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The immune microenvironment of colorectal cancer - Relationship with survival, sidedness, and pre-diagnostic anthropometry

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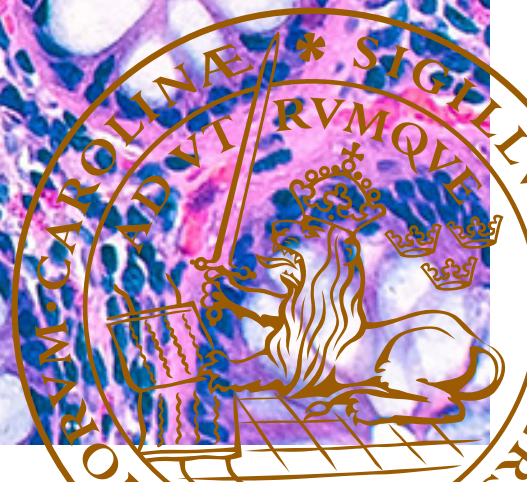
A high-magnification histological section of colorectal tissue, stained with hematoxylin and eosin (H&E). The image shows several cross-sections of colonic crypts, which are glandular structures lined by columnar epithelial cells. The nuclei of the cells are stained blue, while the cytoplasm and surrounding connective tissue are stained pink. The crypts are arranged in a regular, organized pattern, typical of normal colonic mucosa.

The immune microenvironment of colorectal cancer

Relationship with survival, sidedness, and pre-diagnostic anthropometry

JONNA BERNTSSON

DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY





Jonna Berntsson has during her doctoral studies investigated the relationship between pre-diagnostic anthropometry and the immune microenvironment of colorectal cancer, as well as the prognostic value of different immune cell subsets, with particular reference to primary tumour location.



The immune microenvironment of colorectal cancer

Relationship with survival, sidedness, and pre-diagnostic
anthropometry

Jonna Berntsson



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DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended in Hornbergssalen, Kulturen Restaurang & Konferens

Tegnérsplatsen 6, Lund

Friday October 11, 2019 at 9.15 a.m.

Faculty opponent

Professor Richard Palmqvist

Department of Medical Biosciences, Umeå University

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| Title: The immune microenvironment of colorectal cancer: relationship with survival, sidedness, and pre-diagnostic anthropometry | | |
| <p>Abstract</p> <p>Colorectal cancer (CRC) is the third most common cancer worldwide. Increasing evidence suggests that CRC should be considered a heterogeneous disease, with multiple differences between proximal and distal tumours. The immune system may, depending on the context, promote or inhibit tumour growth, and different immune cell subsets have been found to be associated with impaired or improved prognosis in CRC. The major aim of this thesis was to investigate the prognostic impact of different immune cell signatures in CRC, with particular reference to primary tumour location, and, furthermore, to perform a characterization of immune cell signatures in relation to anthropometric factors.</p> <p>The study cohort for Papers I-III consists of all 626 cases of CRC in the prospective, population-based cohort Malmö Diet and Cancer Study (MDCS) from 1991 up until December 31, 2008, of which tumours from 557 cases were available for tissue microarray construction, including 201 (36.2%) right-sided and 145 (26.1%) left-sided colon cancers, and 209 (37.7%) rectal cancers. Immunohistochemistry was applied to assess the density of tumour-infiltrating immune cells. For Paper IV, the analyses were restricted to the 584 cases included in the European Prospective Investigation into Cancer (EPIC) cohort, of which the MDCS forms part. Anthropometric measurements were taken at baseline. Cox proportional hazards regression models were applied to study the hazard ratios for survival, and the risk of CRC with particular immune cell compositions.</p> <p>Paper I shows that dense infiltration of B cells is an independent favourable prognostic factor in CRC.</p> <p>Paper II demonstrates that high infiltration of cytotoxic T cells is an independent favourable prognostic factor only in right-sided colon cancer, whereas high infiltration of regulatory T cells is an independent prognostic factor only in rectal cancer. Moreover, re-analysis of the data from paper I revealed that the prognostic impact of B cells is only evident in right-sided tumours.</p> <p>Paper III demonstrates that high expression of programmed cell death ligand 1 (PD-L1) on immune cells is an independent favourable prognostic factor only in patients with right-sided and left-sided colon cancer.</p> <p>Paper IV shows that obesity, indicated by several anthropometric factors, is associated with risk of CRC with high infiltration of B cells and cytotoxic T cells but with low infiltration of regulatory T cells in both sexes, albeit with weaker associations in women. Moreover, the results show that obesity is associated with risk of CRC with low PD-L1 expression on immune cells in men, but with high PD-L1 expression on immune cells in women.</p> <p>These results show that the prognostic impact of tumour-infiltrating immune cells in CRC differs according to primary tumour location. It is also demonstrated that obesity might influence the immune microenvironment of CRC. In summary, the findings indicate that primary tumour location, anthropometric factors, and sex are all important factors to include in future studies on the tumour microenvironment of CRC.</p> | | |
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Relationship with survival, sidedness, and pre-diagnostic
anthropometry

Jonna Berntsson



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To my family

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List of papers

Papers included in the thesis

The thesis is based on studies reported in the following papers, and are referred to in the text by their respective Roman numerals:

- I. **Berntsson J**, Nodin B, Eberhard J, Micke P, Jirstrom K: Prognostic impact of tumour-infiltrating B cells and plasma cells in colorectal cancer. *International journal of cancer* 2016;139(5):1129-39
- II. **Berntsson J**, Svensson MC, Leandersson K, Nodin B, Micke P, Larsson AH, Eberhard J, Jirstrom K: The clinical impact of tumour-infiltrating lymphocytes in colorectal cancer differs by anatomical subsite: A cohort study. *International journal of cancer* 2017;141(8):1654-66
- III. **Berntsson J**, Eberhard J, Nodin B, Leandersson K, Larsson AH, Jirstrom K. Expression of programmed cell death protein 1 (PD-1) and its ligand PD-L1 in colorectal cancer: Relationship with sidedness and prognosis. *Oncoimmunology* 2018;7(8):e1465165
- IV. **Berntsson J**, Eberhard J, Nodin B, Leandersson K, Larsson AH, Jirstrom K. Pre-diagnostic anthropometry, sex, and risk of colorectal cancer according to tumour-infiltrating immune cell composition. *Oncoimmunology (forthcoming)*
doi: 10.1080/2162402X.2019.1664275

Papers not included in the thesis

- **Berntsson J**, Lundgren S, Nodin B, Uhlén M, Gaber A, Jirstrom K. Expression and prognostic significance of the polymeric immunoglobulin receptor in epithelial ovarian cancer. *Journal of Ovarian Research* 2017;7:26

- Lundgren S, **Berntsson J**, Nodin B, Micke P, Jirström K. Prognostic impact of tumour-infiltrating B cells and plasma cells in epithelial ovarian cancer. *Journal of Ovarian Research* 2016;9:21
- Fristedt R, Borg D, Hedner C, **Berntsson J**, Nodin B, Eberhard J, Micke P, Jirström K. Prognostic impact of tumour-associated B cells and plasma cells in oesophageal and gastric adenocarcinoma. *Journal of Gastrointestinal Oncology* 2016;7:848-859
- Murphy N, (...), Jirström K, **Berntsson J**, Xue X, Riboli E, Cross AJ, Gunter MJ. Heterogeneity of Colorectal Cancer Risk Factors by Anatomical Subsite in 10 European Countries: A Multinational Cohort Study. *Clinical Gastroenterology Hepatology* 2019;17:1323-1331.e6

Abbreviations

| | |
|---------|--|
| 5-FU | 5-fluorouracil |
| AJCC | American Joint Committee on Cancer |
| ALASCCA | Adjuvant Low dose ASpirin in Colorectal Cancer |
| APC | adenomatous polyposis coli |
| APE | abdominoperineal excision |
| ASA | acetylsalicylic acid |
| BFP | body fat percentage |
| BMI | body mass index |
| BRAF | V-raf murine sarcoma viral oncogene homolog B |
| CAPOX | capecitabine + oxaliplatin |
| CEA | carcinoembryonic antigen |
| CIMP | CpG island methylator phenotype |
| CIN | chromosomal instability |
| CME | complete mesocolic excision |
| CMS | consensus molecular subtypes |
| COX-2 | cyclooxygenase 2 |
| cCR | clinical complete response |
| pCR | pathological complete response |
| CRC | colorectal cancer |
| CRT | chemoradiotherapy |
| CTLA-4 | cytotoxic T-lymphocyte associated protein 4 |
| DC | dendritic cell |
| DFS | disease-free survival |

| | |
|-----------|--|
| DIA | digital image analysis |
| EGFR | epidermal growth factor receptor |
| ELAPE | extralevator abdominoperineal excision |
| EPIC | European Prospective Investigation into Cancer and Nutrition |
| ESMO | European Society of Medical Oncology |
| FAP | familial adenomatous polyposis |
| FDA | Food and Drug Administration |
| FOLFIRI | fluorouracil/leucovorin + irinotecan |
| FOLFOX | fluorouracil/leucovorin + oxaliplatin |
| FOLFOXIRI | fluorouracil/leucovorin + oxaliplatin + irinotecan |
| FoxP3 | Forkhead box P3 |
| GDP | guanosine diphosphate |
| GTP | guanosine triphosphate |
| Gy | Gray |
| HIPEC | hyperthermic intraperitoneal chemotherapy |
| HNPCC | hereditary nonpolyposis colorectal cancer |
| HR | hazard ratio |
| HRT | hormone replacement therapy |
| IFN | interferon |
| IGF-1 | insulin-like growth factor 1 |
| IGKC | immunoglobulin kappa C |
| IHC | immunohistochemistry |
| IL | interleukin |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| LAR | lower anterior resection |
| LV | leucovorin |
| MAP | <i>MUTYH</i> associated polyposis |
| MDCS | Malmö Diet and Cancer Study |
| MHC | major histocompatibility complex |

| | |
|----------|--|
| MMR | mismatch repair |
| dMMR | mismatch repair deficient |
| pMMR | mismatch repair proficient |
| MSI | microsatellite instability/unstable |
| MSS | microsatellite stability/stable |
| NK | natural killer |
| NKG2D | NK receptor member D |
| NKT | natural killer T |
| NRAS | <i>V-RAS</i> oncogene homolog |
| OS | overall survival |
| PD-1 | programmed cell death protein 1 |
| PD-L1 | programmed cell death protein ligand 1 |
| PFS | progression free survival |
| SCREESCO | Screening of Swedish Colons |
| SD | standard deviation |
| Th | T helper |
| TMA | tissue microarray |
| TME | total mesorectal excision |
| TNF | tumour necrosis factor |
| TNM | tumour-node-metastasis |
| Tregs | regulatory T cells |
| VEGF | vascular endothelial growth factor |
| WHR | waist-hip ratio |

Background

Epidemiology

With an annual incidence of more than 1.8 million new cases every year, colorectal cancer (CRC) is the second most common cancer in women and the third most common cancer in men worldwide (1). The incidence varies greatly throughout the world, with rates tending to rise uniformly with increasing human development index (2). The highest incidence rates are found in Australia and New Zealand, Northern America, Eastern Asia, and parts of Europe (Norway, the Netherlands, Hungary, Slovenia, and Slovakia), whereas the lowest rates are found in Southern Asia and most regions in Africa (1). Incidence is higher in men than in women, with an increasing male-to-female incidence rate ratio from the caecum to the rectum (3).

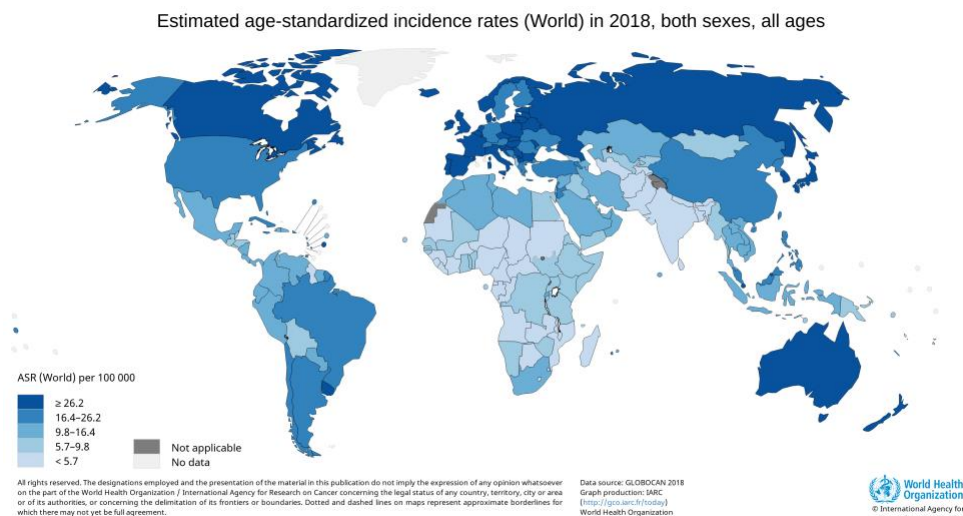


Figure 1. Global variations in CRC incidence (4). Reproduced with permission from GLOBOCAN.

Overall, CRC is the second most common cause of cancer-related mortality (1). Although with both incidence and mortality increasing in some regions (Eastern Europe, Latin America, and some Asian areas), the mortality is steadily decreasing

in others, also in areas with increasing incidence, e.g. Northern and Southern Europe and North America (5). In Sweden, the incidence has slowly increased during the last 30 years, with more than 6800 new cases in 2018, whereas mortality rates have steadily declined (6).

The rises in incidence have been attributed to obesity, dietary patterns, and other lifestyle factors, whereas the decreasing mortality reflects improvements in treatment (5). The introduction of organized or opportunistic CRC screening programs has also led to substantial decreases in CRC incidence and mortality (7).

Colorectal carcinogenesis

Colorectal carcinogenesis was first described as a step-wise accumulation of genetic and epigenetic changes, with loss of tumour suppressor genes, inactivation of genes involved in DNA repair, and alterations in oncogenes. According to this model, these events lead to the formation of adenoma and carcinoma (8, 9), as illustrated in Figure 2.

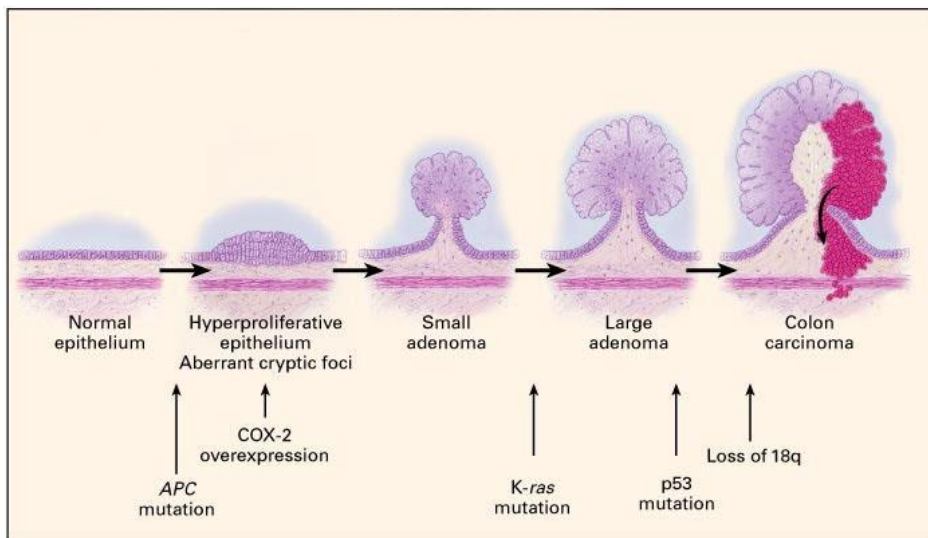


Figure 2. The adenoma-carcinoma sequence (10). Reproduced with permission from *The New England Journal of Medicine*.

Nevertheless, subsequent research has shown that these alterations do not necessarily occur sequentially, but can be acquired through different pathways.

Three major mechanisms have been delineated: chromosomal instability (CIN), CpG island methylator phenotype (CIMP), and microsatellite instability (MSI).

CIN is the most common type of genomic instability in CRC, with defects in chromosomal segregation, telomere stability, and the DNA damage response, and mutations in checkpoint genes (11, 12). The Adenomatous Polyposis Coli (*APC*) gene is most commonly the initial gene mutated, resulting in sustained activation of the Wnt/ β -catenin signalling pathway, that normally regulates cell growth, differentiation, and apoptosis. Thus, mutations of *APC* result in accumulation of undifferentiated cells in colonic crypts and the formation of a polyp (13). Accumulation of subsequent mutations in the proto-oncogene *KRAS* (Kirsten rat sarcoma viral oncogene homolog) (14) and the tumour suppressor gene *TP53*, known as the “guardian of the genome” (15), may eventually result in carcinoma (12). CIN accounts for approximately 85% of CRC, and these tumours are more frequent in the left colon or rectum, and tend to have lower densities of tumour-infiltrating immune cells (16).

The mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* encode a system repairing so-called base-base mismatches in DNA, errors that occur during the normal replication of DNA. Inactivation of these genes causes MMR deficiency (dMMR), which leads to accumulation of short, repetitive DNA sequences or microsatellites, and hence, MSI (17). Germline mutations in MMR genes cause Lynch syndrome, a hereditary form of CRC, whereas the majority of sporadic dMMR/MSI CRC results from methylation of *MLH1* (18). dMMR is present in approximately 15% of all CRC, although less frequently in more advanced tumours, and is associated with poor differentiation, *BRAF* (V-raf murine sarcoma viral oncogene homolog B) mutation, proximal tumour location, and high infiltration of immune cells (17).

CIMP is observed in approximately 15% of all CRC, and in nearly all tumours with aberrant methylation of *MLH1* (19). DNA methylation causes epigenetic silencing of genes, and in the normal genome, methylation of cytosine, one of the four main bases in DNA, occurs in areas of repetitive DNA sequences (20). In CRC, the DNA methylation occurs in so called CpG-islands, found in the promoter regions of approximately 50% of all protein-encoding genes, possibly causing silencing of tumour suppressor genes (21). CIMP-high tumours are more common in women and in older patients, and are more commonly poorly differentiated, dMMR/MSI-high, *BRAF* or *KRAS* mutated, and located in the proximal colon (22, 23).

Based on the aforementioned pathways, and the heterogeneity of CRC at the gene-expression level, four different consensus molecular subtypes (CMS) have been proposed by the Colorectal Cancer Subtyping Consortium (24), as illustrated in Figure 3.

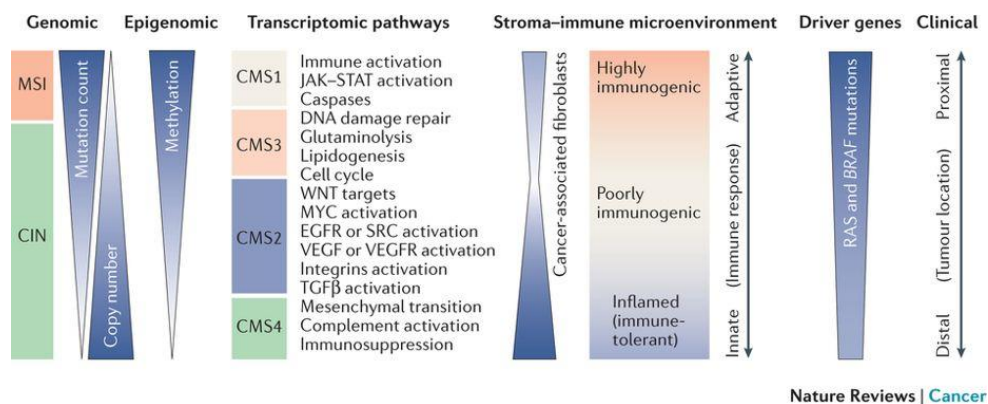


Figure 3. Consensus molecular subtypes (CMS) in colorectal cancer (25). Reproduced with permission from Springer Nature.

CMS1, representing approximately 15% of all CRC, is characterized by dMMR/MSI, hypermutation and hypermethylation, and high density of tumour-infiltrating immune cells. CMS1 tumours more commonly occur in the right colon, in older patients, and in females. CMS2 accounts for over 35% of early-stage tumours and represents the largest subtype, with CIN and losses in tumour suppressor genes being more frequent. Both this subtype and CMS4, accounting for approximately 20% of all CRC, present with microsatellite stability (MSS) and low levels of DNA methylation, and are more commonly distally located. CMS4 tumours are also characterized by a more inflamed microenvironment (25), with upregulation of immunosuppressive factors, known to be carcinogenic (26). CMS3 represents approximately 15% of early-stage tumours, and present with dMMR/MSI and hypermutation in up to 30% of cases. Metabolic factors are thought to play an important role in the carcinogenesis of CMS3 tumours (25).

Aetiology and risk factors

The aetiology of CRC is considered to be multifactorial. Risk factors are either modifiable, i.e. lifestyle factors, or non-modifiable, e.g. family history, genetics, sex, and ethnicity. Age is one of the strongest risk factors, with three out of four patients being older than 65 years at diagnosis (27).

Chronic inflammation

In CRC, as well as in several other types of cancer, chronic inflammation has been found to foster proliferation, survival, and migration of cancer cells (28). For

example, patients with inflammatory bowel disease, i.e. ulcerative colitis or Crohn's disease, have been demonstrated to have a five to sevenfold increased risk of CRC (29). In contrast, long-term users of aspirin or non-steroidal anti-inflammatory drugs have been found to have a reduced risk of CRC (30). Furthermore, retrospective studies suggest that adjuvant treatment with common acetylsalicylic acid (ASA) significantly improves survival for CRC patients with alterations in *PIK3CA*, the gene encoding phosphatidylinositol-4,5-bisphosphonate 3-kinase, catalytic subunit alpha polypeptide (31, 32). The Swedish randomized multicenter study ALASCCA (Adjuvant Low dose ASpirin in Colorectal Cancer) is currently investigating the effect of adjuvant treatment with ASA in patients with *PIK3CA* mutated CRC (33).

Hereditary factors

Although approximately 20% of patients with CRC have a first and/or second degree relative diagnosed with the same disease, only 2-4% of cases are caused by a well-defined genetic syndrome (34).

The most common form of hereditary CRC, accounting for approximately 3% of CRC cases (34), is Lynch syndrome, or hereditary nonpolyposis colorectal cancer (HNPCC). HNPCC is caused by germline mutations in MMR genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*), leading to dMMR, with autosomal dominant inheritance (35). The lifetime risk of CRC is reported to be between 30-80%, with a median age at diagnosis of 45 years, and the syndrome is also associated with increased risk of endometrial, ovarian, and gastric cancer (36). The majority of HNPCC patients present with tumours in the proximal colon, and the tumours are often poorly differentiated and display mucinous or signet ring cell histology. Synchronous or metachronous tumours are common (34). Systematic endoscopy screening is recommended from the age of 20-25 to reduce the risk of CRC and increase survival rates, and prophylactic subtotal colectomy might be discussed in selected cases (37).

Familiar adenomatous polyposis (FAP) is the second most common inherited CRC syndrome, accounting for about 1% of CRC cases (38). FAP is caused by a germline mutation in the *APC* gene. Although described as an autosomal dominant disorder, about 20% of cases emerge as de novo mutations (39). Characterized by many hundreds of adenomatous colorectal polyps, the lifetime risk of CRC is nearly 100% (38). The majority of cases present with rectal cancer or left-sided colon cancer (40). Patients undergo annual endoscopy screening from the age of 12, and prophylactic colectomy is recommended, usually around the age of 20 (41). Evidence also suggests that selective cyclooxygenase 2 (COX-2) inhibitors may be used as adjunctive therapy (42).

MUTYH associated polyposis (MAP) is an autosomal recessive disorder with germline variants in the *MUTYH* gene, a base excision repair gene (43). Described

as an attenuated FAP, MAP is characterized by ten to hundreds of adenomas, predominantly in the right colon (44). For patients with biallelic *MUTYH* mutations, clinical management should be similar to that of patients with FAP (43, 44).

Finally, several hamartomatous polyposis syndromes have been described (45). Inherited in an autosomal dominant manner, these syndromes are characterized by hamartomatous polyps in the gastrointestinal tract, but also present with several extra-intestinal findings (45). Although accounting for less than 1% of CRC cases, the importance of identifying these patients for inclusion in systematic screening has been pinpointed (45).

Obesity

Obesity has been demonstrated to be a major risk factor for several types of cancer, including CRC, where every 5 kg/m² increase in body mass index (BMI) has been found to increase the risk by 30% (46), although with weaker associations for rectal cancer (47). Obesity at earlier age is also reported to increase the risk of CRC later in life (48), and longer duration of adulthood overweight has been found to further increase this risk (49). For women, the association between BMI and colon cancer risk has been demonstrated to be weaker than for men (50), and some studies report no significant increase in risk of rectal cancer in obese women (50, 51). Although it has been reported that the positive association between BMI and colon cancer is restricted to MSS tumours (52, 53), other studies found no effect on risk by MMR status (54-56), and, contrastingly, one study demonstrated an association between obesity and dMMR/MSI-high CRC in women (55). It should however be pointed out that, although being the most commonly used anthropometric factor to denote obesity, BMI may not be optimal. Epidemiological data suggest that abdominal obesity, rather than overall obesity, may be more predictive of CRC risk, also in women (51).

There have been several hypothesized explanations for the effect of obesity on cancer risk. Firstly, obesity is associated with low-grade chronic inflammation, an established mediator of cancer development and progression (57). Pro-inflammatory gene expression and the numbers of macrophages in adipose tissue have been found to be positively associated with adipocyte size (58), which increases with obesity. Adipose tissue also releases cytokines, e.g. leptin, stimulating the production of pro-inflammatory mediators and further spurring the low-grade inflammation (59). High levels of leptin have been associated with increased CRC risk (60). Secondly, chronic hyperinsulinemia is associated with increased activity of insulin-like growth factor 1 (IGF-1), which has been demonstrated to stimulate cell proliferation and inhibit apoptosis (61), and to be associated with CRC risk (62). Thirdly, the adipose tissue and adipocytes have been reported to increase the proliferation of colon cancer cells *in vitro* (63), and enzymes

participating in the metabolism of fatty acids are upregulated in CRC (64). Finally, hormonal factors may influence CRC risk, with obese men tending to have lower androgen levels, and studies reporting lower androgenicity to increase men's risk of CRC (65, 66).

Physical activity

Large meta-analyses suggest occupational (67) and recreational (68) physical activity to be associated with decreased CRC risk, with stronger evidence for colon cancer than for rectal cancer (68) and in individuals with higher BMI (69). Although not fully understood, possible explanations include reduced insulin resistance and altered IGF-1 levels, reduced gut transition time with decreased exposure of colon mucosa to carcinogens, and favourable modulation of colonic inflammation genes (70). Regular physical activity has also been found to stimulate lymphocyte proliferation and to increase the activity of natural killer (NK) cells, thus enhancing immunological surveillance (71).

Diet

High intake of red and processed meat has been demonstrated to be associated with increased risk of CRC (72, 73). This association appears to be stronger for distal CRC (74, 75). Consumption of red meat has also been reported to be associated with risk of dMMR tumours (76), however, other studies have shown conflicting results (77) or no associations between diet and MMR status (78).

There are several proposed explanations for the impact of red meat on CRC risk. Heme iron, levels of which are high in red meat, has been found to damage the colonic mucosa and to stimulate compensatory hyperproliferation of the epithelium in animal models (79), and has also been associated with increased risk of colon cancer (80). Processed red meat, and meat cooked at high temperatures, also contains potential mutagens and carcinogens, e.g. heterocyclic amines and polycyclic aromatic hydrocarbons (81, 82). Finally, oncogenic bovine viruses, surviving high temperatures, have been suggested to be involved in CRC carcinogenesis (83).

Contrastingly, high intake of dietary fibre (84) and fish (85) has been shown to be inversely associated with CRC. Dietary fibre has been proposed to bind carcinogens, increase the faecal bulk and shorten gut transit times, and further alter the gut microflora and lower faecal pH in the colon (86). Omega-3, levels of which are high in fish, has in animal models and in *in vitro* studies been shown to inhibit carcinogenesis (87), and food with a high content of vitamin D might also have beneficial effects (88).

Smoking

Smokers, either former or current, have been demonstrated to have an increased risk of CRC (89), although this association appears to be stronger for rectal cancer than colon cancer (90). Studies also report this association to be stronger for dMMR/MSI-high tumours or tumours with high-degree CIMP, suggesting that smoking might induce epigenetic changes promoting carcinogenesis (91). Finally, smokers have been demonstrated to have an increased risk of tumours with low infiltration of T cells, possibly indicating that smoking also induces carcinogenesis through suppression of T cell-mediated anti-tumour immunity (92).

Alcohol

High alcohol consumption is an established risk factor for CRC, with no apparent difference according to tumour subsite (93, 94). A J-shaped association has been demonstrated, with light/moderate alcohol intake being protective of CRC risk compared to no alcohol consumption (93), although others report no significant associations between moderate levels of alcohol consumption and CRC risk (94). It has been reported that acetaldehyde, the major product from ethanol metabolism, causes colon mucosal damage and stimulates cell proliferation, possibly explaining the association between alcohol consumption and risk of developing CRC (93).

Hormonal factors

Reproductive and hormonal factors have been suggested to partly explain the differences in CRC incidence between men and women, with associations between postmenopausal hormone replacement therapy (HRT) (95, 96) as well as oral contraceptive use (97) and decreased CRC risk. High parity has also been reported to be inversely associated with CRC risk (96). Possible explanations for the auspicious effect of female hormones have been decreased levels of IGF-1 (98), reduced secretion of bile acids (99), believed to be carcinogenic (100), and furthermore, oestrogen receptor-induced apoptosis in malignant cells (101).

Clinical aspects

Screening

Screening programs, either opportunistic or organized, have been implemented in numerous countries, with population-based randomized studies demonstrating a

decrease in relative mortality by 15-20% (102-104). Methods used are faecal occult blood test, faecal immunochemical test, or sigmoidoscopy/colonoscopy. In Sweden, the SCREESCO (Screening of Swedish Colons) program is currently investigating the optimal screening method, randomising individuals to either one-time colonoscopy, repeated faecal blood tests, or no screening, with a follow-up period of 15 years (105).

Diagnosis

The presentation of symptoms differs with tumour location. Proximal colon tumours rarely produce symptoms until relatively advanced, whereas pain, cramps, blockage, or blood in stool might herald the presence of distal colon cancer or rectal cancer. About 20% of cases present as an acute colonic obstruction (106).

Colonoscopy and rectoscopy with biopsy are used for diagnosing colon and rectal cancer, respectively. CT scan of the abdomen and thorax is performed for detection of potential distant metastases, most commonly located in the liver, lungs, or peritoneum. Patients with rectal cancer also undergo a pelvic MRI, for assessment of local growth in relation to the mesorectal fascia and adjacent organs in the pelvis.

Staging

Table 1.

The TNM staging system according to the American Joint Committee on Cancer (AJCC), 8th edition (107).

| Primary tumour (T) | | Regional lymph node metastasis (N) | | Distant metastasis (M) | |
|--------------------|---|------------------------------------|--|------------------------|--|
| TX | Primary tumour cannot be assessed | NX | Regional lymph nodes cannot be assessed | M0 | No distant metastasis |
| T0 | No evidence of primary tumour | N0 | No region lymph node metastasis | M1a | Metastasis to one site or organ |
| Tis | Carcinoma <i>in situ</i> , intramucosal carcinoma | N1a | Metastasis in one regional lymph node | M1b | Metastasis to two or more sites or organs |
| T1 | Tumour invades the submucosa | N1b | Metastasis in two or three regional lymph node | M1c | Metastasis to the peritoneal surface, with or without other site or organ metastases |
| T2 | Tumour invades the muscularis propria | N1c | Tumour deposits without regional lymph node metastasis | | |
| T3 | Tumour invades through the muscularis propria into pericolorectal tissues | N2a | Metastasis in four to six regional lymph nodes | | |
| T4a | Tumour invades through the visceral peritoneum | N2b | Metastasis in seven or more regional lymph nodes | | |
| T4b | Tumour directly invades or adheres to adjacent organs or structures | | | | |

Disease staging is the basis on which treatment decisions are made, and it is also the strongest predictor of survival for CRC patients. The TNM staging system, by the American Joint Committee on Cancer (AJCC), is based on the size and extent of the tumour (T), the involvement of lymph nodes (N), and the presence or absence of distant metastasis (M) (107), as illustrated in Table 1.

Prognostic factors

Combining the T, N, and M parameters, tumours are designated an overall stage of I-IV. Stage I thus represents the least advanced tumours with excellent 5-year survival rates, whereas patients with stage IV tumours have a dismal prognosis, as demonstrated in Table 2.

Table 2.

Survival according to TNM stage (AJCC cancer staging manual, 8th edition) (107).

| Stage | TNM | | | 5-year overall survival (%) |
|-------|--------|--------|-----|-----------------------------|
| I | T1-T2 | N0 | M0 | 98 |
| IIA | T3 | N0 | M0 | 83 |
| IIB | T4a | N0 | M0 | 77 |
| IIC | T4b | N0 | M0 | 68 |
| IIIA | T1-T2 | N1/N1c | M0 | 65 |
| IIIB | T1 | N2a | M0 | 60 |
| | T3-T4a | N1/N1c | M0 | |
| | T2-T3 | N2a | M0 | |
| IIIC | T1-T2 | N2b | M0 | 45 |
| | T4a | N2a | M0 | |
| | T3-T4a | N2b | M0 | |
| | T4b | N1-N2 | M0 | |
| IVA | Any T | Any N | M1a | 8 |
| IVB | Any T | Any N | M1b | 0 |
| IVC | Any T | Any N | M1c | - |

Emergency surgery is associated with a significantly reduced 5-year overall survival (OS) compared to elective surgery, also after adjustment for clinical stage (108, 109), and should be included as a factor in treatment decisions. After surgery, the tumour is examined by a pathologist. The extent of surgical resection has a considerable prognostic impact, and in case of macroscopic or microscopic residual tumour, additional treatment might be considered (110). Furthermore, the differentiation grade is determined as low or high grade, the former including highly and moderately differentiated tumours and the latter including poorly or undifferentiated tumours, according to the *WHO Classification of Tumours of the Digestive System 2010* (111). Tumours with mucinous or signet ring cell histology should also be regarded as high-grade (111). Tumour grade is a well-known stage-independent prognostic factor, with high grade, i.e. poorly differentiated tumours,

being an adverse prognostic factor. Moreover, the presence of vascular and perineural invasion (112, 113), or tumour budding (114) are harbingers of decreased survival. Finally, resection of less than 12 lymph nodes predicts poorer prognosis, due to risk of underestimation of disease stage (115).

Prognostic and treatment predictive biomarkers

Kirsten rat sarcoma viral oncogene homolog (KRAS)

KRAS is a proto-oncogene in the RAS/RAF/MEK/ERK pathway. The *KRAS* protein cycles between an inactive and an active state, binding to guanosine diphosphate (GDP) and guanosine triphosphate (GTP), respectively. When bound to GTP, *KRAS* sends extracellular signals regulating proliferation, differentiation, apoptosis, and cell migration (14). Mutations, predominantly affecting codons 12 and 13, lead to a protein insensitive to inactivation, and are found in 30-50% of all CRC (116, 117). *KRAS* mutation is a negative predictor of response to treatment targeting the epidermal growth factor (EGFR) (118, 119), and *KRAS* mutations have also been associated with increased risk of recurrence and death in CRC (116, 118). The mutation rate is reported to decrease in tumours from the caecum to the left colon, but to increase again in tumours of the rectum (120).

V-raf murine sarcoma viral oncogene homolog B (BRAF)

The proto-oncogene *BRAF* acts downstream of RAS in the RAS/RAF/MEK/MAPK pathway, regulating signal transduction between the extracellular environment and the nucleus. Mutations, predominantly a V600E substitution, lead to a sustained activation (121), and occur in approximately 10-15% of all CRC (117). Concomitant mutations in *BRAF* and *KRAS* are rare (122). *BRAF* mutations are more frequent in right-sided than in left-sided CRC (120, 123), and have been reported to be associated with poor prognosis, particularly in patients with MSS tumours (124), and to be predictive of decreased response to anti-EGFR treatment (125).

Microsatellite instability (MSI)

As described previously, dMMR or MSI results in genomic instability, and is present in approximately 15% of all CRC (126), however in less than 5% of stage IV tumours (127). dMMR/MSI has been shown to be associated with a lower recurrence rate (128) and prolonged survival (129), although with conflicting results in stage IV patients (129, 130), and may be used to identify stage II CRC patients with very low risk of recurrence, who are not likely to benefit from adjuvant chemotherapy (131). MSI status is also, since 2017, the first Food and Drug Administration (FDA) approved tissue-agnostic biomarker for selection of cancer patients most likely to benefit from immunotherapy (132).

Carcinoembryonic antigen (CEA)

CEA is a blood-based biomarker that is recommended to be measured preoperatively in patients with non-metastatic CRC, or before treatment start in patients with advanced disease (133, 134). Preoperative elevated CEA has been associated with metastasis and recurrence (135). However, postoperative CEA has been reported to be a better prognostic indicator than preoperative CEA (136), with a significantly lower disease-free survival (DFS) for patients with elevated postoperative levels than for patients with normal values (137).

Treatment

The treatment of CRC has improved over the past decades, with refined surgical techniques and the use of neoadjuvant radiotherapy as well as neoadjuvant and adjuvant chemotherapy reducing mortality and morbidity. The prognosis for CRC patients has further improved after introduction of targeted therapies. This multimodal treatment is thus a multidisciplinary teamwork, including surgeons, radiologists, pathologists, and oncologists. All CRC cases should therefore be discussed at multidisciplinary team meetings (138).

Surgery

The primary treatment of CRC is surgery, and curative resection is the most important factor for patient survival. Similar recurrence rates have been demonstrated after laparoscopic surgery and open surgery (139), and either approach is therefore acceptable. The aim of surgery is to remove the primary tumour with negative margins, including the lymphatic drainage and regional lymph nodes of the mesentery, and to resect its vascular supply (140).

The extent of resection for a colon cancer is based on the colonic blood supply, assuring both adequate margins and sufficient blood supply. For tumours of the caecum, ascending colon, right flexure, and the proximal part of the transverse colon, a right hemicolectomy should be performed, including ligation of the ileocolic, right colic, and right branch of the middle colic vessels. Tumours of the transverse colon might also be treated with a transverse colectomy, or with an extended right hemicolectomy with ligation of the ileocolic and middle colic arteries. Splenic flexure tumours are managed by either an extended right hemicolectomy, or by an extended left hemicolectomy with ligation of the inferior mesenteric vessels, depending on the blood supply. A left hemicolectomy is also performed for tumours of the descending colon or the proximal sigmoid colon, whereas a sigmoid resection, ligating the left colic artery, is required for mid and distal sigmoid colon cancers. Total or subtotal colectomy is performed in case of synchronous tumours in the right and left colon, as well as for patients with HNPCC or FAP (37, 41). The complete mesocolic excision (CME), based on the total

mesorectal excision (TME) for rectal cancer as later described, has been demonstrated to improve recurrence rates and overall survival (141).

In patients with early rectal tumours, approximately 20-40% of all cases, surgery alone is sufficient to provide local control. For tumours of the middle or upper third of the rectum, a lower anterior resection (LAR) is performed, whereas an abdominoperineal excision (APE) is used for the most distal tumours. TME is golden standard for rectal cancer surgery worldwide, a technique that includes removal of the rectum as well as the entire rectal mesentery as an intact unit (142). The introduction of the TME technique has improved the results after APE; however, the risk of local recurrence and mortality is still higher than after LAR, possibly due to the anatomic reduction of the mesorectal tissue around the distal rectum. A more radical approach, the extralevator APE (ELAPE) technique, has recently been introduced for tumours with direct and/or possible invasion of the anal sphincter (143).

Up to 20% of all CRC cases undergo surgery in an acute setting, due to obstruction, perforation, or major bleeding (106). Obstructive tumours are more common in the left colon (106). Emergency resection of the tumour is not appropriate for patients needing neoadjuvant oncological treatment, which is often the case for rectal cancer and locally advanced colon cancer.

The presence of distant metastases previously excluded patients from curative surgery. However, for a selected but increasing number of stage IV patients with limited disease in the liver (144), lung (144, 145), or peritoneum (146), long-term survival can be achieved with a combination of surgery and chemotherapy (147). Patients with isolated peritoneal metastases might also undergo hyperthermic intraperitoneal chemotherapy (HIPEC), although recent results from the PRODIGE 7 trial demonstrated no survival benefit of the addition of HIPEC to cytoreductive surgery (148).

Radiotherapy

In more advanced rectal tumours, the risk of local recurrence is substantial, and preoperative radiotherapy has been shown to reduce this risk by 50-70% (149, 150). For intermediate rectal cancer, representing 40-60% of all new cases, short-course radiotherapy 5 Gray (Gy) x 5 is administered one week before surgery. About 10-20% of all new cases are locally advanced, and as the risk of non-radical resection is high for these patients, chemoradiotherapy (CRT) is recommended. CRT is delivered as 45-50.4 Gy in fractions of 1.8-2 Gy, with capecitabine as a radiosensitiser. Surgery is performed after 6-8 weeks, or after up to 12 weeks if needed to achieve resectability (151). The optimal neoadjuvant treatment is debated, and in the ongoing international RAPIDO trial, patients with locally advanced rectal cancer are randomised to either standard CRT followed by selective postoperative

adjuvant chemotherapy, or short-course radiotherapy followed by 6 cycles of capecitabine and oxaliplatin before surgery (143, 152).

In approximately 15-25% of patients who have undergone CRT and surgery, no residual tumour is found in the resection specimen, i.e. a pathological complete response (pCR) (153). Hence, a so called watch-and-wait policy was investigated, where patients with distal rectal cancer who had achieved a clinical complete response (cCR) after CRT were closely followed by clinical, endoscopic, and radiological assessment, and did not undergo surgery (154). This organ-preserving strategy was demonstrated to be associated with similar 5-year OS and DFS rates as for patients who had undergone CRT and surgery, also confirmed in the large database “International Watch & Wait Database” (155). According to guidelines from European Society of Medical Oncology (ESMO), the watch-and-wait approach may be considered for intermediate or “bad” tumours with cCR after CRT, or for early tumours in fragile patients or in patients rejecting radical surgery (151).

For elderly or frail patients with low performance status, not suitable for chemotherapy, short-course radiotherapy with delayed surgery is an option. Radiotherapy is also an option in the palliative setting, for reducing symptoms such as bleeding, soiling, and pain (143).

Radiotherapy is not commonly used in colon cancer. However, CRT might be considered for locally advanced colon cancers with direct invasion into non-resectable tissue (143).

Chemotherapy

The risk of recurrence and death within 5 years has been reported to be as high as 40-60% for stage III colon cancer patients (156). To reduce this risk, adjuvant chemotherapy is recommended, and includes 3-6 months treatment with 5-fluorouracil (5-FU)/leucovorin (LV) plus oxaliplatin (FOLFOX), or capecitabine plus oxaliplatin (CAPOX), with initiation within 8 weeks of surgery (131).

In stage II colon cancer, the risk of recurrence and death varies, and it has been debated whether or not adjuvant chemotherapy should be recommended to these patients. The risk of recurrence in high-risk stage II colon cancer has been reported to be as high as 30-40%, and for patients with at least one risk factor, adjuvant therapy should therefore be discussed. Risk factors include T4 tumour, resection of less than 12 lymph nodes, emergency surgery due to obstruction or perforation, poorly differentiated histology, vascular or perineural invasion, and tumour budding (131, 157).

Patients with dMMR/MSI-high colon cancer have been demonstrated to have improved DFS and OS rates compared to patients with pMMR/MSS tumours, also after adjusting for stage (129). Furthermore, dMMR tumours have been reported to

indicate resistance to 5-FU based chemotherapy (158, 159), however, findings are inconclusive (160). Nonetheless, MMR status may help guide the adjuvant treatment decision, and given the excellent prognosis for stage II dMMR tumours, adjuvant chemotherapy is generally not recommended to this group of patients (131).

For rectal cancer patients, there are conflicting opinions on the beneficial effect of adjuvant treatment. In clinical practice, rectal cancer is often treated similarly to colon cancer, however, adjuvant chemotherapy is generally not recommended to patients who have received neoadjuvant CRT (151).

For patients with incurable disease, the choice of treatment is based on tumour burden, disease aggressiveness, and the patient's general condition and preference. 5-FU is used alone or in combination with irinotecan or oxaliplatin, with combination therapy being superior to 5-FU/LV alone in terms of response rate, progression-free survival (PFS) and OS (161). Triplet chemotherapy, with 5-FU/LV plus oxaliplatin plus irinotecan (FOLFOXIRI), has been reported to give even higher response rates, albeit with increased toxicity, and is recommended as first line palliative treatment for fit patients with *BRAF*-mutated tumours (162). In addition, FOLFOXIRI is used in the neoadjuvant setting for patients with *RAS*-mutated tumours when downsizing of the tumour is needed to enable surgery (162).

Targeted therapy

In addition to chemotherapy, the introduction of targeted drugs has improved the outcome for patients with metastatic CRC.

Bevacizumab is a monoclonal antibody targeting the vascular endothelial growth factor (VEGF) A, a growth factor protein stimulating angiogenesis, whereas aflibercept is a fusion protein that in addition to binding to VEGF-A also targets VEGF-B and placental growth factor. Another angiogenesis inhibitor is ramucirumab, that binds to VEGF receptor 2, which subsequently inhibits activation of the receptor and downstream signalling. Bevacizumab is used in the palliative setting in combination with 5-FU-based chemotherapy, and aflibercept and ramucirumab may be used as second line treatment (143, 162). In recent years, new therapies targeting angiogenesis have been developed, including the tyrosine kinase inhibitor regorafenib, however, with modest effect on PFS and OS (163).

Another target for anti-tumour therapy is EGFR. In contrast to bevacizumab, the anti-EGFR antibodies cetuximab and panitumumab can be used either as single agents or in combination with FOLFOX or FOLFIRI, both with documented beneficial effects on survival (164, 165). However, these therapies are only effective in tumours without mutations in the *RAS* gene (164), and therefore, analysis of *KRAS* and neuroblastoma V-*RAS* oncogene homolog (*NRAS*) status is necessary before treatment initiation. Furthermore, recent retrospective analyses from large

phase III trials demonstrate that patients with right-sided tumours have less benefit from the addition of EGFR-targeted therapy (166-168).

Checkpoint inhibitors

In recent years, advances in our understanding of the relationship between the immune system and cancer has led to substantial developments in cancer treatment. Several antibodies blocking immune checkpoint proteins have been introduced, with nivolumab, pembrolizumab, and lambrolizumab targeting programmed cell death protein 1 (PD-1); atezolizumab, avelumab, and durvalumab targeting programmed cell death ligand 1 (PD-L1); and ipilimumab targeting cytotoxic T-lymphocyte antigen 4 (CTLA-4). Although demonstrating promising results in several human malignancies, such as melanoma (169, 170), non-small cell lung cancer (171, 172), and renal cell carcinoma (173, 174), the results in CRC have been less convincing (175, 176). However, after studies reporting tumours with dMMR/MSI-high showing greater sensitivity to PD-1/PD-L1 blockade (177, 178), the FDA granted pembrolizumab the first tissue-agnostic approval for treatment of unresectable dMMR/MSI-high solid tumours in 2017 (132). Nivolumab was later granted an accelerated approval for treatment of dMMR/MSI-high metastatic CRC, and in 2018, the combination of nivolumab and ipilimumab was approved, after reports of a more beneficial effect compared to anti-PD-1 monotherapy (179).

Sidedness

Differences between proximal and distal CRC have been reported since the 1980s (180-182), and in the 1990s, the existence of three distinct categories of CRC according to primary tumour location was proposed (183, 184). Although further discussed (185-191), this concept did not gain widespread impact until new interest was sparked by observations that primary tumour location, or sidedness, was associated with prognosis and response to EGFR-targeted therapies (166-168, 192-194).

Proximal colon cancer is defined as occurring in the caecum, the ascending colon, the hepatic flexure, or the proximal two thirds of the transverse colon, whereas distal colon cancer is defined as occurring in the splenic flexure, the descending colon, or the sigmoid colon, as illustrated in Figure 4.

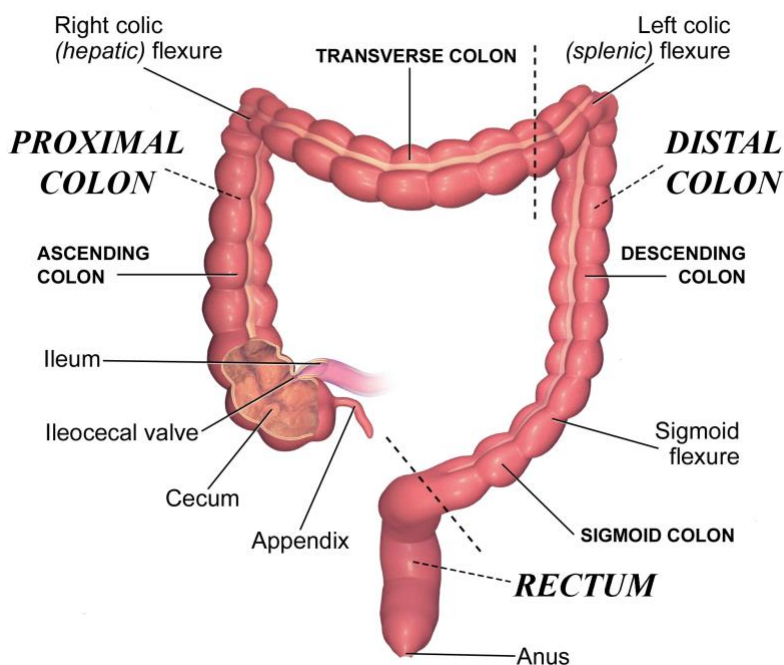


Figure 4. Definitions of the proximal colon (caecum, ascending colon, hepatic flexure, and proximal two thirds of transverse colon), the distal colon (splenic flexure, descending colon, and sigmoid colon), and the rectum.

Embryology, physiology, and immunology

The colon develops from two different embryonic areas of the primitive gut. The midgut gives rise to the small intestine and the proximal part of the colon up until approximately two thirds of the transverse colon, whereas the distal third of the transverse colon through the upper anal canal originates from the hindgut. Furthermore, additional changes in gene expression occur in postnatal development, with more than 1000 genes being differently expressed in the proximal and the distal colon, generally with higher levels of expression in the distal colon (195). There are also vascular differences, in that the proximal colon is supplied by the superior mesenteric artery and the capillary network is multi-layered, whereas the distal colon is perfused by the inferior mesenteric artery and has a single-layered capillary network (196, 197).

As the faecal content is degraded by the microbiota during the colonic passage, the production of short-chain fatty acids and metabolites varies between the proximal and the distal colon and the rectum (198). The number of bacteria increases from the proximal colon to the rectum (199), and the frequency of several bacterial enzymes involved in the production of mutagenic or carcinogenic metabolites is higher in the distal than in the proximal colon (200). Furthermore, levels of pro-carcinogenic metabolites, e.g. N-nitroso compounds, have been demonstrated to be higher in the distal colorectum than in the proximal colon (201). On the other hand, the proximal colon is more exposed to bile acids and secondary bile acids (202), suggested to promote colorectal carcinogenesis (100, 203). Finally, the overall immune activity is reported to be higher in the proximal colon compared to the rectum (204), and the number of intraepithelial T cells decreases from the proximal colon to the rectum (205).

Cancer

Clinicopathological differences

There are also numerous histological and molecular differences between colorectal adenocarcinomas according to their anatomical location. While distal, or left-sided, tumours have a more polypoid growth, proximal, or right-sided, tumours are often flat, and thus more difficult to detect by colonoscopy (206), possibly explaining the fact that these tumours are often diagnosed in more advanced stages (190, 207). Right-sided tumours are also more often poorly differentiated, and have a higher frequency of peritoneal carcinomatosis, whereas hepatic and pulmonary metastases are more common in left-sided tumours (190).

Furthermore, the carcinogenic pathways and molecular characteristics also differ according to location of the bowel. As beforementioned, dMMR/MSI-high and CIMP-high CMS1 tumours typically occur in the proximal colon, whereas CIN-high CMS2-4 tumours are more commonly located distally (24). Although generally categorized into right-sided and left-sided colon cancer and rectal cancer, several studies have found that molecular features gradually change along bowel subsites. For instance, the rates of *BRAF* mutation and dMMR/MSI increase from the caecum to the ascending colon, then steadily decrease towards the rectum (120, 208), and the rate of *KRAS* mutations decreases from the caecum to the descending colon, however, increases again in the sigmoid colon and the rectum (120). Also when dichotomizing colon cancer cases into proximal and distal tumours, the hypermutated pattern of more proximal tumours has been demonstrated (209, 210). Furthermore, the microbiota, possibly playing a part in colorectal carcinogenesis, has been shown to differ according to tumour location, with the frequency of *Fusobacterium nucleatum*-positive tumours being higher in the proximal colon than in the distal colon and the rectum (211). Finally, the density of tumour-infiltrating immune cells is higher in proximal colon cancers than in distal colon cancers (212).

Clinical presentation also differs, with right-sided tumours more often presenting with iron deficiency anaemia, whereas hematochezia and changes in bowel habits are more prevalent in patients with left-sided colon tumours or rectal tumours (213).

Epidemiology

The incidence rates of right-sided and left-sided colon cancers are approximately 35-40% and 60-65%, respectively. Nonetheless, the incidence of right-sided tumours has steadily increased over the last decades (214, 215), possibly due to environmental factors. As previously described, the impact of several lifestyle factors on CRC risk is reported to differ according to tumour location, with smoking (90) and high consumption of red meat (74) being more strongly associated with risk of left-sided colon cancer or rectal cancer, and obesity (47) and physical activity (68) being associated with risk of colon cancer rather than rectal cancer. The proportion of right-sided CRC is higher among women than men (190, 216), and increasing age is also associated with a shift of the subsite of CRC from the left to the right side of the colon (189, 217).

Prognosis and treatment prediction

Retrospective analyses of large randomised trials demonstrate that primary tumour location is predictive of treatment response to EGFR-targeted therapy in patients with RAS wild-type tumours, with left-sided colon cancer patients having a clear survival benefit whereas patients with right-sided tumour derive limited benefit

from cetuximab (166-168). Contrastingly, anti-VEGF treatment has been reported to be more effective in right-sided than in left-sided CRC (193).

Colonoscopy is generally considered to decrease the risk of CRC mortality, however, research demonstrates that this favourable impact is significantly higher in (218), or limited to (219), patients with distal cancer. Furthermore, patients with right-sided colon cancer have been demonstrated to have a significantly poorer prognosis than patients with left-sided colon cancer, also after adjusting for known prognostic factors (220). In combined analysis of early stage colon cancer, the association between tumour location and prognosis has been reported to be non-significant (221). However, in subgroup analysis according to stage, patients with right-sided stage II colon cancer have been reported to have a significantly prolonged survival compared to patients with left-sided stage II colon cancer, whereas the opposite was seen for patients with stage III tumours (207), suggesting additional alterations between proximal and distal CRC during tumour progression.

The immune system in cancer

The hypothesis that the immune system might prevent neoplasia was first proposed in 1909 (222). The concept of immune surveillance was later presented, suggesting that the immune system is constantly monitoring the body for tumour cells and destroying these cells before a tumour is established (223, 224). This was also demonstrated in animal models in the early 2000s, with the development of spontaneous tumours in immunodeficient mice (225). Dunn and Schreiber suggested a broader concept of cancer immunoediting, describing a dual relationship between the immune system in both preventing and shaping neoplasia (226). Evasion of immune response has since been added as a hallmark of cancer (227), supplementing the original six hallmarks published by Hanahan and Weinberg (228), and immuno-oncology, focusing on the interactions between the immune system and the tumour, has evolved as a new field in cancer research. Moreover, several types of immunotherapies, harnessing the body's immune response in the fight against cancer, have been introduced during the last decade.

Numerous different immune cells are involved in the anti-tumour immunity, as illustrated in Figure 5. Herein, some are briefly presented.

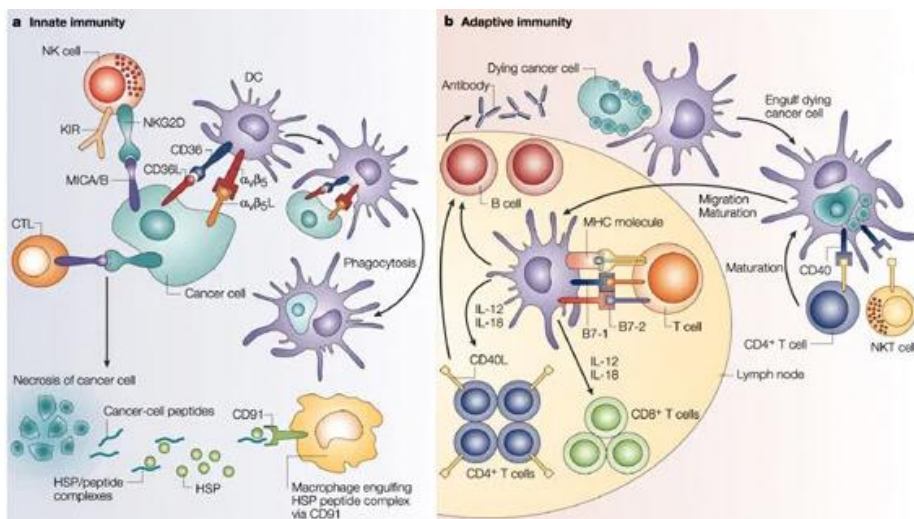


Figure 5. Cancer recognition by innate and adaptive immunity (229). Reproduced with permission from Springer Nature.

Innate immunity

The innate immune system is composed of NK cells, macrophages, monocytes, dendritic cells (DCs), neutrophils, eosinophils, basophils, and mast cells. Recognizing different proteins on cancer cells, they generate a non-specific, but important, anti-tumour immune response (230).

Natural killer cells

NK cells are important players in the anti-tumour immunity, and are regulated by inhibitory and activating receptors. Activating receptors include e.g. NKG2D (NK receptor member D of the lectinlike receptor family), that binds different ligands overexpressed on cancer cells, whereas inhibitory receptors recognize major histocompatibility (MHC) class I molecules, expressed on all nucleated cells (231). Consequently, NK cells can also be activated by down-regulated expression of MHC class I molecules on cancer cells (232). NK cell activity is enhanced by cytokine interleukin (IL) 2 (233). NK cells eliminate tumour cells by releasing cytoplasmic granules containing proteins for cell lysis, by expressing tumour necrosis factor (TNF) α and TNF receptors, shown to induce tumour-cell apoptosis, and by producing cytokines, e.g. interferon (IFN) γ , which enhances the anti-tumour immune response (234). In CRC, dense infiltration of NK cells has been reported to be associated with improved patient outcome (235).

Macrophages

Macrophages are a heterogeneous population of immune cells, involved in both the innate and the adaptive immune responses, and their main functions are phagocytosis, endocytosis, secretion, and microbial killing. Classical activation of macrophages by IFN- γ , pattern recognition receptors, or granulocyte macrophage colony-stimulating factor, results in production of pro-inflammatory cytokines including IL-12, which further stimulate NK cell and T helper (Th) 1 cell development and production of IFN- γ (236). Alternatively activated macrophages develop in response to IL-4 or IL-13, from Th2 cells, which upregulate the expression of MHC class II molecules, stimulating endocytosis and antigen presentation (237). Tumour-associated macrophages are generally alternatively activated macrophages, that can secrete a variety of cytokines known to enhance tumour growth and progression (238). Although generally being associated with an impaired prognosis (239), increased densities of macrophages in CRC have been found to signify a favourable prognosis (240).

Dendritic cells

DCs are professional antigen-presenting cells, that bridge between the innate and the adaptive immune systems. So-called danger signals are recognized by DCs, which capture and process tumour antigens. The activated DCs migrate to the lymph nodes, where they present tumour antigens for CD4⁺ and CD8⁺ T cells as well as B cells, thus inducing cellular and humoral immunity (241). Several clinical trials have investigated the efficacy of DC-based immunotherapy in CRC, with varying results (242).

Adaptive immunity

The adaptive immune system generates a specific anti-tumour response. In short, adaptive immunity encompasses components capable of forming immunological memory due to specific immune responses targeting the antigens.

T cells

Three signals are required for activation of CD4⁺ Th cells and CD8⁺ cytotoxic T cells. Signal one is recognition of antigen, e.g. tumour antigen, presented by antigen-presenting cells, and binding of the T cell receptor and CD4 and CD8 molecules to the MHC complex. Binding of CD28 on the T cells to B7 molecules on the antigen-presenting cells constitutes the second signal for Th cells, whereas cytotoxic T cells require signals from other co-stimulatory molecules, e.g. CD70 and CD137 (243). The third signal is exposure to cytokines (243), promoting the CD4⁺ T cells to differentiate into different subsets, e.g. Th1, Th2, Th17, follicular Th cells, and regulatory T cells (Tregs), depending on the cytokine milieu (244). Proliferation of T cells is, as for NK cells, stimulated by IL-2 (245).

Tregs are recognized by expression of CD4, CD25, and FoxP3 (Forkhead box P3), with the latter being crucial in regulating the development and function of Tregs. Tregs modulate and suppress the immune response by other T cells (246), and it has been reported that the up-regulation of FoxP3⁺ Tregs is dependent on CD8⁺ T cells, hence maintaining homeostasis and self-tolerance (247). Despite associations with an impaired prognosis in e.g. breast, gastric, and ovarian cancer (248), high Treg densities have been found to be associated with a prolonged survival in CRC (249, 250).

Activated and antigen-specific cytotoxic CD8⁺ T cells can recognize and lyse tumour cells, and high infiltration of CD8⁺ T cells, both within the tumour and in the peri-tumoural stroma, has been demonstrated to be associated with a favourable

prognosis in CRC (251-253). Furthermore, it has been reported that high T cell infiltration is a more powerful prognostic factor than traditional staging (253), and a so-called immunoscore, based on the density of CD3⁺ and CD8⁺ T cells, has been suggested to be incorporated into routine protocols for prognostic classification of CRC (254).

B cells

Acting as antigen-presenting cells, B cells present antigens on the MHC class II molecules to CD4⁺ T cells, which, after co-stimulatory signals, results in activation of the B cells (255). Active B cells undergo extensive proliferation and immunoglobulin class switch, and terminally differentiate into antibody-secreting plasma cells (256). Similar to T cells and NK cells, B cell proliferation is stimulated by IL-2 (257). B cells are identified by CD20, a marker expressed by all mature B cells except plasma cells. Interestingly, B cells have been demonstrated to either facilitate (258) or inhibit anti-tumour immunity (259), or even to promote de novo carcinogenesis (260), depending on the composition of B cell subsets. Nevertheless, B cells also exert direct cytotoxicity (261, 262), and can attract and stimulate DCs, T cells, and other immune cells by secreting chemokines (263). Furthermore, B cells have been shown to mediate their anti-tumour effects through production of autoantibodies, which may promote anti-tumour immunity by opsonization of tumour antigens, destruction of tumour cells mediated by the complement system, or by antibody dependent cytotoxicity (256). As a consequence, dense infiltration of CD20⁺ B cells has been reported to be associated with an improved prognosis in e.g. breast cancer (264), gastric cancer (265), and non-small cell lung cancer (266), and high densities of CD20⁺ B cells in liver metastases of CRC patients have been shown to signify a lower risk of disease recurrence and prolonged survival (267).

Plasma cells

After having undergone activation, B cells derive into plasma cells, with the main function of secreting antibodies. Immunoglobulin kappa C (IGKC) may be used as a marker to distinguish plasma cells from B cells, as IGKC is expressed in abundance on plasma cells, but not on CD20⁺ cells. IGKC has in CRC, as well as in non-small cell lung cancer and breast cancer, been associated with improved prognosis and is reported to be as predictive of outcome as the entire B cell metagene (268).

Immune checkpoints

Immune checkpoint proteins are key modulators of the anti-tumour immune response, and their interaction promotes either inhibitory or activating signalling pathways. Among the inhibitory immune checkpoints, shown to induce a negative signal to T cells, are CTLA-4 and the PD-1/PD-L1 pathway (269).

CTLA-4 is upregulated on T cells after activation, and attenuates T cell activation and expansion by outcompeting CD28 for ligand binding with B7 molecules on antigen-presenting cells, and by downregulating IL-2 production (270, 271). By blocking the interaction of CTLA-4 with its ligands, treatment with the CTLA-4 antibody ipilimumab result in unrestrained T cell proliferation (272), and has shown promising results in several types of cancer, although with an anti-tumour activity that is inferior to PD-1 blockade (170, 273).

PD-1 is not only expressed by T cells, but also by B cells, NKT cells, activated monocytes, DCs, and macrophages, suggesting a wider role in immune regulation than for CTLA-4 (274). Although its ligand PD-L1 is expressed at higher levels than PD-L2, both PD-L1 and PD-L2 expression can be found on a variety of immune cells, e.g. T cells, B cells, macrophages, and DCs, with their expression being induced by IFN- γ and other cytokines (274, 275). Furthermore, both PD-L1 and PD-L2 can be expressed on tumour cells from various origins, and PD-L1 expression has also been observed on endothelial cells in the heart, β -cells in the pancreas, and muscle cells (274). Up-regulated PD-1 expression has been demonstrated as a feature of T cell exhaustion (276), and in tumour cells, PD-L1 has at the gene expression level been demonstrated to be upregulated by multiple oncogenic signalling pathways, or by exposure to IFN- γ or other inflammatory cytokines produced by immune cells (247, 277). It has also been suggested that induction of the PD-1/PD-L1 pathway represents a mechanism of immune resistance to the adaptive anti-tumour activity (278). Engagement of PD-1 with its ligands PD-L1 or PD-L2 results in inhibited production of IL-2 and T cell receptor signalling, thus impairing the activation of T cells, and also plays a role in differentiating naïve T cells into Tregs, consequently further hampering the T cell response (277). Hence, the PD-1/PD-L1 pathway mediates cancer immune evasion, and targeting this pathway results in a potentiation of the anti-tumour capacities of T cells (279, 280), with promising results in several types of cancer (169-173), including dMMR/MSI-high CRC (177, 178).

Aims of the thesis

The general aim of this thesis was to study the prognostic impact of different immune cells in CRC, with particular reference to sidedness, and to investigate the association between pre-diagnostic obesity, measured as several anthropometric factors, and CRC risk according to tumour-infiltrating immune cell composition.

The specific aims of each paper are listed below:

- To investigate the prognostic impact of B cells and plasma cells in CRC (Paper I)
- To investigate the prognostic impact of regulatory and cytotoxic T cells in CRC, with particular reference to sidedness (Paper II)
- To re-examine the prognostic impact of B cells and plasma cells in relation to sidedness of CRC (Paper II)
- To investigate the prognostic impact of PD-1 and PD-L1 expression in CRC, with particular reference to sidedness (Paper III)
- To investigate the relationship between pre-diagnostic anthropometry and CRC risk according to tumour-infiltrating immune cell composition and PD-L1 expression, with particular reference to potential sex differences (Paper IV)

Patients

Study cohort

The Malmö Diet and Cancer Study (MDCS) is a prospective, population-based cohort study with the primary aim to investigate associations between various dietary factors and cancer incidence (281). Subjects were recruited using both a personal letter of invitation and community direct invitation (282). Between 1991 and 1996, 18 326 (60.2%) women, born between 1923 and 1955, and 12 120 (39.8%) men, born between 1923 and 1945, were enrolled. Taken together, a total number of 30 446 participants from a background population of 74 138 were recruited, resulting in a participation rate of 41% (283). The MDCS forms part of the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, from which 2348 cases from the MDCS were excluded due to incomplete data, leaving 17 035 (60.6%) women and 11 063 (39.4%) men, with a total of 28 098 individuals (284).

Baseline examinations

Baseline examinations were conducted between March 1991 and September 1996. Questionnaires concerning demographic, socioeconomic, reproductive, and various lifestyle factors, e.g. dietary habits, were filled out by all participants. Blood samples were taken, and anthropometrics were measured by a trained nurse (281).

Weight (multiples of 0.1 kg) and height (to the nearest 0.005 m) were measured, and BMI was calculated as kg/m^2 . Waist circumference was measured at the midpoint between the lower ribs and the iliac crest, and hip circumference was measured at the level of greatest lateral extension, both measurements estimated to the nearest 0.01 m. The waist and hip circumferences of each participant were used to calculate waist-hip ratio (WHR). Using a single frequency bio-impedance methodology (BIA 103, RLJ-systems, Detroit, MI, USA) with tetra polar electrode placement and subjects in a supine position, lean body mass and fat mass were determined and served to calculate bodyfat percentage (BFP). The BIA method has previously been validated in Swedish middle-aged and elderly adults (285).

Follow-up

Information on CRC incidence was obtained through the Swedish Cancer Registry up until December 31, 2007, and from the Southern Swedish Regional Tumour Registry for the period of January 1, 2008 to December 31, 2008. Median time from baseline until diagnosis was 8.6 (standard deviation [SD]=4.3) years, and the median follow-up time in the entire cohort was 13.7 (SD=3.2) years.

Information on vital status and cause of death was obtained from the Swedish Cause of Death Registry up until December 31, 2013. Follow-up began at CRC diagnosis and ended at death, emigration, or December 31, 2013, whichever came first. Median follow-up time was 5.97 (range 0-21.69) years for the entire cohort (n=626) and 10.05 (range 5.03-21.69) years for patients alive (n=274).

Study population

For papers I-III, a total number of 626 CRC cases were identified in the MDCS cohort. Median age at diagnosis was 71 (range 50-86) years. Clinical and treatment data were obtained from medical charts. Histopathological data were obtained from pathology records. TNM staging was performed according to the AJCC. Cases with insufficient tumour material were excluded, whereby a total of 557 (89.0%) cases were available for tissue microarray (TMA) construction, as illustrated in Figure 6.

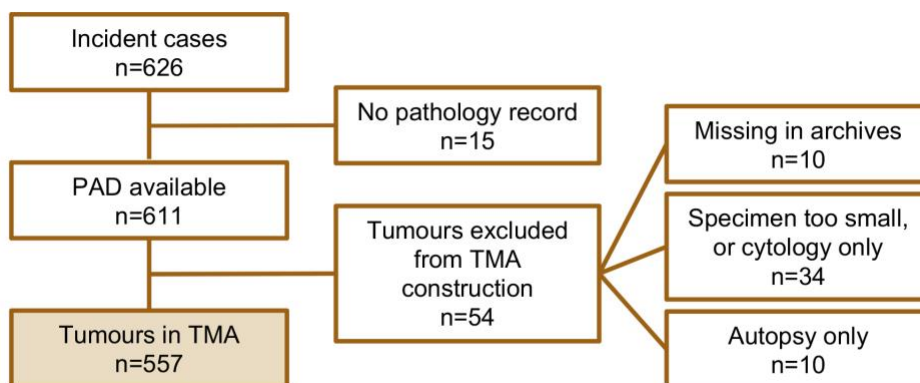


Figure 6. Flowchart outlining the study cohort from a total of 626 incident cases of CRC from the Malmö Diet and Cancer Study, up until December 31, 2008.

Right colon was defined as the appendix, caecum, ascending, and proximal two thirds of the transverse colon, whereas left colon was defined as the left colic flexure, descending and sigmoid colon, corresponding to the midgut vs hindgut fetal origin as well as innervation and blood supply. Information on tumour location was available for 555 (99.6%) cases. There were 201 (36.2%) right-sided and 145 (26.1%) left-sided colon cancers, and 209 (37.7%) rectal cancers.

For paper IV, a total of 584 cases of incident CRC in the EPIC cohort were identified. Eight cases were re-classified as intramucosal carcinoma and excluded from the analyses. Prevalent CRC was denoted in 66 participants, none of whom had incident CRC, and they were also excluded from the analyses.

Ethical considerations

All EU and national regulations and requirements for handling human samples have been fully complied with during the conduct of this project; i.e., decision no. 1110/94/EC of the European Parliament and of the Council (OJL126 18,5,94), the Helsinki Declaration on ethical principles for medical research involving human subjects, and the EU Council Convention on human rights and Biomedicine. Ethical permission for the MDCS (LU 90–51) and the present study (LU 530–2008) was obtained from the Ethics Committee at Lund University. Written informed consent was obtained from each subject at study entry.

Methods

Tissue microarray

The TMA technique, first introduced in 1998, is a high-throughput method for evaluation of protein expression that has become an important tool in biomarker research. TMAs are constructed by taking multiple cylindrical tissue cores, typically 0.6-2 mm, from representative parts of a formalin-fixed, paraffin embedded tumour, and inserting these cores into a common receiver paraffin block (286). The recipient block is sliced into 4-5 μm sections that are mounted on microscope glass slides, and subsequently subjected to immunohistochemistry (IHC) (286, 287). The TMA construction is illustrated in Figure 7.

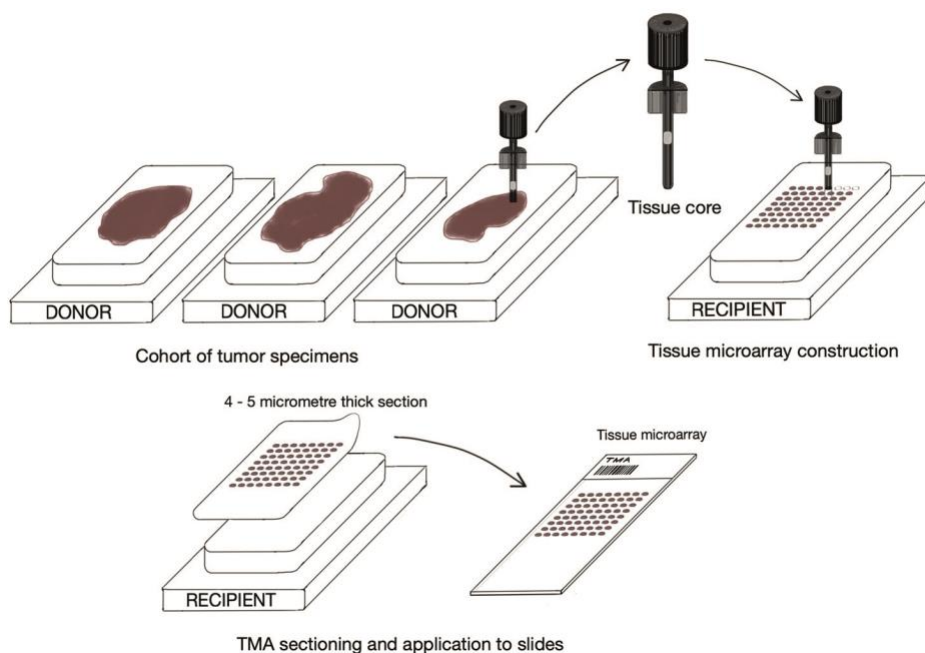


Figure 7. Schematic illustration of the TMA technique. Reproduced courtesy of Gustav Andersson.

Using only a fraction of antibody compared to full-face tissue sections, the technique enables simultaneous screening of hundreds of tissue samples simultaneously. Furthermore, one TMA block can be sectioned up to 300 times, thus conserving valuable tissue material. One concern that has been raised regarding the TMA technique is the issue of tumour heterogeneity, and that the tissue cores might not be representative of the entire tumour. Nevertheless, previous studies have demonstrated that a high degree of concordance with full face sections is accomplished by retrieving duplicate or triplicate cores (288, 289), and that the TMA technique might provide equal, or even superior, prognostic information than full-face tissue sections (287, 290).

Immunohistochemistry

IHC is a widely used technique to determine the distribution of antigens in tissue samples by the use of antibodies. First introduced in the early 1940s (291), the technique has since been further developed, e.g. by the introduction of antibodies labelled with enzymes in the 1960s (292).

As tissue samples are usually fixated in formalin and embedded in paraffin, the tissue is pre-treated with heat and an antigen retrieval solution that breaks the cross-links formed by the formalin fixation. Thereafter, a primary antibody is applied, which reacts with the tissue antigen. By adding a secondary antibody labelled with an enzyme, e.g. peroxidase, or a fluorescent agent, the reaction can be visualised in a light microscope (293).

The antibodies can be polyclonal or monoclonal, the latter being more specific as they bind only to one epitope. To ensure reliable results, it is essential to use antibodies that are validated regarding their specificity (294). Western blot is an appropriate first validation step, whereby proteins are separated based on their molecular weight, and an antibody is considered validated if it produces a single band of the expected molecular weight (295).

The antibodies used in this thesis are described in the papers I-III.

Digital image analysis

Classical manual microscopy of IHC stained tissue sections has several limitations, with high interobserver variation being one of the main issues. To reduce these problems, digital image analysis (DIA) was developed. DIA was used to analyse the density of CD3⁺ and CD8⁺ T cells in paper II.

Digital images of IHC stained tissue sections are acquired by whole slide imaging scanners or microscope-based systems, and the images are thereafter pre-processed by combining spectral, local spatial, and local morphological information. The taxonomy is then defined for the system by digitally painting examples of the defined structures, and this algorithm is then applied to identify IHC stained cells.

Although DIA has indeed been demonstrated to be superior to manual scoring in terms of interobserver variation (296), the technique does not come without limitations. Sections need to be carefully produced to avoid folds and other unequal thickness, which might otherwise cause problems in the segmentation. Varying intensity and quality of the staining might also be difficult to compensate for. Finally, indistinct cell borders pose a challenge in separating individual cells for DIA cell counting (297).

Statistical analyses

For papers I-III, associations between immune cell density and clinicopathological parameters as well as other investigated biomarkers were analysed using Mann-Whitney U test and Pearson's chi-square test. Spearman's rho test was used to analyse the interrelationship between investigated immune cells. Classification and regression tree analysis was used to determine an optimal prognostic cut-off for dichotomisation into high and low immune cell density. Kaplan-Meier analysis and log rank test were applied to illustrate differences in survival according to immune cell density and PD-1 and PD-L1 expression. Hazard ratios (HRs) for survival were calculated by Cox regression proportional hazards models in unadjusted analysis, without taking other factors into account, and in multivariable analysis, adjusting for age, sex, T stage, N stage, M stage, differentiation grade, and vascular invasion. The proportional hazards assumption was tested using Cox regression with a time-dependent covariate analysis, whereby the proportional hazards assumption was considered to be satisfied when the factor x time interaction was non-significant. The proportional hazards assumption was also evaluated graphically using log-log plots.

In paper IV, Pearson's chi-square test was applied to compare the distribution of well-established and potential risk factors for CRC between cases and the rest of the cohort. Cox proportional hazards models were used to assess the impact of different anthropometric factors on risk of CRC defined by low and high immune cell density, and PD-L1 expression. In the multivariable Cox analysis, potential confounders were included, i.e. age (years), smoking habits (yes regularly, yes occasionally, former smoker, or never smoker), alcohol consumption (g/day), and educational level (≤ 8 years, 9–10 years, 11–13 years of education, or university degree).

Anthropometric factors were examined as continuous variables as well as quartiles, and trend was calculated as linear trend over quartiles. A case-to-case analysis examined the heterogeneity of immune cell densities regarding their associations to anthropometric factors using an unconditional logistic regression model. In order to assess any potential interaction between each anthropometric factor and sex, an interaction term was added to the logistic regression model.

All statistical calculations were performed using IBM SPSS Statistics Versions 22-24 (SPSS Inc., Chicago, IL, USA). All tests were two-sided and a p -value <0.05 was considered significant.

Results and Discussion

The detailed results are presented in the original papers. Therefore, the principal findings are herein only briefly summarized and discussed.

Paper I

This was the first TMA-based study to investigate the clinicopathological correlates and prognostic impact of B cells and plasma cells in CRC. One previous study had demonstrated B cells to be associated with an improved patient outcome in metastatic CRC (240). Herein, significant associations were found between CD20⁺ B cell infiltration and lower T-stage ($p<0.001$) and negative M-stage ($p<0.001$). Furthermore, patients with tumours displaying high density of CD20⁺ B cells had a significantly prolonged 5-year OS, also after adjustment for age, sex, TNM stage, differentiation grade, and vascular invasion (HR=0.63).

There are several potential explanations for the favourable impact of B cell infiltration. In vivo, B cell MHC class II expression has been demonstrated to be required for maximal expansion, memory formation, and cytokine production of CD4⁺ T cells (298, 299), the latter being important factors in mediating anti-tumour immunity (300), e.g. by secreting IFN- γ and recruiting NK cells and macrophages to tumour sites (301). Moreover, activated B cells have been found to promote tumour cell lysis, further hampering tumour progression (302). Finally, by their function as APCs and by providing costimulatory signals to T cells, B cells also contribute to T cell activation and expansion (298, 303), facilitating the activation of the cell-mediated anti-tumour immune response (258, 304). Although mainly acting as non-direct effector cells, B cells thus seems to play a pivotal role in anti-tumour immunity. However, it should also be considered that the presence of B cells, amongst other immune cells, primarily mirrors the overall immunity and condition of the patient (305).

By producing antibodies, plasma cells have the ability to activate antigen-dependent cellular cytotoxicity, with NK cells as the main effector cells. Plasma cells can also activate the complement system, which generates anaphylatoxins, membrane attack complex, and opsonizes targeted tumour cells (306). Herein, the expression of

CD138 and IGKC, both expressed on plasma cells, was analysed. Expression of CD138 did not carry any independent prognostic value, neither on immune cells nor on tumour cells. This is in line with results from a meta-analysis on the prognostic significance of CD138 in CRC (307), but in contrast with findings from breast cancer (308), ovarian cancer (309), and lung cancer (310). Nevertheless, some studies have not discriminated between immune cell-specific and tumour cell-specific expression of CD138. IGKC, which is exclusively expressed on plasma cells, was significantly associated with a prolonged 5-year OS, however, only in univariable analysis (HR=0.67), which is in line with previous results in CRC (268).

Previous research has largely focused on the role of the T cells in anti-tumour immunity. Taken together, these results demonstrate an important role also for the B cell lineage in CRC.

Paper II

This study was the first to investigate the prognostic impact of CD3⁺, CD8⁺, and FoxP3⁺ T cells in CRC, with particular reference to primary tumour location. A re-analysis of the results from paper I according to primary tumour location was also performed.

In line with previous research (251-253), high density of CD8⁺ T cells was overall significantly associated with a prolonged 5-year OS in multivariable analysis, after adjusting for age, sex, TNM stage, differentiation grade, and vascular invasion. However, when taking primary tumour location into account, high infiltration of CD8⁺ T cells was only an independent predictor of a prolonged 5-year OS in patients with right-sided colon cancer (HR=0.53), with a significant prognostic interaction between CD8⁺ T cells and right-sidedness (p for interaction=0.031). When MSI status was included in the adjusted model, high infiltration of CD8⁺ T cells remained significantly associated with an improved OS in patients with right-sided tumours (HR=0.42). Neither CD3⁺ nor FoxP3⁺ T cells were prognostic in univariable analysis in any tumour subsite. However, in multivariable analysis, high infiltration of both CD3⁺ and FoxP3⁺ T cells were significantly associated with a prolonged 5-year OS in patients with rectal cancer (HR=0.45 and HR=0.54, respectively).

Re-analysis of the results from paper I demonstrated that high infiltration of CD20⁺ B cells was significantly associated with an improved 5-year OS in patients with right-sided as well as left-sided tumours in univariable analysis; however, only in patients with right-sided colon cancer did this association remain significant after adjusting for known prognostic factors (HR=0.38). High density of CD138⁺ plasma cells was an independent predictor of a prolonged 5-year OS only in patients with left-sided tumours (HR=0.48).

Taken together, both CD8⁺ T cell and CD20⁺ B cell infiltration was only prognostic in patients with right-sided colon cancer, and not in patients with left-sided colon cancer or rectal cancer. Previous studies demonstrate immune cell infiltration to be higher in dMMR/MSI-high tumours (311), more commonly found with proximal location (17). Interestingly, in contrast to the expected, the number of CD8⁺ T cells was in this study found to be significantly lower in right-sided colon cancers than in left-sided colon cancers or rectal cancers ($p=0.004$). *Fusobacterium nucleatum* is a common oral bacterium that has been found to be enriched in CRC (312), and appears to potentiate CRC neoplasia development by modulating the inflammatory response, e.g. by generating a pro-inflammatory microenvironment (313, 314) and by inhibiting T cell and NK activity (315). A higher proportion of *F. nucleatum* have been demonstrated in dMMR/MSI-high (316) and proximal tumours (211), and is also reported to downregulate the total number of T cells in CRC (317), however, only in dMMR/MSI-high tumours (318). Thus, the microbiota might partly explain the decreased number of CD8⁺ B cells in right-sided tumours in this cohort. Furthermore, an enhanced cytotoxic function in T cells has been identified in patients with right-sided colon cancer rather than left-sided colon cancer and rectal cancer (319), possibly explaining why cytotoxic T cells was only prognostic in patients with right-sided tumours.

Tregs have been associated with an unfavourable prognosis in several types of cancer, in line with their suppressive effect on anti-tumour immunity (320). However, in CRC, Tregs have been associated with a favourable prognosis (249, 250), further confirmed in the present study. Although validity studies are needed to further confirm whether the prognostic value of Tregs is limited to rectal cancer, one explanation for the auspicious impact of Tregs may be related to the gastrointestinal microbiota, as Tregs have been suggested to prevent bacteria-driven inflammation and carcinogenesis (320), and bacterial population densities are higher in the rectum than in the colon (202). Furthermore, Tregs are known to inhibit activation and function of pro-inflammatory Th17 cells (321), which have been shown to promote cancer growth (322) and to be associated with an impaired prognosis in CRC (323). Although not investigated herein, it is tempting to postulate that the number of Th17 cells might be higher in rectal cancer than in colon cancer, therefore explaining the beneficial role of Tregs only in patients with rectal cancer.

In conclusion, this was the first study to demonstrate that the prognostic impact of T cells as well as B cells in CRC differs according to primary tumour location, with both cytotoxic T cells and B cells only being associated with a favourable prognosis in patients with right-sided colon cancer, further indicating the heterogeneous nature of CRC.

Paper III

In line with previous studies (324-327), high expression of PD-L1 on tumour-infiltrating immune cells was independently associated with an improved prognosis in the entire cohort. Nonetheless, this was the first study to investigate the prognostic impact of PD-1 and PD-L1 expression in CRC with particular reference to primary tumour location.

When taking tumour location into account, high PD-L1 expression on immune cells was significantly associated with an improved 5-year OS in patients with right-sided or left-sided colon cancers, but not in patients with rectal cancer, also in multivariable analysis (HR=0.47 and HR=0.28, respectively). Furthermore, there was a significant interaction between high PD-L1 expression on immune cells and tumour location in the colon versus the rectum (p for interaction=0.019). Finally, high expression of PD-1 was significantly associated with a prolonged 5-year OS, only in patients with right-sided tumours, and only in univariable analysis.

Since cancer cells are capable of up-regulating the expression of PD-L1 and thereby inhibiting the anti-tumoural T cells response (328), it is not surprising that PD-L1 expression within the tumour microenvironment has been associated with an impaired prognosis in numerous human cancers (329). In CRC, results regarding the prognostic impact of PD-L1 have been conflicting, with reports on associations with both improved (324) and impaired (330) prognosis, and others reporting no significant associations with survival (326, 331). Herein, tumour cell-specific PD-L1 expression was not prognostic, neither in the full cohort nor according to tumour location.

As previously mentioned, dMMR/MSI-high tumours are vigorously infiltrated by immune cells (332-334). However, these tumours also demonstrate highly upregulated expression of immune checkpoint proteins, e.g. CTLA-4, PD-1, and PD-L1 (332). Therefore, the increased expression of PD-L1 might merely reflect a general activation of the anti-tumoural immune response, also indicated herein by the significant association between immune-cell specific PD-L1 expression and density of CD8⁺ T cells (p<0.001). Nevertheless, the prognostic impact of PD-L1 was independent of CD8⁺ T cell density and MSI status. Another possible explanation may be that PD-L1 expression mirrors infiltration of IFN- γ producing Th cells, since PD-L1 gene expression has been found to correlate with IFN- γ gene expression in CRC (324). Additionally, an IFN- γ induced tumour microenvironment in CRC has been reported to be associated with an auspicious outcome (335). Nonetheless, the favourable prognostic impact of immune cell-specific PD-L1 expression in CRC may also be explained by regulatory and hitherto undefined mechanisms.

As the first study to investigate the prognostic impact of PD-1 and PD-L1 in CRC in relation to primary tumour location, the results from this study further indicate that sidedness may be an important factor to take into consideration in therapeutic decisions, possibly also for eligibility for immunotherapy.

Paper IV

Obesity is a well-established risk factor for CRC but its association with the tumour microenvironment has been sparsely described. This study investigated the relationship between several pre-diagnostic anthropometric variables (height, weight, BMI, waist and hip circumference, WHR, and BFP) and risk of CRC according to tumour immune cell composition.

In the full cohort, obesity, defined by several anthropometric factors, was significantly associated with risk of CRC with high infiltration of CD8⁺ T cells and CD20⁺ B cells, but with low infiltration of FoxP3⁺ T cells and low expression of PD-L1 on tumour cells. In separate analysis according to sex, the associations between obesity and risk of CD8 high and CD20 high tumours remained significant in men, but, were weaker or non-significant in women. Furthermore, in men, all anthropometric factors except height were significantly associated with risk of CRC with low PD-L1 expression on immune cells, whereas in women, weight and BMI were associated with risk of CRC with high PD-L1 expression on immune cells.

Although hitherto only sparsely investigated, previous studies demonstrate somewhat conflicting results, with two studies showing inverse associations between BMI and CD3⁺ (336) and CD8⁺ (337) infiltration, and another reporting no relationship with lymphocyte infiltration (338). However, there may have been a misclassification of risk, as these studies only used BMI as an indicator of obesity, and body fat distribution seems to be a more accurate indicator of obesity when assessing the allover risk for CRC incidence (51). Moreover, none of these studies considered possible sex differences, which has been reported in both the innate and adaptive immune system, with immune gene expression (339), T cell blood count (340), and immune cell activation (341) differing between men and women. It has also been reported that men have a greater benefit from PD-1/PD-L1 blockade than women (342), and furthermore, that obese patients have an improved response to PD-1/PD-L1 blockade (343). The observed association between obesity and immunotherapy response may have clinical implications and thus merits further study, also regarding potential sex differences, not least given the finding of contrasting associations between obesity and immune cell-specific PD-L1 expression in women and men in the present study. To the best of our knowledge,

this is the first study to describe the relationship between obesity and expression of PD-L1 in the tumour microenvironment of CRC.

Moreover, obesity has been associated with greater abundances of T cells, B cells, and macrophages in adipose tissue (344-346). Leptin, released from the adipose cells, has also been demonstrated to have effects on immune function, with deficiency being associated with impaired T cell proliferation and cytokine production (347). Nevertheless, the increased infiltration of T cells has been shown to contribute to production of proinflammatory mediators (348) and recruitment of macrophages (344), enhancing adipose inflammation which may confer an increased cancer risk (57). Additionally, obesity is known to be involved in suppression of both the innate and the adaptive immune systems, and accelerated immune ageing (349, 350). Thus, obesity might have paradoxical effects on the immune response in CRC, and the results from the present study further emphasize that anthropometric factors may be important to consider in studies on the prognostic or predictive value of immune biomarkers.

Taken together, this study demonstrated that the association between obesity and risk of CRC with a particular immune microenvironment differed according to sex. Nonetheless, no significant interactions were found between sex and each anthropometric factor for any of the investigated immune cells subsets, and further studies are therefore merited to examine the effect of sex and obesity on the tumour microenvironment in CRC.

Strengths and limitations

The MDCS is a large prospective population-based cohort, which has been well validated in numerous studies. All non-cases represent controls in paper IV, and as this should ideally represent the studied population, this may contribute to a higher reliability of the results. Nonetheless, due to a participation rate of 40% in the MDCS, a potential selection bias compared with the general population must be taken into consideration, with overall cancer incidence being higher in non-participants during recruitment, and mortality being higher in non-participants both during and following the recruitment period (283). Furthermore, participation in the MDCS might *per se* be associated with a certain body type, also posing a potential selection bias. Another potential selection bias is the exclusion of cases due to insufficient tumour material and, hence, the validity of the TMA results. Finally, neoadjuvant treatment, e.g. radiotherapy for rectal cancer patients, may induce molecular changes not naturally occurring, and may also eliminate most or all tumour cells in resected specimens, which also might pose as a possible selection bias. Nevertheless, in the present cohort, only 61 (29.8%) of patients with rectal cancer received neoadjuvant treatment, and the results did not differ in any of the studies when these cases were excluded. Furthermore, as the vast majority of cases had not received targeted therapies, the cohort may be regarded as more treatment-naïve than subsequent and more modern cohorts.

One of the major criticisms of the TMA technique is that only a small fraction of the tissue specimen is used, and that this fraction might not be representative of the whole tissue section. Nonetheless, the representativeness increases by including several samples from the same tissue specimen, and studies have also concluded that all findings based on various methods on full-face sections were indeed reproducible in TMA-based studies (287, 290). This was also evaluated in paper III, wherein a high rate of concordance between PD-1 and PD-L1 expression in TMAs and full-face sections was demonstrated.

Some of the previous studies regarding the effect of obesity on CRC subtype have used self-reported anthropometric measurements. In the herein used cohort, all anthropometric measurements were obtained by trained nurses, and although there is a potential risk for interobserver variation, there were strict recommendations for how the participants should be dressed and positioned for examination, and for how the measurements should be taken. There is, however, a possibility of

misclassification of risk for CRC subtype, as anthropometric data were only obtained at baseline, and individuals might have gained or lost weight thereafter. As the number of years from baseline until diagnosis varies, further misclassification of risk cannot be ruled out. There is also a possibility that obesity or body constitution are merely inferior surrogate markers for other factors, e.g. degree of physical activity and metabolic imbalance. In the MDCS, the data regarding physical activity are not reliable, and have thus not been included in the multivariable analysis. However, future studies should take this information into consideration.

Due to multiple testing, there is an inherent risk of type I errors. As all included studies are exploratory, nominal p-values are throughout presented without adjustment for multiple testing. As the number of cases in some groups is relatively small, there is also a risk of type II errors. Finally, the use of classification and regression tree analysis to decide the optimal cut-off for prognostic analyses poses a risk of potential statistical error, as this might overfit the model. Therefore, the results need to be validated in additional studies.

Conclusions and future perspectives

High infiltration of tumour-infiltrating B cells was an independent favourable prognostic factor in colorectal cancer, however, then taking primary tumour location into account, only in patients with right-sided colon cancer.

High infiltration of cytotoxic T cells was independently associated with improved outcome only in patients with right-sided colon cancer, whereas Tregs was an independent favourable prognostic factor only in patients with rectal cancer.

High PD-L1 expression on immune cells was an independent favourable prognostic factor in patients with both right-sided and left-sided colon cancer, but not in patients with rectal cancer. Tumour cell-specific PD-L1 expression was not prognostic, in any tumour subsite.

Obesity was associated with risk of CRC with high density of B cells and cytotoxic T cells in both sexes, but the associations were stronger and indicated by more anthropometric factors in men than in women. Furthermore, obesity was associated with risk of CRC with low infiltration of Tregs and low expression of PD-L1 on tumour cells. Regarding immune-cell specific PD-L1 expression, obesity was associated with risk of tumours with low expression in men, but with high expression in women.

The results from this thesis indicate that the prognostic impact of the immune microenvironment of CRC differs according to primary location of the tumour. Furthermore, the results show that obesity might influence the tumour immune landscape of CRC, possibly in a sex-dependent manner. Taken together, these findings demonstrate that tumour location, anthropometric factors, and sex are all important factors to take into consideration in future studies on the tumour immune environment as a target for prevention and treatment of CRC.

Populärvetenskaplig sammanfattning

Tjock- och ändtarmscancer är den tredje vanligaste cancerformen hos män och den näst vanligaste cancerformen hos kvinnor i världen. I Sverige diagnosticerades 2018 cirka 4700 patienter med tjocktarmscancer och drygt 2100 patienter med ändtarmscancer. Tidigare forskning pekar mot att rött kött och fiberfattig kost, liksom låg fysisk aktivitet, rökning och hög alkoholkonsumtion ökar risken för tjock-och ändtarmscancer. Vidare har en stor mängd studier visat att övervikt också är en riskfaktor, även om resultaten inte är lika övertygande hos kvinnor.

Sjukdomen delas in i kliniska stadier (I-IV) vid diagnos, information som sedan används för att bedöma vilken behandling patienten ska få. Behandlingen består av kirurgi, och i mer avancerade fall ges tilläggsbehandling med cellgifter för att minska risken för återfall. Det har dock visat sig att den traditionella stadiindelningen inte är tillräcklig för att förutspå sjukdomsförlopp och prognos hos enskilda patienter. Därför är det av yttersta vikt att utveckla kunskaperna kring andra prognostiska faktorer.

Allt större vetenskapligt underlag pekar nu mot att tjock- och ändtarmscancer bör betraktas som en heterogen cancertyp, med avsevärda skillnader beroende på var i tjock- eller ändtarmen tumören har uppstått. I dagens forskningsläge delas tjocktarmscancer upp i högersidiga och vänstersidiga tumörer, där de tumörer som benämns som högersidiga återfinns i den första delen och de vänstersidiga i den senare delen av tjocktarmen. Den första delen av tjocktarmen har ett annat ursprung i fosterutvecklingen än vad den senare delen av tjocktarmen samt ändtarmen har, och även kärlförsörjningen skiljer sig åt mellan dessa delar. Ändtarmscancer, som uppstår i tarmens sista 15 centimeter, behandlas i regel som en egen undergrupp, främst på grund av skillnader i behandling och prognos.

Det har visat sig att högersidiga tjocktarmstumörer oftare drabbar kvinnliga och äldre patienter, och det är vanligare med genmutationer i dessa tumörer än i vänstersidiga tumörer. Högersidiga tumörer diagnosticeras också oftare vid mer avancerade stadier, men oavsett stadium har dessa patienter en klart sämre prognos. Dessutom har det visat sig att behandlingssvaret vid målinriktade cancerterapi påverkas av var i tjock- eller ändtarmen tumören har uppstått.

Det har länge varit känt att immunförsvaret spelar en viktig roll i både utvecklingen av och försvaret mot cancer. Hittills har de flesta studier fokuserat på betydelsen av

så kallade mördar-T-celler, som har visat sig kunna förgöra tumörceller och därmed förbättra prognosen. B-celler, som är en annan typ av vita blodkroppar vars funktion främst är att känna igen och bilda antikroppar mot exempelvis bakterier och tumörceller, har visat sig antingen förbättra eller försämma prognosen, beroende på cancerform. Under de senaste åren har ett flertal olika typer av immunterapier godkänts för behandling. De verkar genom att stimulera kroppens immunförsvar till att angripa cancerceller, främst genom hämning av så kallade checkpoint-proteiner, som normalt fungerar som bromsar gentemot mördar-T-celler. För upptäckten av dessa bromsar tilldelades James P. Allison och Tasuku Honjo Nobelpriset i fysiologi eller medicin 2018. Genom att blockera dessa bromsar blir mördar-T-cellerna mer aggressiva mot cancer, och sådan typ av behandling har visat enastående resultat vid bland annat malignt melanom och en viss typ av lungcancer. I USA är dessa typer av läkemedel sedan 2017 godkända för behandling av en undergrupp av tjock- och ändtarmscancer med förekomst av mutationer i reparationssystemet för arvsmassan.

Mot ovanstående bakgrund har syftet med detta avhandlingsarbete varit att undersöka mängden av olika typer av immunceller i tjock- och ändtarmscancer och hur de är kopplade till kliniska faktorer och överlevnad, samt eventuella skillnader mellan höger- och vänstersidig tjocktarmscancer respektive ändtarmscancer. Därutöver har vi även undersökt hur olika antropometriska faktorer, det vill säga mått på kropps-konstitution, påverkar risken för tjock- och ändtarmscancer med en viss sammansättning av immunceller.

Som underlag till studierna användes ett stort datamaterial från Malmö Kost Cancer-studien. Malmö Kost Cancer-studien initierades i början av 1990-talet och hade som mål att undersöka kopplingar mellan kosten och efterföljande cancer-risk. Totalt rekryterades 17 000 kvinnor och 11 000 män, födda 1923-1950, från befolkningen i Malmö, motsvarande en deltagandefrekvens på 40 procent. Förutom uppgifter om matvanor lämnade deltagarna också information om tidigare sjukdomar, medicinering, motion och andra livsstilsvanor. I samband med det första studiebesöket togs även olika mått på kropps-konstitution: längd, vikt, body mass index (BMI), midje- och höftmått, en kvot på midje- och höftmått (waist-to-hip ratio, WHR), samt kroppsfett i procent. Fram till och med 2008 hade 626 fall av tjock- och ändtarmscancer identifierats, varav tumörer fanns tillgängliga från 557 fall. Med hjälp av immunohistokemisk analys, som kortfattat går ut på att målsökande antikroppar fäster sig vid proteiner vilket ger en färgreaktion som kan studeras i mikroskop, kunde antalet av olika immunceller i tumörens närmiljö undersökas.

I det första delarbetet undersöktes närvaron av B-celler och plasmaceller, en typ av högspecialiserade B-celler, med hjälp av immunohistokemisk analys av proteinerna CD20, CD138 och IGKC. Vi fann att mängden av både B-celler och plasmaceller

var högre i mindre aggressiva tumörer utan djupväxt i tarmen. Vi kunde också se att de patienter vars tumörer innehöll många B-celler levde betydligt längre än de patienter vars tumörer innehöll få B-celler.

Det andra delarbetet fokuserade på närvaron av mördar-T-celler och regulatoriska T-celler, vilka reglerar och dämpar den immunologiska aktiviteten, och deras prognostiska betydelse. Tidigare studier har visat att en hög närvaro av mördar-T-celler i tumörmiljön är kopplat till en förlängd överlevnad, men man har aldrig tidigare undersökt om den gynnsamma kopplingen till prognos varierar beroende på var i tjock- eller ändtarmen tumören har uppstått. Vi kunde i denna studie visa att patienter vars tumörer innehöll många mördar-T-celler hade en bättre prognos, men detta samband sågs endast vid högersidig tjocktarmscancer, inte vid vänstersidig tjocktarmscancer eller ändtarmscancer. Vi fann också att patienter med ändtarmscancer vars tumörer innehöll många regulatoriska T-celler levde längre än de patienter vars tumörer innehöll få regulatoriska T-celler. Detta samband sågs inte hos patienter med tjocktarmscancer. I detta delarbete analyserade vi även om resultaten från det första delarbetet, för att undersöka om den prognostiska effekten av B-celler varierade beroende på var tumören hade uppstått. Vi kunde då visa att den gynnsamma prognostiska effekten av B-celler endast gällde för patienter med högersidig tjocktarmscancer, och inte för patienter med vänstersidig tjocktarmscancer eller ändtarmscancer.

I avhandlingens tredje delarbete undersöktes förekomsten av två immunccheckpoint-proteiner ("bromsar" för immunsystemet), programmed cell death-protein 1 (PD-1) och programmed cell death-ligand 1 (PD-L1) och kopplingen till prognos. Proteinet PD-1 finns på vissa immunceller, medan PD-L1 kan uttryckas på både immunceller och tumörceller. När PD-1 binder till receptorn PD-L1 bromsar detta mördar-T-cellernas aktivitet. Patienter vars tumörer har ett högt uttryck av PD-L1 har visat sig ha en sämre prognos än patienter vars tumörer har ett lågt uttryck. Däremot har tidigare forskning visat att högt uttryck av PD-L1 på celler i immunsystemet är kopplat till bättre prognos i flertalet cancerformer, inklusive tjock- och ändtarmscancer. Inga studier har dock tidigare undersökt om denna koppling varierar beroende på var tumören har uppstått. Vi fann att ett högt uttryck av PD-L1 på immunceller var kopplat till bättre prognos, oberoende av kända prognostiska faktorer, men bara hos patienter med högersidig eller vänstersidig tjocktarmscancer, och inte hos patienter med ändtarmscancer.

Det är välkänt att fetma är kopplat till en ökad risk för att drabbas av tjock- och ändtarmscancer, men endast ett fåtal studier har undersökt hur fetma påverkar sammansättningen av immunceller i dessa cancerformer. Därför undersökte vi i det fjärde delarbetet förhållandet mellan fetma, indikerat av olika antropometriska faktorer, och risken att drabbas av tjock- och ändtarmscancer med olika sammansättningar av immunceller. Vi fann samband mellan övervikt och risk för

tjock- och ändtarmscancer med en hög mängd av B-celler och mördar-T-celler. Vidare fann vi att övervikt hos kvinnor var kopplat till risk för tjock- och ändtarmscancer med ett högt uttryck av PD-L1 på immunceller, medan övervikt hos män var kopplat till risk för tjock- och ändtarmscancer med ett lågt uttryck av PD-L1 på immunceller. Dessa resultat pekar mot att både övervikt och kön kan påverka sammansättningen av immunceller i tjock- och ändtarmscancer.

Sammanfattningsvis har detta avhandlingsarbete kunnat visa att tumörlokaliseringen har betydelse för den prognostiska effekten av olika immunceller i tjock- och ändtarmscancer. Vidare har vi visat att fetma inte bara ökar risken att drabbas av tjock- och ändtarmscancer utan även påverkar sammansättningen av immunceller i tumörerna, och att denna effekt tycks skilja sig mellan könen.

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