survival in patients given first-line chemotherapy was 9 mo for patients with MSI-H tumors and 18 mo for patients with MSS tumors. C, Median progression free survival in patients given first-line chemotherapy was 4 mo for patients with MSI-H tumors and 8 mo for patients with MSS tumors. n, number of patients; e, number of events, HR, Hazard Ratio, CI, confidence interval

adjusting for other prognostic variables ($P = 0.037$, Table 3). When dividing patients into four subgroups according to *BRAF* and MSI status, median OS and PFS after first-line chemotherapy was statistically significantly different (Figure 4E-G, Table 2). Patients with MSI-H/wtBRAF had the worst prognosis compared to the other groups with only 2 months median OS and PFS, but we had only five patients with this molecular tumor characteristic. The negative prognostic value of MSI-H status was seen regardless of *KRAS* status, and in patients with wild-type *KRAS* and wt*BRAF* (double wildtype) tumors, but we had very few patients in some of the subgroups (Supplementary Table S4).

In multiple Cox regression analyses, including known clinical prognostic factors for mCRC survival, mut*BRAF* was the only molecular tumor marker significantly associated with reduced OS. For patients who received first-line chemotherapy mut*BRAF*, mut*KRAS* and MSI-H were all significantly associated with shorter OS and PFS (Table 3).

4 | DISCUSSION

This is, as far as we know, the largest population-based study reporting on MSI and *BRAF* status and its effect on treatment and survival in mCRC. The general poor survival in our cohort is comparable to that seen in Scandinavian cancer registries (10 months median OS) (8) and the American SEER database (1 year survival rate 47%) [25] during the same time period, reflecting our real-world cohort of patients and not poor treatment, as patients receiving combination chemotherapy had the same OS as in clinical trials, including patients during the same time period. We report 7% MSI-H tumors, almost twice as high as most previous reports of mCRC.[1-5] We believe this is due to the unselected nature of the cohort with many elderly patients, patients with poor PS, never included in clinical trials, and many mut*BRAF* cases. Tran et al observed 8% MSI-H in a trial population group and 13% in a general population group, also showing higher presence of MSI-H in patients outside clinical trials.[6] A recently published study on genomic profiling of 8887 mCRC patients reported 7% MSI-H cases,[26] in accordance with our result. In this study, we also confirm our previously published result showing a much higher frequency of mut*BRAF* tumors (20%) compared to previous studies.[7] The same frequency is reported in a recent Nordic phase II trial of elderly vulnerable patients with mCRC.[27] The relatively higher frequency of MSI-H and mut*BRAF* in the general mCRC population implicates that more patients than previously expected could benefit from immunotherapy, or being candidate for adding *BRAF*-inhibitor combinations to standard treatment (Figure 3). This stresses the importance of MMR and *BRAF* testing in all mCRC patients. Figure 3 illustrates the distribution of the tumor molecular alterations, their survival and possible treatment options.

TABLE 2 Median (Med) overall survival and progression free survival (months) after different treatment regimens in a population-based Scandinavian cohort of metastatic colorectal cancer patients (n = 583) according to MSI status (left side) and MSI and *BRAF*-mutation status combined (right side)

n (%) Survival time	All patients 583 (100) Med (95% CI)	MSI-H 40 (7) Med (95% CI)	MSS 543 (93) Med (95% CI)	*P*-value	MSI-H/mut*BRAF* 33 (6) Med (95% CI)	MSI-H/wt*BRAF* 5 (1) Med (95% CI)	MSS/mut*BRAF* 84 (15) Med (95% CI)	MSS/wt*BRAF* 447 (79) Med (95% CI)	*P*-value
OS									
All patients	11 (9.6, 12.4)	6 (2.9, 9.1)	11 (9.6, 12.4)	0.004	6 (2.6, 9.4)	2 (0.0, 4.1)	8 (3.9, 12.1)	12 (10.5, 13.5)	<0.001
n/e	583/562	40/39	543/523		33/33	5/5	84/83	447/428	
1st-line chemotherapy	17 (14.9, 19.1)	9 (6.8, 11.2)	18 (15.8, 20.2)	0.010	11 (7.0, 15.0)	4 {4, 7}	14 (11.4, 16.6)	19 (16.4, 21.6)	0.001
n/e	364/347	20/20	344/327		17/17	2/2	51/50	287/271	
1st-line combination chemotherapy	20 (17.3, 22.7)	8 (6.2, 9.8)	20 (17.3, 22.7)	0.015	8 (5.8, 10.2)	4 {4, 7}	14 (10.5, 17.5)	21 (18.1, 23.9)	<0.001
n/e	278/262	14/14	264/248		11/11	2/2	36/35	225/210	
Best supportive care only	3 (2.2, 3.8)	2 (0.3, 3.7)	3 (2.2, 3.8)	0.025	3 (1.1, 4.9)	1 {1, 1, 2}	1 (0.3, 1.7)	4 (3.2, 4.8)	0.001
n/e	216/215	19/19	197/196		16/16	3/3	33/33	158/157	
PFS									
1st-line chemotherapy	8 (7.2, 8.4)	4 (1.0, 6.2)	8 (7.2, 8.6)	0.101	5 (0.6, 8.6)	2 {2, 2}	7 (5.5, 8.3)	8 (7.3, 8.7)	0.007
n/e	363/351	20/20	343/331		17/17	2/2	50/49	287/276	
1st-line combination chemotherapy	8 (7.5, 8.8)	3 (0.2, 5.9)	8 (7.8, 9.0)	0.110	5 (0.8, 8.4)	2 {2, 2}	7 (6.7, 8.1)	9 (7.9, 9.2)	0.002
n/e	278/267	14/14	264/253		11/11	2/2	36/35	225/215	
2nd-line chemotherapy	5 (3.7, 5.3)	4 (0.4, 7.9)	5 (3.7, 5.7)	0.578	3 (0.5, 4.9)	5 {5}	4 (1.3, 6.6)	5 (3.6, 5.9)	0.779
n/e	200/197	7/7	193/190		6/6	1/1	25/25	165/162	

Abbreviations: All patients, patients with MSI status available; CI, confidence interval; e, number of events; MSI-H, microsatellite instable high; MSS, Microsatellite stable; mut*BRAF*, *BRAF* mutation; n, number of patients; OS, overall survival; PFS; progression free survival; *P*-value, log-rank test; wt*BRAF*, *BRAF* wildtype.

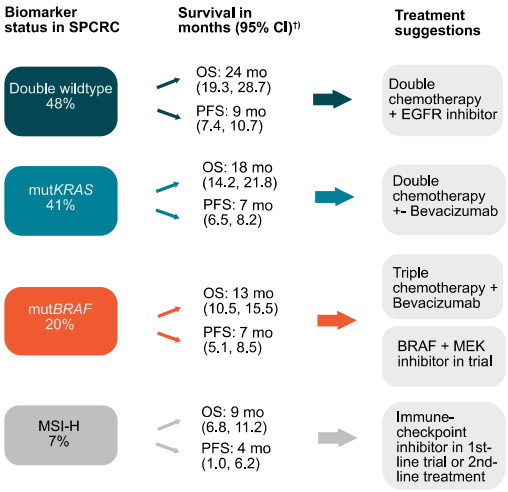

FIGURE 3 Frequency of molecular alterations and survival data after first-line chemotherapy in a Scandinavian population-based cohort of metastatic colorectal cancer (SPCRC) with suggestions on choice of treatment for the specific patient groups. Double wildtype, *BRAF* and *KRAS* wildtype; mut*BRAF*, *BRAF* mutation; mut*KRAS*, *KRAS* mutation; MSI-H, microsatellite instability-high; OS, median overall survival; PFS, median progression-free survival; CI, confidence interval; †) after first-line chemotherapy

In primary, non-metastatic CRC, MSI-H is known to be a good prognostic factor.[28,29] In mCRC, on the other hand, these patients belong to the poor prognostic group, in accordance with previous studies.[2,3,5,6,14] MSI-H and mut*BRAF* were clearly associated, and in multivariate analysis both tumor markers were independent poor prognostic predictors for OS and PFS in patients treated with first-line chemotherapy. In subgroup analysis, we found that the negative impact of MSI-H on survival only reached statistical significance in wt*BRAF* patients, and the negative impact of mut*BRAF* on survival was only seen in MSS patients. Previous studies on this matter have shown contradictory results.[3-6,16,30] None of the other studies are entirely population based, and a limitation of all studies, including our own, is the limited number of patients in some subgroups. In our cohort, patients with MSI-H/wt*BRAF* tumors had the worst prognosis, in accordance with the randomized COIN trial.[3] A recent study reports poor prognosis in mCRC patients with ALK, ROS1, and NTRK rearrangements in tumor, and these cases were associated with MSI-H (48%) and were almost exclusively wt*BRAF* (96%).[31] This might explain the particularly poor prognosis we see in this subpopulation, but the very limited number of patients in this group precludes firm conclusions and the results need verification in the larger studies.

Our cohort of MSI-H patients had substantially less benefit from chemotherapy compared to MSS patients, and very few made it to the second- and third-line of treatment. Recent third-line immunotherapy trials in MSI-H mCRC patients have shown ORR 31% (nivolumab monotherapy) and 55% (dual checkpoint inhibition), with median PFS and OS not reached at 12 months.[19,20] These impressive results are in great contrast to the treatment benefit (ORR 5%, PFS 4 months) and survival (OS 9 months) seen in our unselected MSI-H patients treated with first-line chemotherapy. However, the patient populations differ greatly and there are probably several reasons for this vast difference in prognosis. More than 45% of our MSI-H patients had PS > 1, while immunotherapy trials only include patients with PS 0-1. Most MSI-H patients in our cohort were also mut*BRAF* (87%) (Figure 1C), markedly in contrast to the immunotherapy trials, where only 0%, 16%, and 24% were mut*BRAF*[18-20] (Figure 1D-F). Other population-based cohorts of mCRC have shown different frequencies of mut*BRAF* in MSI-H tumors, ranging from 25%-60%, but with limited number of patients.[4,6,16,29,32] Patients with Lynch syndrome tumors are essentially MSI-H/wt*BRAF* and often diagnosed at a younger age, which could explain the high frequency of these patients in clinical studies. In our study, we found only five cases with this molecular feature. MSI-H/mut*BRAF* tumors develop in the serrated pathway and belong to the consensus molecular subtype 1 classification of CRC, associated with poor survival after relapse.[33] In the two immunotherapy trials including patients with mut*BRAF* tumors, the response rate and survival did not significantly differ according to *BRAF* status, but the numbers were limited. Additional data on immunotherapy in the non-Lynch group in a general patient population is warranted, to further evaluate if the benefit of immunotherapy in MSI-H patients may vary according to *BRAF* status.

In our study, MSI-H/mut*BRAF* cases were more often seen in elderly patients (>75 years), and median age of MSI-H cases was 75 years, in great contrast to the recent third-line immunotherapy trials (46-58 years).[18-20] MSI-H status was less important for survival in elderly patients, but this might in part reflect the low treatment frequency in this subgroup. Elderly cancer patients in general receive less palliative chemotherapy and treatment recommendations for the elderly are uncertain as they usually are not included in clinical trials.[8] In a recent retrospective study, elderly patients (>62 years) with malignant melanoma had a better response to anti-PD-1 therapy compared to younger patients,[34] believed to be due to decreased intertumoral Tregs and increased $CD8^+$:Treg ratio in the elderly patients. Considering the high age of most mCRC patients with MSI-H tumors, future studies on immunotherapy in elderly patients are important.

The marked difference between our MSI-H patients and third-line immunotherapy trials reported so far should be taken into consideration when transferring results from these studies to the general population. There is reason to believe

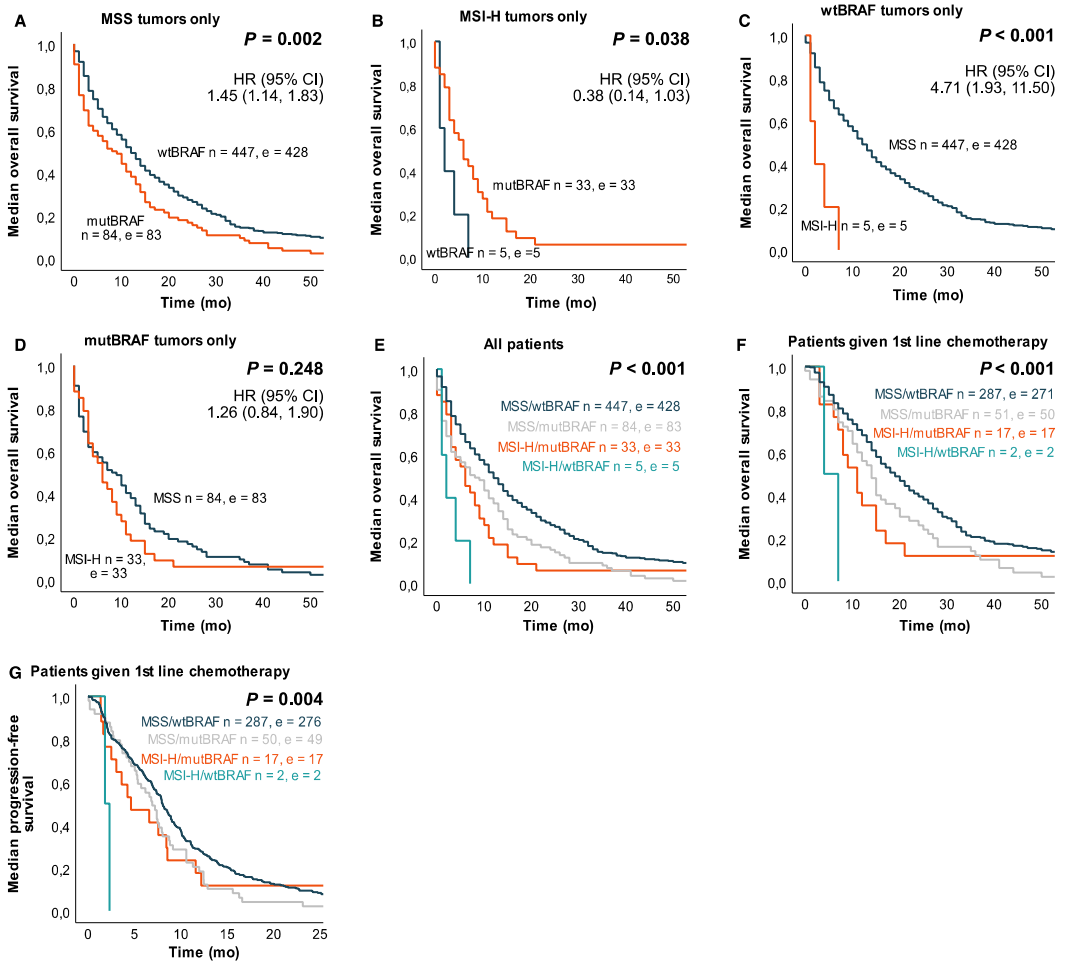

FIGURE 4 Survival in a population-based Scandinavian cohort of metastatic colorectal cancer patients according to MSI and *BRAF* status. Kaplan-Meier curves was calculated with log-rank test for p-value and univariate Cox regression for HR and 95% CI. A, Median overall survival for patients with MSS tumors was 8 mo if mut*BRAF* and 12 mo if wt*BRAF*. B, Median overall survival for patients with MSI-H tumors was 6 mo if mut*BRAF* and 2 mo if wt*BRAF*. C, Median overall survival for patients with *BRAF*-wildtype tumors was 2 mo if MSI-H and 12 mo if MSS. D, Median overall survival for patients with *BRAF*-mutated tumors was 6 mo if MSI-H and 8 mo if MSS. E, Median overall survival for all patients in subgroups of MSI and *BRAF* status. F, Median overall survival for patients given first-line chemotherapy. G, Median progression free survival for patients given first-line chemotherapy. n, number of patients; e, number of events

that future immunotherapy trials in first-line may recruit a more heterogeneous MSI-H population, with for instance more aggressive disease and more mut*BRAF* cases.

At present, the use of immune-checkpoint inhibitors is recommend as second-line treatment for MSI-H mCRC patients.[21] Our data, however, show that a substantial number of MSI-H patients never get to second-line treatment and the benefit of first-line chemotherapy is very limited. Both these factors indicate that checkpoint inhibitors should probably be given as first-line treatment and such studies are ongoing. A recent abstract from the Checkmate 142 trial with first-line dual checkpoint inhibition in 45 patients reported 60% ORR, in line with third-line immunotherapy trials.[35] The very poor response rate in our cohort of 5% in MSI-H compared to 40% in MMS is of particular concern if the patient has a potentially resectable disease, and first-line treatment with checkpoint inhibitors should be considered in such cases.

TABLE 3 Results from multiple Cox regression of overall survival and progression free survival in a population-based Scandinavian cohort of metastatic colorectal cancer patients (n = 798) diagnosed from October 2003 to August 2006 and followed until 4 February 2014

	Overall survival all patients[a]) (n = 360, e = 343)			Overall survival in patients given 1st-line chemotherapy (n = 248, e = 233)			Progression free survival after 1st-line chemotherapy (n = 247, e = 237)		
Variable	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	**p-value**	**HR**	**95% CI**	***P*-value**
Female	0.85	0.68-1.06	0.156	0.77	0.59-1.02	0.063	0.97	0.74-1.26	0.805
Age >75 y	1.05	0.75-1.48	0.757	1.39	0.89-2.16	0.147	1.54	0.99-2.38	0.055
PS ECOG > 1	1.86	1.42-2.44	<0.001	2.17	1.50-3.13	<0.001	2.05	1.42-2.96	<0.001
Right-sided tumor	1.11	0.86-1.43	0.407	0.96	0.71-1.31	0.815	0.79	0.58-1.08	0.135
Tumor grade 3	1.81	1.34-2.45	<0.001	1.76	1.22-2.55	0.003	1.65	1.15-2.37	0.007
Primary tumor resected	1.04	0.60-1.83	0.879	0.80	0.43-1.49	0.480	1.48	0.83-2.63	0.185
Synchronous metastases	0.70	0.55-0.88	0.002	0.78	0.59-1.04	0.087	0.77	0.58-1.02	0.066
> 1 organ metastases	1.55	1.14-2.10	0.005	1.79	1.22-2.63	0.003	1.48	1.01-2.16	0.044
Liver only	1.44	1.00-2.08	0.051	1.44	0.92-2.26	0.113	1.34	0.86-2.09	0.198
Curative metastasis surgery	0.29	0.18-0.47	<0.001	0.33	0.20-0.55	<0.001	0.38	0.24-0.62	<0.001
ALP high	2.00	1.57-2.54	<0.001	1.95	1.45-2.63	<0.001	1.59	1.48-2.13	0.002
First-line chemotherapy	0.38	0.27-0.54	<0.001			ni			ni
MSI-H	1.42	0.86-2.37	0.174	2.34	1.18-4.64	0.015	2.13	1.08-4.18	0.028
BRAF mutation	1.86	1.29-2.69	0.001	1.94	1.23-3.05	0.004	1.62	1.04-2.53	0.034
KRAS mutation	1.22	0.94-1.57	0.135	1.39	1.02-1.90	0.038	1.52	1.12-2.08	0.008
Interactions[b]									
MSI-H effect in wt*BRAF*	4.46	1.83-10.86	0.001						
MSI-H effect in mut*BRAF*	1.20	0.80-1.80	0.386						
mut*BRAF*-effect in MSS	1.44	1.14-1.83	0.002						
mut*BRAF*-effect in MSI-H	0.40	0.16-1.04	0.059						

Abbreviations: ALP high, Alkaline Phosphatase >105 U/L; CI, confidence interval; e, number of events; HR, hazard ratio; MSI-H, microsatellite instable high; MSS, microsatellite stable; mut*BRAF*, *BRAF* mutated; n, number of patients; ni, not included; PS ECOG, performance status score developed by Eastern Cooperative Oncology Group; *P*-value, from likelihood ratio test; wt*BRAF*, *BRAF* wildtype.

[a]CEA >4 and LDH high was also statistically significant when included in the multiple regression model, but were excluded from the analysis due to many missing values.

[b]Testing the hypothesized interaction between MSI and *BRAF* showed significantly higher effect of MSI-H among those with wt*BRAF* (HR = 4.46) than in those with mut*BRAF* (HR = 1.20) tumors and higher effect of mut*BRAF* among those with MSS (HR = 1.44) compared to MSI-H (HR = 0.40) tumors (interaction HR = 0.28, 95% CI: 0.11-0.74, P = 0.010), after adjusting for all other covariates it was still statistically significant (interaction HR = 0.20, 95% CI: 0.94-0.91, *P* = 0.037).

5 | LIMITATIONS OF STUDY

This is a prospectively collected study; the analyses, however, were done retrospectively. The patients were treated more than 10 years ago, and although the treatment options for mCRC have not changed much in the past decade, we possibly treat more patients with more intensive/combination regimens as well as metastasectomy, resulting in improved survival. Our study is population-based and therefore includes more patients with older age, worse PS, comorbidity and less treatment compared to clinical trials, however, patients without available or sufficient tumor tissue for analysis could not be included and these patients have a particularly poor prognosis.[7] Despite this being the largest population-based study reporting on MSI and BRAF status and its effect on survival in mCRC, the number of patients in some of the MSI/*BRAF* subgroups was still limited.

6 | CONCLUSIONS

In a population-based cohort of mCRC patients, MSI-H and mut*BRAF* were more common than previously reported, and consequently more patients could benefit from

immunotherapy and *BRAF*-inhibitor treatments. Our unselected cohort of MSI-H patients differed considerably from patients included in recent immunotherapy trials as they were older, had worse PS and most of them also had mut*BRAF* tumor (non-Lynch). Further studies are needed to evaluate the effect of immunotherapy in these subgroups of patients. Patients with MSI-H tumors had worse survival, very poor response rate and few received second-line treatment, indicating that these patients should probably be considered for immunotherapy as the first-line treatment.

ACKNOWLEDGEMENTS

We thank Randi Eikeland (Haukeland University Hospital, Bergen, Norway) for data management.

CONFLICT OF INTEREST

The authors of this manuscript declare no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

HS: conceptualization, data curation, methodology, project administration, supervision, writing—original draft and writing—review and editing. PP: conceptualization, data curation, methodology, project administration, supervision and writing—review and editing. BG: conceptualization, data curation, methodology, project administration, supervision, resources, writing—original draft and writing—review and editing. AD: investigation, methodology, validation and writing—review and editing. FP: investigation, methodology, resources, validation and writing—review and editing. PHE: investigation, methodology, validation and writing—review and editing. AM: investigation, methodology, validation and writing—review and editing. MS: investigation, methodology, validation and writing—review and editing. KA: formal analysis, visualization, writing—original draft and writing—review and editing. GEE: formal analysis, methodology and writing—review and editing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients seen at the clinics. The study, including molecular classification of all tumors in the three regions, was approved by the regional ethical committees in Norway (Regional Committee for Medical and Health Research Ethics—REC West), Sweden (Regional Ethical Committee Uppsala) and Denmark (The Regional Scientific Ethical Committees for Southern Denmark).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Aasebø KØ, Dragomir A, Sundström M, et al. Consequences of a high incidence of microsatellite instability and *BRAF*-mutated tumors: A population-based cohort of metastatic colorectal cancer patients. *Cancer Med*. 2019;8:3623–3635. https://doi.org/10.1002/cam4.2205

frontiers in Oncology

ORIGINAL RESEARCH
published: 11 February 2020
doi: 10.3389/fonc.2020.00008

CDX2: A Prognostic Marker in Metastatic Colorectal Cancer Defining a Better *BRAF* Mutated and a Worse *KRAS* Mutated Subgroup

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Gastrointestinal Cancers, a section of the journal Frontiers in Oncology

Received: *30 October 2019*
Accepted: *06 January 2020*
Published: *11 February 2020*

Citation:
Aasebø K, Dragomir A, Sundström M, Mezheyeuski A, Edqvist P-H, Eide GE, Ponten F, Pfeiffer P, Glimelius B and Sorbye H (2020) CDX2: A Prognostic Marker in Metastatic Colorectal Cancer Defining a Better BRAF Mutated and a Worse KRAS Mutated Subgroup. Front. Oncol. 10:8. doi: 10.3389/fonc.2020.00008

Background: Survival of metastatic colorectal cancer (mCRC) patients has improved, but mainly for trial patients. New predictive and prognostic biomarkers validated in the general mCRC population are needed. Caudal-type homeobox 2 (CDX2) is an intestine-specific transcription factor with potential prognostic and predictive effect, but the importance in mCRC has not been fully investigated.

Methods: Immunohistochemistry analysis of CDX2 was performed in a Scandinavian population-based cohort of mCRC (n = 796). Frequency, clinical and tumor characteristics, response rate, progression-free survival, and overall survival (OS) were estimated.

Results: Loss of CDX2 expression was found in 87 (19%) of 452 stained cases, in 53% if *BRAF* mutated (*BRAF*mut) and in 9% if *KRAS* mutated (*KRAS*mut). CDX2 loss was associated with microsatellite instability, *BRAF*mut, and poor differentiation and inversely associated with *KRAS*mut. Patients with CDX2 loss received less first-line (53 vs. 64%, p = 0.050) and second-line (23 vs. 39%, p = 0.006) chemotherapy and secondary surgery (1 vs. 9%, p = 0.019). Median progression-free survival and OS for patients given first-line combination chemotherapy was 4 and 10 months if CDX2 loss vs. 9 and 24 months if CDX2 expressed (p = 0.001, p < 0.001). Immediate progression on first-line combination chemotherapy was seen in 35% of patients with CDX2 loss vs. 10% if CDX2 expressed (p = 0.003). Median OS in patients with *BRAF*mut or *KRAS*mut and CDX2 expressed in tumor (both 21 months) was comparable to wild-type patients (27 months). However, if CDX2 loss, median OS was only 8 and 11 months in *BRAF*mut and *KRAS*mut cases, respectively, and 10 months in double wild-type patients. In multivariate analysis, CDX2 loss (hazard ratio: 1.50, p = 0.027) and *BRAF*mut (hazard ratio: 1.62, p = 0.012) were independent poor prognostic markers for OS.

Conclusion: In a population-based cohort of mCRC patients, CDX2 loss is an independent poor prognostic marker. Expression of CDX2 defines a new subgroup of *BRAF*mut cases with a much better prognosis. Loss of CDX2 defines a small group of *KRAS*mut cases with a worse prognosis. Patients with CDX2 loss receive less palliative chemotherapy with less benefit and rarely reach secondary surgery.

Keywords: caudal type homeobox transcription factor, CDX2, colorectal cancer, metastatic disease, stage 4 colorectal cancer, prognosis, population based

INTRODUCTION

Colorectal cancer (CRC) is one of the major cancer types worldwide. Globally, there are 1.4 million new cases and 0.7 million deaths in 2012 (1, 2). Approximately 25% of patients present with metastatic CRC (mCRC) at diagnosis, and another 20% will eventually develop metastasis. Despite progress over the past decades, with median overall survival (OS) up to 30 months in clinical trials, prognosis for patients in population-based cohorts is still poor with a median OS of 10–15 months (3, 4). Patients included in clinical trials are highly selected with, for instance, better performance status, younger age, and less or no comorbidity, and cannot be compared to the general mCRC patients. There is a need for predictive and prognostic markers validated in population-based cohorts to guide treatment selection and improve survival for mCRC patients.

Caudal-type homeobox 2 (CDX2) is an intestine-specific transcription factor and one of the most sensitive and specific markers of intestinal differentiation (5). Immunohistochemistry (IHC) analysis for CDX2 is implemented in the clinical diagnostics as a biomarker for intestinal differentiation in tumors of unknown origin. It is deregulated in a subset of patients with CRC, and downregulation has been associated with poor prognosis (6–13). Loss of CDX2 expression has also been associated with other poor prognostic features such as advanced stages, poor differentiation, *BRAF* mutation (*BRAF*mut), and microsatellite instability (MSI) (12, 14, 15). The negative prognostic effect of CDX2 could therefore be related to the known associations between these features and poor survival. No previous studies have fully explored if the negative prognostic effect of CDX2 loss in mCRC could be confounded by these associations. Two recent retrospective studies have reported CDX2 loss as a predictive biomarker for treatment benefit of chemotherapy in stage II (7) and stage III (8) CRC. This has so far not been demonstrated in mCRC (8, 9, 11). Recently, CDX2 loss was reported to be an independent negative prognostic marker in mCRC patients undergoing curative liver metastasis resection, indicating CDX2 loss as a potential biomarker to identify patients with limited benefit from surgery (11). It is therefore important to know the proper frequency of CDX2 loss and its relations to outcome in unselected mCRC patients. The aim of this study was to determine the prevalence, prognostic, and predictive effect of CDX2 loss in unselected patients of mCRC in relation to tumor differentiation, *BRAF, KRAS*, and MSI status.

METHODS

Patient Cohort

A prospectively collected cohort was established of all non-resectable mCRC patients referred to the oncology units of three regional hospitals in Scandinavia: Odense University Hospital (Denmark) ($n = 325$), Uppsala University Hospital (Sweden) ($n = 155$), and Haukeland University Hospital (Norway) ($n = 316$) during 2003–2006 with last follow-up in 2014. These hospitals cover all oncology treatment in their region. Cases not referred in the region were identified through the national (Norway and Sweden) and regional (Denmark) cancer registries ($n = 49$). The cohort consists of 796 patients (**Figure 1**).

Tissue Retrieval and Tissue Microarray Generation

Paraffin-embedded tissue blocks were retrieved from the primary tumor in the majority of cases or from a metastatic lesion (six cases), and corresponding hematoxylin–eosin stained glass slides were examined. Tissue microarray (TMA) generation had been performed previously in 460 (58%) cases (16) according to standards used in the Human Protein Atlas (17), with two 1-mm diameter tumor cores extracted per patient. TMA was generated from tissue blocks from surgical resection of primary tumor in 419 of 460 (91%) TMA cases, the remaining 41 from biopsies (35 cases from primary tumor and 6 cases from metastatic lesion).

Tumor Analyses

Results on gene analysis of *KRAS*, *BRAF*, and MSI in *BRAF*mut tumors and IHC analysis on *BRAF* and MMR were available and performed as described previously (16, 18). IHC for CDX2 was performed for all patients included in the TMA cohort ($n = 460$) using a mouse-monoclonal antibody, #NCL-CDX2, from Leica Biosystems (formerly Novocastra), diluted 1:50. Automated IHC was performed using an Autostainer 480 instrument (Thermo Fischer Scientific, Waltham, MA, United States), with diaminobenzidine (Thermo Fisher Scientific) as chromogen. High-resolution images of the IHC staining were obtained by scanning with an Aperio AT2 slide scanner (Aperio, Vista, CA, United States) at 200× magnification. Semiquantitative assessment of immunoreactivity in all tumor cells was assessed independently by two pathologists (AD, FP) without knowledge of clinicopathological data. Annotation discrepancies were re-evaluated to reach consensus. Immunoreactivity was scored for nucleus on a four-tier intensity scale (1 = negative, 2 = weak, 3 = moderate, or 4 = strong), and the estimated fraction of

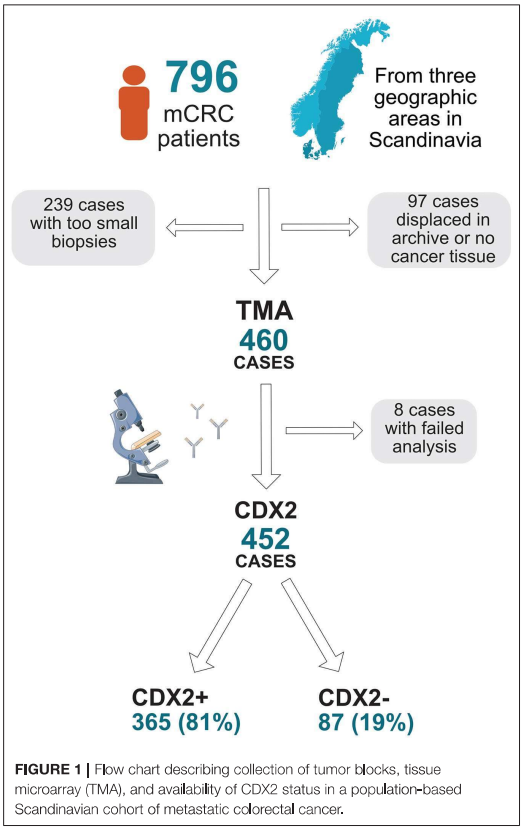

FIGURE 1 | Flow chart describing collection of tumor blocks, tissue microarray (TMA), and availability of CDX2 status in a population-based Scandinavian cohort of metastatic colorectal cancer.

stained tumor cells was denoted as 1 (0–1%), 2 (2–10%), 3 (11–25%), 4 (26–50%), 5 (51–75%), and 6 (>75%) (**Figure 2**). Loss of CDX2 expression (CDX2 loss) was defined as tumors with nuclear fraction staining <10% regardless of intensity, as recommended in the interpretation of IHC of tumor markers in CRC (19). This cutoff was chosen according to previous literature, and the distribution of expression across the cohort. CDX2 expression was defined as tumors with nuclear fraction staining >10% regardless of intensity. CDX2 status was evaluable in 452 cases (**Figure 1**).

Statistics

Exact chi-square test was used for group comparisons. Multiple binary logistic regression was used for dichotomous outcome variables, and results are reported as odds ratios (ORs) with 95% confidence intervals (CIs). OS was the interval from the date of metastatic disease to the date of death and censored if the patient was alive on February 4th, 2014. Progression-free survival (PFS) was the interval from the date of first administration of chemotherapy to the date of progression (on CT scan) or death and censored if the patient was alive without progression on February 4th, 2014. OS and PFS were analyzed using the Kaplan–Meier method with log rank test and Cox multiple regression. Cox regression was performed with backward stepwise selection of covariates to the final model. At the first step, we included all relevant covariates. From this model, we removed the variable with the largest *p*-value. In the second step, we removed the covariate with largest $p > 0.05$ among the remaining variables from the first step. The process continued until all remaining variables were significant at level 0.05, and a final model was obtained. Results are reported as hazard ratios (HRs) and 95% CIs. All analyses were performed with the statistical program SPSS v22. All statistical tests were two-tailed using 5% significance level.

RESULTS

Study Population

Of the 796 patients included, 460 patients had TMA generated; reasons for no TMA were too small biopsies with no resection of primary tumor performed ($n = 239$) and missing or no cancer tissue ($n = 97$) (**Figure 1**). Cases with lacking TMA was evenly distributed between the three regions of inclusion (45% from Haukeland, 36% from Uppsala, and 43% from Odense University Hospital, $p = 0.190$). For patients with CDX2 status available, median OS was 11 months (9.4, 12.6) for all patients ($n = 452$), 18 months (15.0, 21.0) if given first-line chemotherapy ($n = 281$) and 21 months (17.1, 24.9) if given first-line combination chemotherapy ($n = 217$). As first-line treatment, 52 patients received irinotecan-based and 168 oxaliplatin-based combination chemotherapy, and 57 received 5-fluorouracil monotherapy. Twenty-one had bevacizumab and 20 had cetuximab combination treatment. Combination chemotherapy was mainly doublet; only three patients received triplet chemotherapy. There was no significant difference in treatment schedules given between patients with loss or expression of CDX2 (**Table S1**). For patients with CDX2 status available, 21% had a *BRAF* V600E mutation (*BRAF*mut), 41% a *KRAS* mutation (*KRAS*mut), 8% were MSI high (MSI-H), and 38% double (*KRAS* and *BRAF*) wild-type tumor (**Table 1**, **Figure 3A**).

Patient Characteristics and Treatment

Eighty-seven (19%) of 452 patients had CDX2 loss (**Figure 3A**). Frequency of CDX2 loss was similar (20%) among the subgroup of elderly patients (>75 years). In patients with MSI-H, *BRAF*mut and *KRAS*mut tumors, 58, 53, and 9% had CDX2 loss, respectively. A Venn diagram (**Figure 3A**) illustrates the frequency of CDX2 loss, MSI-H, *BRAF*mut, and *KRAS*mut, and their interrelations. CDX2 loss was associated with right-sided primary tumor, poor differentiation, MSI-H, *BRAF*mut, and *KRAS* wild type (*KRAS*wt) (**Table 1**, **Figure 3B**). In subgroup analyses, cases with CDX2 loss were mostly *BRAF*mut/MSS (42%), and cases with CDX2 expression were mostly *BRAF*wt/MSS (87%) (**Figure 3C**). Patients with CDX2 loss had less often lung metastases and liver-only disease, but more often distant lymph node metastases (**Table 1**). In multiple

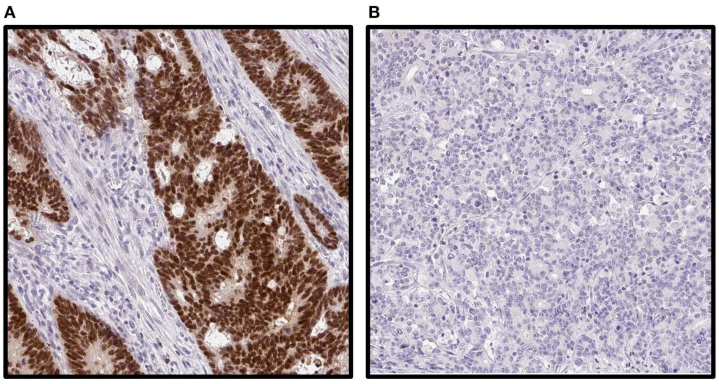

FIGURE 2 | Immunohistochemical staining images of caudal-type homeobox 2 (CDX2) on tumor tissue microarray in a population-based Scandinavian cohort of metastatic colorectal cancer patients. **(A)** Strong staining in all cells. **(B)** Completely negative staining.

logistic regression, *BRAF*mut and poor differentiation were significantly correlated to CDX2 loss (**Table S2**). Patients with CDX2 loss received less first- (53 vs. 64%, $p = 0.050$) and second-line chemotherapy (23 vs. 39%, $p = 0.006$) and rarely had secondary surgery (1 vs. 9%, $p = 0.019$) compared to patients with CDX2 expressed.

Survival and Response

Median OS in the whole cohort (untreated and treated with chemotherapy) was 6 months if tumor had CDX2 loss and 13 months if tumor showed CDX2 expression ($p < 0.001$). For patients given first-line chemotherapy, median OS was 11 months if CDX2 loss ($n = 46$) and 21 months if CDX2 expressed ($n = 235$) ($p < 0.001$). For patients given first-line combination chemotherapy, median OS was 10 months if CDX2 loss and 24 months if CDX2 expressed ($p < 0.001$) (**Figure 4**). Median PFS after first-line chemotherapy was 5 vs. 8 months ($p = 0.003$) in patients with CDX2 loss vs. expression and 4 vs. 9 months ($p = 0.001$) if given first-line combination chemotherapy (**Figure 4**). We did not observe any survival benefit of combination chemotherapy compared to monotherapy in first-line treatment of patients with tumors demonstrating CDX2 loss (median OS 10 vs. 12 months, $p = 0.979$ and median PFS 5 vs. 4 months, $p = 0.742$), but this was evident in patients with tumors demonstrating CDX2 expression (median OS 12 vs. 24 months, $p < 0.001$ and median PFS 5 vs. 9 months $p < 0.001$) (**Figure 5**). For patients given first-line combination chemotherapy with response registered ($n = 194$ of 217), objective response rate was 35% if the tumor showed CDX2 loss and 49% if CDX2 was expressed. Immediate disease progression was seen in 35% of patients with CDX2 loss compared to 10% with CDX2 expressed (**Table S3**, **Figure 5E**).

In further subgroup survival analyses, we selected patients given first-line combination chemotherapy to eradicate potential treatment selection bias (**Figure 4D**). In patients with double (*KRAS* and *BRAF*) wild-type tumor, median OS was 10 months if CDX2 loss ($n = 10$) compared to 27 months if CDX2 expressed ($n = 77$) ($p = 0.576$). Patients with *KRAS*mut tumor had median OS of 11 months if CDX2 loss ($n = 4$) compared to 21 months if CDX2 expressed ($n = 83$) ($p = 0.007$). Patients with *BRAF*mut tumors had median OS of 8 months if CDX2 loss ($n = 18$) compared to 21 months if CDX2 expressed ($n = 21$) ($p = 0.008$). Owing to selection of patients treated with combination chemotherapy, we had few patients in some subgroups, particularly of cases with *KRAS*mut and CDX2 loss. However, when analyzing all *KRAS*mut cases, regardless of treatment, median OS was only 2 months if CDX2 loss ($n = 16$) compared to 13 months if CDX2 expressed ($n = 163$) ($p < 0.001$). The negative prognostic effect of CDX2 loss was also significant when analyzing all *BRAF*mut cases, with 6 months median OS if CDX2 loss ($n = 51$) compared to 10 months if CDX2 expressed ($n = 45$) ($p = 0.008$) (**Figure S1**). In patients treated with first-line chemotherapy, the negative prognostic potential of CDX2 loss was seen regardless of *BRAF*, *KRAS*, and MSI status (**Figure S2**), however with few patients in some of the subgroups. CDX2 loss was a poor prognostic marker regardless of tumor side (left-sided 12 vs. 25 months and right-sided 8 vs. 22 months median OS, $p < 0.001$) and tumor grade (poorly differentiated 6 vs. 20 months, $p < 0.001$ and well/moderately differentiated 14 vs. 25 months median OS, $p = 0.002$) in patients treated with first-line combination chemotherapy. Elderly given first-line chemotherapy had a median OS of 9 months if CDX2 loss compared to 15 months if CDX2 expressed ($p = 0.048$).

In multiple Cox regression analyses, with known prognostic factors for mCRC survival, both CDX2 loss and *BRAF*mut (among others) were statistically significant associated with reduced OS. CDX2 loss, MSI-H, *BRAF*mut, and *KRAS*mut (among others) with shorter PFS (**Table 2**).

TABLE 1 | Patient characteristics according to caudal-type homeobox 2 status in a population-based cohort of metastatic colorectal cancer with CDX2 status available ($n = 452$).

Characteristics*	Patients with CDX2 status	Missing	CDX2–	CDX2+	CDX2– vs. CDX2+ *p*-value
Total number (%)	452		87 (19)	365 (81)	
Age in years, median (95% CI)	70 (68.0, 70.2)		70 (66.5, 71.5)	70 (67.8, 70.3)	0.957
Age > 75 years, *n* (%)	155 (34)		31 (36)	124 (34)	0.802
Female, *n* (%)	229 (51)		50 (58)	179 (49)	0.189
PS WHO > 1, *n* (%)	152 (34)		37 (43)	115 (32)	0.058
Right sided, *n* (%)	177 (40)	7	52 (60)	125 (35)	< 0.001
Liver metastases, *n* (%)	287 (64)		49 (56)	238 (65)	0.137
Liver only, *n* (%)	118 (26)		14 (16)	104 (29)	0.018
Lung metastases, *n* (%)	113 (25)		14 (16)	99 (27)	0.038
Lymph node metastases, *n* (%)	131 (29)		37 (43)	94 (26)	0.003
Peritoneal metastases, *n* (%)	88 (20)		23 (26)	65 (18)	0.072
>1 metastatic site, *n* (%)	262 (58)		55 (63)	207 (57)	0.280
Synchronous metastases, *n* (%)	244 (54)		54 (62)	190 (52)	0.095
ALP high, *n* (%)	222 (56)	55	43 (60)	179 (55)	0.513
Primary tumor resected, *n* (%)	414 (92)		80 (92)	331 (92)	1.000
Tumor grade 1, *n* (%)	55 (13)	15	7 (8)	48 (14)	< 0.001
2, *n* (%)	288 (66)		39 (47)	249 (70)	
3, *n* (%)	94 (22)		37 (45)	57 (16)	
KRAS mutation, *n* (%)	179 (41)	15	16 (19)	163 (46)	< 0.001
BRAF mutation, *n* (%)	96 (21)	9	51 (59)	45 (12)	< 0.001
Double wild type, *n* (%)	164 (38)	15	18 (21)	146 (41)	0.001
MSI-H, *n* (%)	35 (8)	11	21 (26)	14 (4)	< 0.001
*BRAF*mut/MSI-H, *n* (%)	30 (7)	16	18 (23)	12 (3)	< 0.001
*BRAF*mut/MSS, *n* (%)	66 (15)		33 (42)	33 (9)	
*BRAF*wt/MSI-H, *n* (%)	5 (1)		3 (4)	2 (1)	
*BRAF*wt/MSS, *n* (%)	336 (77)		25 (32)	311 (87)	
Curative metastasis surgery, *n* (%)	33 (7)	1	1 (1)	32 (9)	0.019
First-line chemotherapy, *n* (%)	281 (62)		46 (53)	235 (64)	0.050
Combination, *n* (%)	217 (77)		34 (74)	183 (78)	0.567
Monotherapy, *n* (%)	64 (23)		12 (26)	52 (22)	
Second-line chemotherapy, *n* (%)	162 (36)	1	20 (23)	142 (39)	0.006
Third-line chemotherapy, *n* (%)	72 (16)	1	2 (2)	70 (19)	< 0.001
BSC only, *n* (%)	170 (38)		41 (47)	129 (35)	0.049

CDX2–, CDX2 loss; CDX2+, CDX2 expression; MSI-H, microsatellite instable high; MSS, microsatellite stable; PS ECOG, performance status score developed by Eastern Cooperative Oncology Group; Right sided, site of colon cancer in ascending colon and transversum; Left sided, site of colon cancer in descending colon, sigmoid, and rectum; Metastases, at time of diagnosis of metastatic disease; Synchronous metastases, within 6 months after initial diagnose; ALP high, alkaline phosphatase >105 U/L; Double wild type, both BRAF and KRAS wild type; BRAFmut, BRAF mutated; BRAFwt, BRAF wild type; BSC, best supportive care; p-value, chi-square test except for age (t-test).

**Percentage is calculated without missing values. Owing to rounding, not all percentages are 100 in total.*

DISCUSSION

This is the largest study of incidence of CDX2 loss and its correlation to treatment and survival in a population-based cohort of mCRC and the first study that also corrects for the prognostic markers MSI, *BRAF*, and *KRAS* status in the analyses. The generally poor survival in our cohort is comparable to Scandinavian cancer registries (3) and the American SEER database during the same time period (20), reflecting our unselected population of mCRC. A recent study confirms the low median OS of mCRC patients in the general population compared to phase III trial results (4). Our real-world data shows that mCRC patients who have non-resectable metastatic disease with CDX2 loss have a worse prognosis, receive less first- and second-line chemotherapy with less benefit and rarely receive secondary surgery.

The incidence of CDX2 loss was 19%, comparable to previous published results on stage IV CRC patients in population-based cohort studies (8, 15). Others have reported lower frequency of CDX2 loss, but most of these studies have very few patients with stage IV disease (21, 22). Two recent mCRC studies showed only 3–6% CDX2 loss, probably reflecting the selection of patients with better prognosis into clinical trials (10) and patients treated at the Mayo Clinic (9) compared to our population-based cohort. CDX2 loss was significantly associated with poor prognostic markers such as MSI-H, *BRAF*mut, right-sided tumors, and poor differentiation, in accordance with previous reports (8, 12, 15). In our study, we demonstrate that CDX2 loss is an independent negative prognostic tumor marker, even after correcting for these known prognostic factors.

*BRAF*mut is considered the clinically most important negative prognostic marker in mCRC (23, 24). Both *BRAF*mut and *KRAS*mut are poor prognostic markers after liver (25–27) and lung surgery (28) and after cytoreductive surgery with hyperthermic intraperitoneal chemotherapy (29). *BRAF*mut has therefore been suggested to be one of the factors to consider before metastatic surgery. However, in the clinic, it has been observed that some patients with *BRAF*mut tumors have a relatively long survival, despite the mutational status. This was recently explored in study of 395 *BRAF*mut mCRCs, where clinical prognostic markers defined three vastly different prognostic groups (30). Our study may, at least in part, explain this as it demonstrates that CDX2 expression defines a new prognostic subgroup in patients with *BRAF*mut (53%) with a much better prognosis, comparable to wild-type patients. We also verify that CDX2 loss is a poor prognostic marker in this subgroup, as demonstrated in a very recently published study (31).

Loss of CDX2 expression also defines a smaller group (9%) of *KRAS*mut cases with a worse prognosis, but this needs to be validated due to few cases in our cohort. These results could have clinical implications when considering treatment strategies for such patients, and further studies of patients undergoing curative metastasis surgery should evaluate if CDX2 status could impact on the negative prognosis of *BRAF*mut and *KRAS*mut. CDX2 loss has also recently been demonstrated as a negative prognostic marker after liver metastasectomy, but this study did

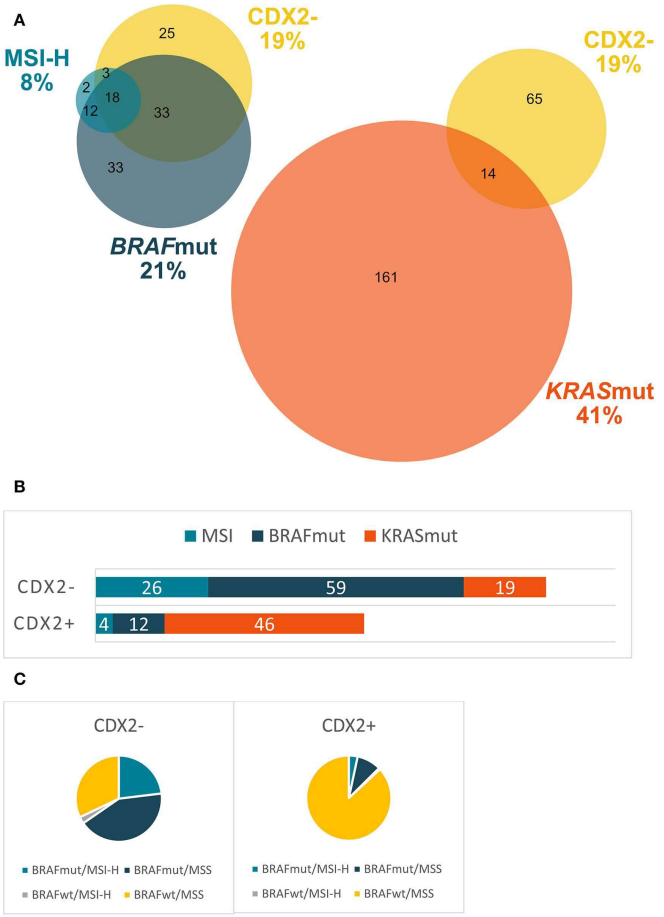

FIGURE 3 | Distribution of different molecular markers in a population-based Scandinavian cohort of metastatic colorectal cancer. **(A)** Frequency (%) of *BRAF*, microsatellite instability (MSI), *KRAS*, and caudal-type homeobox 2 (CDX2) status and their interrelations. **(B)** Frequency (%) of *BRAF*, MSI-H, and *KRAS* mutations in tumors with CDX2 loss and CDX2 expression. **(C)** *BRAF*/MSI subgroups in tumors with CDX2 loss and CDX2 expression. *KRAS*mut, *KRAS* mutation; *BRAF*mut, *BRAF* mutation; MSI-H, microsatellite instable high; MSS, microsatellite stable; double wild type, wild-type *KRAS* and *BRAF*; CDX2–, CDX2 loss; CDX2+, CDX2 expression.

not correct for *KRAS* or *BRAF* mutation status (11). According to our results, it is a reason to believe that CDX2 loss could be a negative prognostic marker for these patients regardless of mutational status, but due to small numbers in our subgroup analysis, particularly in cases with *KRAS*mut and CDX2 loss, this needs to be verified in larger study cohorts.

Recent retrospective studies of stage II–III CRC report CDX2 loss as a potential predictor of benefit from adjuvant chemotherapy (7, 8). In the metastatic situation, the predictive effect of CDX2 loss seems rather to be the opposite (8, 9, 11). In our study, patients with CDX2 loss had poorer survival and response to chemotherapy with more often immediate disease progression on first-line combination chemotherapy, shorter PFS, and few made it to second-line treatment or secondary surgery. This might indicate that patients with CDX2 loss should have a different treatment regimen. An intuitive approach would be, as for *BRAF*mut tumors, to use triple combination therapy (32). Only a few patients were included in our subgroup analyses, however, no increased OS benefit of using doublet chemotherapy vs. monotherapy was seen in patients with CDX2 loss. It is therefore not sure that these patients will benefit from a more intensified chemotherapy regimen. Further studies on new treatment regimens in this subgroup of patients are

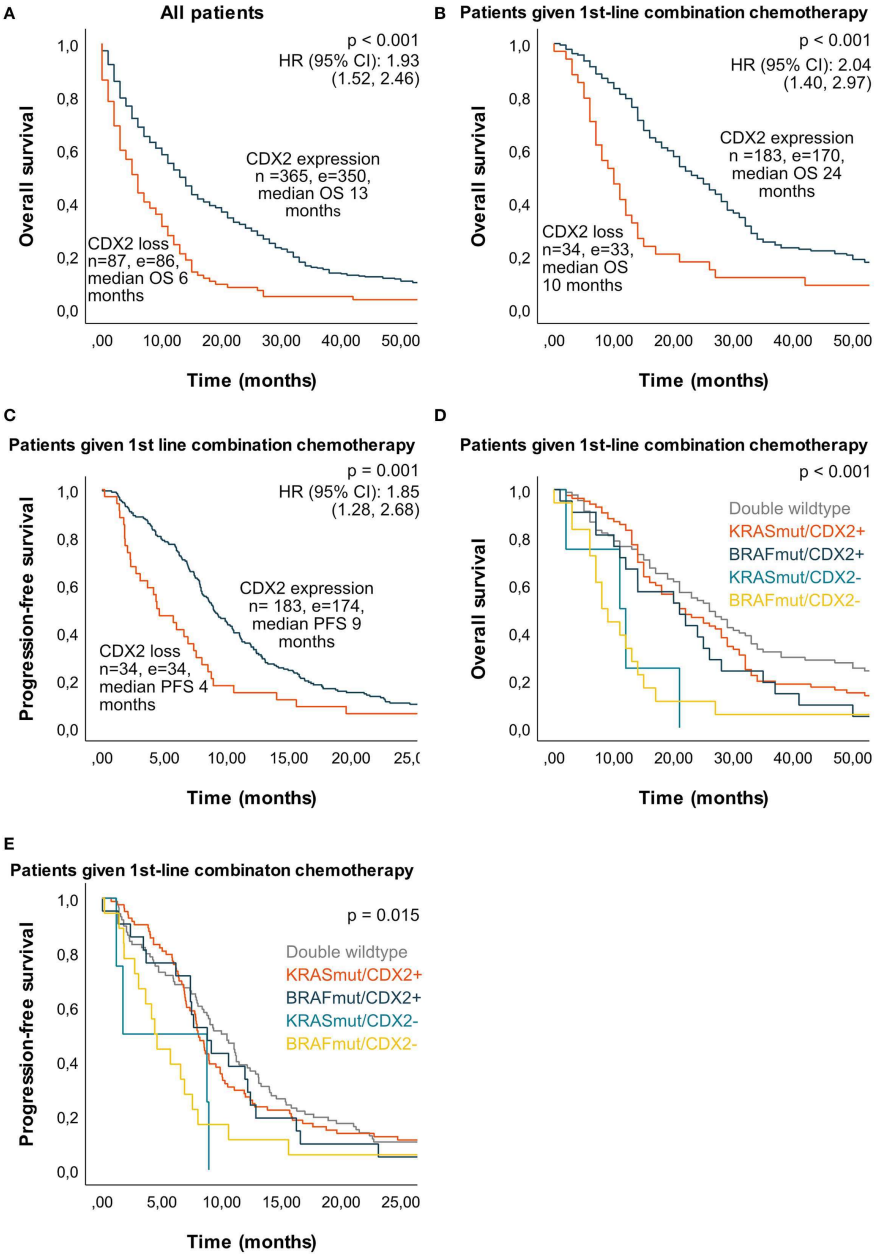

FIGURE 4 | Median overall survival (OS) and progression-free survival (PFS) in a population-based Scandinavian cohort of metastatic colorectal cancer according to tumor molecular alterations. Kaplan–Meier curves were calculated with log-rank test for *p* value and univariate Cox regression for HR and 95% CI. **(A)** Median OS for all patients according to CDX2 status. **(B)** Median OS according to CDX2 status for patients given first-line combination chemotherapy. **(C)** Median PFS according to

(Continued)

FIGURE 4 | CDX2 status for patients given first-line combination chemotherapy. **(D)** Median OS according to tumor molecular alterations in patients given first-line combination chemotherapy: double wild type 26 months ($n = 88$, $e = 80$), *KRAS*mut/CDX2 expressed 21 months ($n = 82$, $e = 77$), *BRAF*mut/CDX2 expressed 21 months ($n = 21$, $e = 20$), *KRAS*mut/CDX2 loss 11 months ($n = 4$, $e = 4$), *BRAF*mut/CDX2 loss 8 months ($n = 18$, $e = 18$). **(E)** Median PFS according to tumor molecular alterations in patients given first-line combination chemotherapy: double wild type 10 months ($n = 88$, $e = 85$), *KRAS*mut/CDX2 expressed 8 months ($n = 83$, $e = 78$), *BRAF*mut/CDX2 expressed 9 months ($n = 21$, $e = 20$), *KRAS*mut/CDX2 loss 2 months ($n = 4$, $e = 4$), *BRAF*mut/CDX2 loss 4 months ($n = 18$, $e = 18$). *n*, number; *e*, events; double wild type: *KRAS* and *BRAF* wild type; *BRAFmut*, *BRAF* mutation; *KRASmut*, *KRAS* mutation; *HR*, hazard ratio; CI, confidence interval; CDX2–, CDX2 loss; CDX2+, CDX2 expression.

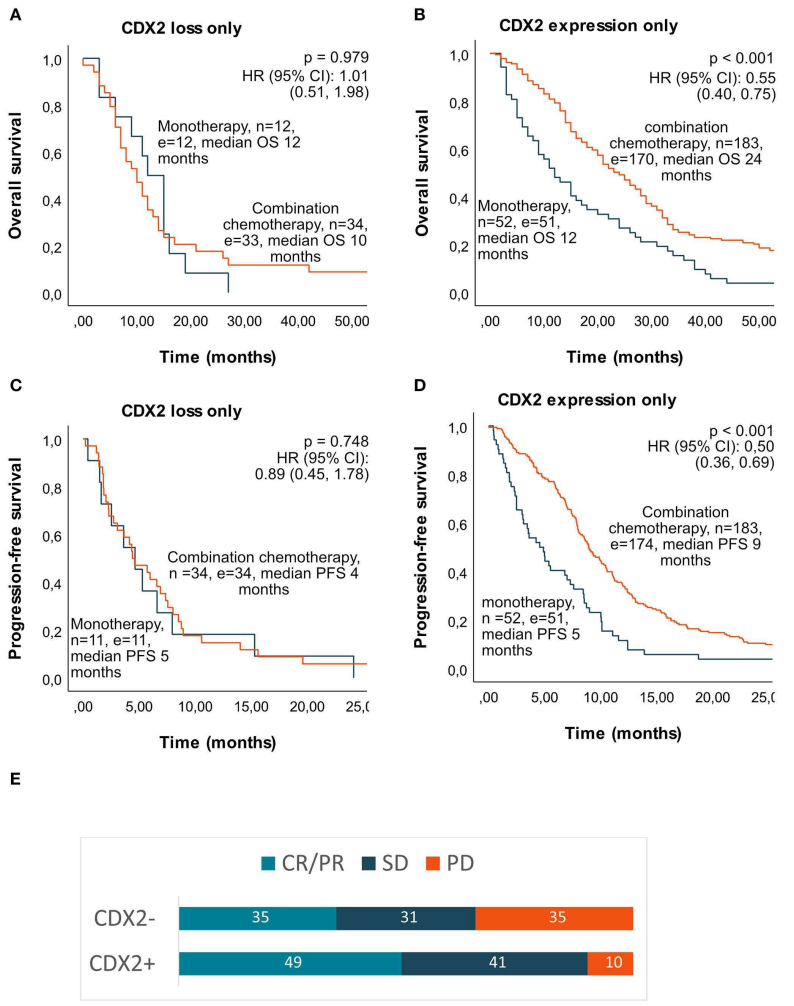

FIGURE 5 | Median overall survival (OS), progression-free survival (PFS), and response rate if given first-line mono- or combination chemotherapy according to tumor CDX2 status in a population-based Scandinavian cohort of metastatic colorectal cancer. Kaplan–Meier curves were calculated with log-rank test for *p*-value and univariate Cox regression for HR and 95% CI. **(A)** Median OS in patients with CDX2 loss. **(B)** Median OS in patients with CDX2 expressed. **(C)** Median PFS in patients with CDX2 loss. **(D)** Median PFS in patients with CDX2 expressed. **(E)** Response rate (%) after first-line combination chemotherapy according to CDX2 status. *n*, number; *e*, events; HR, hazard ratio; CI, confidence interval; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

TABLE 2 | Results from multiple Cox regression of overall survival and progression-free survival in a population-based Scandinavian cohort of metastatic colorectal cancer patients.

	Overall survival* (n = 357, e = 341)			Progression-free survival after first-line chemotherapy (n = 245, e = 235)		
Variable	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Female	0.85	(0.68, 1.03)	0.148	0.94	(0.72, 1.23)	0.655
AGE >75 years	1.04	(0.74, 1.46)	0.844	1.59	(1.02, 2.46)	0.039
PS WHO > 1	1.78	(1.36, 2.34)	<0.001	2.02	(1.39, 2.93)	<0.001
Right-sided tumor	1.06	(0.82, 1.37)	0.662	0.75	(0.55, 1.04)	0.081
Tumor grade 3	1.66	(1.22, 2.25)	0.001	1.55	(1.08, 2.23)	0.019
Primary tumor resected	1.14	(0.65, 2.00)	0.657	1.65	(0.92, 2.97)	0.095
Synchronous metastases	0.70	(0.55, 0.88)	0.002	0.75	(0.56, 1.01)	0.056
>1 organ metastases	1.34	(1.02, 1.76)	0.038	1.47	(1.00, 2.15)	0.049
Liver only	1.19	(0.87, 1.62)	0.271	1.40	(0.91, 2.16)	0.125
Curative metastasis surgery	0.32	(0.20, 0.52)	<0.001	0.38	(0.24, 0.62)	<0.001
ALP high	1.98	(1.55, 2.54)	<0.001	1.55	(1.15, 2.09)	0.004
First-line chemotherapy	0.37	(0.25, 0.53)	<0.001	n.i.		
MSI-H	1.38	(0.83, 2.30)	0.212	2.08	(1.06, 4.08)	0.032
KRAS mutation	1.25	(0.97, 1.62)	0.088	1.62	(1.17, 2.23)	0.003
BRAF mutation	1.62	(1.11, 2.35)	0.012	1.63	(1.04, 2.55)	0.032
CDX2 loss	1.50	(1.05, 2.15)	0.027	1.54	(1.00, 2.35)	0.049

n, number of patients; e, number of events; HR, hazard ratio; CI, confidence interval; p value, from likelihood ratio test; PS ECOG, performance status score developed by Eastern Cooperative Oncology Group; Right-sided tumor, site of colon cancer in ascending colon and transversum; Synchronous metastases, within 6 months after initial diagnose; ALP high, alkaline phosphatase > 105 U/L; MSI-H, microsatellite instable high; n.i., not included.

**CEA > 4 and LDH high was also statistically significant when included in the multiple regression model but were excluded from the analysis due to many missing values.*

clearly warranted, as they might need a completely different treatment approach.

The recently updated National Comprehensive Cancer Network guidelines recommend addition of *BRAF* inhibitors to standard second-line treatment in patients with *BRAF*mut tumors (33). Encouraging data have been published on EGFR-, *BRAF*-, and MEK-inhibitor combination treatment of patients with *BRAF*mut mCRC (34). However, far from all patients benefit substantially from this treatment, and it could be relevant to study if the benefit of this regimen depends on CDX2 status. Tumor MSI status is used as a predictive marker for immunotherapy effect in mCRC patients, and checkpoint inhibitors are currently recommended as second-line treatment for patients with MSI-H tumor according to the National Comprehensive Cancer Network guidelines (33). Since CDX2 loss is associated with MSI-H status and both are poor prognostic markers with poor response to standard chemotherapy in mCRC, future studies should also evaluate if the effect of checkpoint inhibitors may vary according to CDX2 status.

LIMITATIONS

As this is a population-based study, patients with both poorer performance status, older age, and comorbidity are included, and results are therefore difficult to compare with patients included in phase III clinical trials. It is also difficult to assess the predictive effect of CDX2 in our cohort, as patients not given chemotherapy are in the worst prognostic group. Although this is a population-based study, we know that patients who did not have tumor tissue available to perform TMA, and therefore not included in the biomarker analysis, have a particularly poor prognosis (16). To remove treatment selection bias, we chose to select patients treated with first-line and first-line combination chemotherapy for further subgroup survival analyses. In some of the biomarker subgroup analyses, we therefore had few patients, which could affect the results. Our molecular analyses were mainly performed on tissue from primary tumor and not the metastatic site; however, most studies of tumor molecular alterations show high concordance between primary tumor and metastases (35). Furthermore, the evaluation of CDX2 was based on IHC analysis on small TMA sections. Although two samples were taken from each tumor, the possibility of intratumoral heterogeneity cannot be excluded. Finally, the studied patient cohort is more than 10 years old. Treatment options for mCRC patients has, however, not changed much during this time period, although today we treat more fit patients with intensified chemotherapy regimens as well as metastasectomy. There are also recent data from a Dutch population-based synchronic mCRC series reporting no difference in median OS during the past 10 years (4).

CONCLUSIONS

In an unselected cohort of mCRC, CDX2 loss is an independent negative prognostic marker for survival. CDX2 loss indicates poor response and less survival benefits from standard chemotherapy in the metastatic situation, and effort is needed in finding new treatment regimens for this subgroup of patients. Expression of CDX2 defines a new subgroup of *BRAF*mut cases with a much better prognosis. Loss of CDX 2 also defines a smaller group of *KRAS*mut cases with a worse prognosis. CDX2 status may therefore be clinically relevant for a choice of treatment strategy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Regional Committee for Medical and Health Research Ethics—REC West (Norway), Regional Ethical Committee Uppsala (Sweden) and The Regional Scientific Ethical Committees for Southern Denmark (Denmark). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HS, PP, and BG designed the study and was responsible for patient collection. AD investigated all histological slides and was with FP, P-HE, and AM responsible for all IHC annotation. MS was responsible for the molecular analyses. KA and GE made the statistical analyses. KA drafted the manuscript with HS. All authors contributed to manuscript revision and approved the submitted version.

FUNDING

Grants from Lions Cancer Foundation (Uppsala, Sweden), Selanders stiftelse (Sweden), Akademiska Sjukhuset (Uppsala, Sweden), Cancerfonden (Sweden), and kreftforeningen (Norway) have enabled tissue handling, lab equipment, and analysis. University of Bergen was funding the current Ph.D. position of corresponding author and will sponsor an open access publication fee.

ACKNOWLEDGMENTS

We thank Randi Eikeland (Haukeland University Hospital, Bergen, Norway) for data management.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2020.00008/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

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ISBN: 9788230868577 (print)
9788230856956 (PDF)