Kai Klintrup

INFLAMMATION AND INVASIVE MARGIN IN COLORECTAL CANCER
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 1 of Oulu University Hospital, on 21 September 2012, at 12 noon

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Oulu, Finland

Abstract

Prognostic features of colorectal cancer (CRC) are important for determining the optimal treatment for an individual patient. This study was carried out to evaluate the significance of the prognostic significance of inflammatory cell reaction and tumour budding at the invasive front of the tumour in CRC patients, to study the pattern of alterations in the serum cytokine levels of CRC patients compared to healthy controls, and to evaluate whether the patterns of the cytokine levels alter according to the stage of disease.

Study material consists of a series of CRC patients operated in Oulu University Hospital (N=466, studies I-II, and N=148, study III). The intensities of inflammatory cell reaction and tumour budding were estimated and the association of these features with survival was analysed (I-II). Preoperative serum samples were collected from patients and age- and sex-matched controls, and concentrations of 13 cytokines and chemokines were analysed (III).

Inflammatory cell reaction and tumour budding at the tumour invasive margin were independent prognostic markers in CRC. In patients with stage I-II disease, high-grade inflammation was associated with better 5-year survival (87.6%) than low-grade inflammation (47.0%). Tumour budding was present in 24.0% of cases and predicted worse 5-year survival (15.4% in contrast to 63.5%). Serum levels of PDGF, IL-6, IL-7, and IL-8 were higher, and levels of MCP-1 were lower in CRC patients compared to controls. A pattern of five most predictive cytokines reached an excellent capacity in discriminating patients from healthy controls and reached an AUC of 0.890 in the ROC analysis. High-stage CRC was associated with increased levels of IL-6, IL-8 and IL-1ra, and metastasised disease was accompanied by an orientation to Th2 cytokine milieu.

According to this study, patients with CRC can be stratified into different prognostic groups by assessing inflammatory cell reaction and tumour budding at the invasive front of the tumour. Evaluation of these features may give additional information for making treatment decisions. Serum cytokine profile was shown to change during cancer progression and seems promising in separating CRC patients from healthy controls, but its clinical value is yet to be confirmed.

Keywords: budding, chemokine, colorectal cancer, cytokine, inflammation, prognosis, tumour immunology

Tiivistelmä

Kasvaimen ennusteeseen vaikuttavien tekijöiden tunteminen on tärkeää syöpäpotilaan yksilöllisen hoidon suunnittelussa. Tässä tutkimuksessa selvitettiin kasvaimen tulehdusreaktion ja kasvaimen reunan silmiulevan kasvutavan merkitystä kolorektaalisyöpäpotilaiden ennustuksessa. Lisäksi tutkimuksessa mitattiin tulehdusreaktion välittäjäaineiden, seerumin sytokiinin, pitoisuksia potilailla ja verrokeilla sekä näiden pitoisuksien vaihtelua suhteessa syövän levinneisyteen.


Kasvaimen tulehdusreaktio ja silmiuleva kasvutapa osoittautuivat itseäsi kännykkä ennustetekijöiksi. Potilailla oli parempi 5-vuotisennuste (87.6 %), jos kasvaimen tulehdusreaktio oli voimakas verrattuna tapauksiin, joissa tulehdusreaktio oli heikko (47.0 %). 24 %:ssa kasvaimista kasvutapa oli silmiuleva, ja näillä 5-vuotisennuste oli huonompi kuin ei-silmiulevissa (15.4 % vs. 63.5 %). Potilailla todettiin korkeampat seerumin PDGF, IL-6, IL-7, ja IL-8 -pitoisuudet ja matalammat MCP-1 -pitoisuudet kuin verrokeilla. Mittaamalla viiden ennustearvoltaan merkittävimmän sytokiinin pitoisuudet voitiin potilaat luotettavasti erottaa verookeista ROC-analyysin avulla, kun ROC-käyrän alle jäävä pinta-ala oli 0.890 %. Pidemmälle levinneissä taudeissa todettiin korkeampia IL-6, IL-8 ja IL-1ra -pitoisuuksia, ja etäpesäkkeissä taudeissa sytokiineistä paikkaan saapui sekä Th2 - että Th1 -piirteitä.

Tutkimuksen mukaan kasvaimen tulehdusreaktion ja silmiulevan kasvutavan arvioinnilla kolorektaalisyöpäpotilaat voidaan jakaa ennustettuihin ryhmäihin, mitä on mahdollista hyödyntää hoidon suunnittelussa. Seerumin sytokiiniprofileihin otettiin huomioon muuttuvan edelleen edetessä, ja sytyköen pitoisuksia poikkeavat kolorektaalisyöpäpotilailla verrattuna terveisiä merkittävissä arvoihin.

Asiasanat: ennuste, kasvaimen immunologia, kasvaimen reunan silmiuleva kasvutapa, kemokiini, kolorektaalisyövä, sytokiini, tulehdus
To Kata, Aino, Eero and Juho
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Oulu, June 2012

Kai Klintrup
## Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis coli gene</td>
</tr>
<tr>
<td>APR</td>
<td>Abdominoperineal resection</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BRAF</td>
<td>B-raf murine sarcoma oncogene</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CIMP</td>
<td>GpC island methylator phenotype</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosomal instability</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESMO</td>
<td>European Society for Medical Oncology</td>
</tr>
<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>GPS</td>
<td>Glasgow Prognostic Score</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary nonpolyposis colon cancer</td>
</tr>
<tr>
<td>HPP</td>
<td>Hyperplastic polyposis</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon gamma-induced protein</td>
</tr>
<tr>
<td>JPS</td>
<td>Juvenile polyposis syndrome</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma oncogene</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>MAP</td>
<td>MUTYH-Associated Polyposis (MAP)</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MLH1</td>
<td>Mutator L homolog 1 gene</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MSH2 / 6</td>
<td>Mutator S homolog 2 and 6 genes</td>
</tr>
<tr>
<td>MSI</td>
<td>Microsatellite instability</td>
</tr>
<tr>
<td>MUTYH</td>
<td>mutY homolog (E. coli)</td>
</tr>
<tr>
<td>NCCN</td>
<td>National Comprehensive Cancer Network</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroid anti-inflammatory drug</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>Platelet derived growth factor, subtype BB</td>
</tr>
<tr>
<td>PIK3 CA</td>
<td>Phosphatidylinositol 3-kinases, catalytic</td>
</tr>
<tr>
<td>PJS</td>
<td>Peutz-Jeghers syndrome</td>
</tr>
<tr>
<td>PMS2</td>
<td>Postmeiotic segregation increased gene</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristics</td>
</tr>
<tr>
<td>SPP</td>
<td>Serrated polyposis</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumour-associated macrophage</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour node metastasis</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour protein p53 gene</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>UICC</td>
<td>Union for International Cancer Control</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
List of original articles

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals.


* Equal contribution.
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1 Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide (Ferlay et al. 2010). In Finland, more than 2,600 new cases are diagnosed annually. The 5-year survival of the CRC varies from more than 90% in local to under 10% in advanced cases, and the most significant predictor of prognosis is TNM staging (Tables 1 and 2) (Puppa et al. 2010). Postoperative adjuvant chemotherapy has shown to be beneficial for patients with TNM stage III disease, and it is a clinical routine in these patients (Moertel et al. 1995). Of the stage II patients, approximately one third to one fourth die of the disease despite complete resection of the disease. Adjuvant chemotherapy might also be beneficial to these high-risk stage II patients. Predictive markers to identify these patients have been studied, but none of these have shown to be valuable so far.

Tumour-associated inflammation has been recognised as a manifestation of the immune response against cancer cells (Hung et al. 1998). The immune cell response has been shown to influence the behaviour of CRC. Jass et al. included tumour-associated inflammation in his classification system and showed that it predicts survival in CRC (Jass et al. 1987) as well as immune cell-related Crohn’s-like reaction (Graham & Appelman 1990). Although inflammatory cell reaction in and around the tumour has been shown to have prognostic significance in CRC, it has not yet been included in practice, which is largely due to a lack of standardised criteria (Deans et al. 1994, Jass et al. 1996).

The nature of the invasive edge of the tumour has also been included in Jass’ classification (Jass 1987), and tumour border configuration has generated considerable interest as an additional prognostic factor of colorectal cancer (Zlobec & Lugli 2009). Invasive front of the tumour is a tumour-host interaction area where tumour progression and tumour cell dissemination ensue (Zlobec & Lugli 2009). Tumour budding is defined as the presence of isolated cell clusters scattered in the stroma at the invasive margin of the tumour (Ueno et al. 2002). The presence of tumour budding has been linked to poor prognosis in colorectal carcinoma (Hase et al. 1993a).

Cytokines are regulators of immune response as they modulate tumour growth and microenvironment by mediating interactions between cancer cells and infiltrating inflammatory cells. Alteration of cytokine profile from Th1 to Th2 subtypes has been suspected to be associated with tumour progression from adenoma and early cancer stage to advanced cancer stage (Contasta et al. 2003, Pellegrini et al. 1996). Increased serum cytokine concentrations, e.g. elevated...
levels of IL-8 (Ueda et al. 1994), IL-6 (Knupfer & Preiss 2010), and platelet-derived growth factor (PDGF) (Belizon et al. 2009) have been reported in CRC patients compared to healthy individuals. Cytokines, e.g. IL-6, have also been shown to have prognostic value (Knupfer & Preiss 2010). It has been established that relative alterations in cytokine levels can have substantial effects on the immune functions (Commins et al. 2010), and it is likely that an analysis of an extensive set of cytokines would provide more accurate information on the tumour-related immunological responses compared to the use of individual cytokines.

The aim of these studies was to investigate the prognostic information of tumour inflammatory cell reaction and tumour budding in colorectal carcinomas by evaluating these features from the 466 CRC specimens collected from patients operated in Oulu University hospital. We also studied the pattern of alterations in the serum cytokine levels in CRC patients compared to age- and sex-matched healthy controls by analysing the levels of 27 cytokines from a series of 148 CRC patients. An additional goal was to see whether the patterns of the serum cytokine levels change according to the stage of disease.
2 Review of literature

2.1 Colorectal cancer

2.1.1 Epidemiology

More than 1.2 million colorectal cancers (CRC) are diagnosed every year worldwide, accounting for approximately 10% of all cancers (Ferlay et al. 2010). Almost 60% of the cases are found in developed countries. Incidence rates are highest in Australia, New Zealand and Western Europe, while the lowest rates are detected in Africa and South-Central Asia. Incidence rates are substantially higher in men than in women (overall sex ratio 1.4:1). CRC is the third most common cancer in men and the second in women worldwide. Every year about 608,000 deaths from CRC are estimated to occur, making it the fourth most common cause of death from cancer (Ferlay et al. 2010).

In Finland, more than 2,600 new cases of CRC are diagnosed annually and more than 1,000 patients dies from it every year (Finnish Cancer Registry 2011). The incidence of CRC is 27.3/100,000 among men and 18.4/100,000 among women. Incidence rates are higher in southern Finland and in cities compared to rural areas (Fig. 1). It has been estimated that by the year 2020, incidence will rise to more than 3,500 new cases yearly. However, in the USA CRC incidence rates have been decreasing for most of the past two decades (from 66.3 cases per 100,000 persons in 1985 to 45.3 in 2007). In contrast, among adults younger than 50 years, colorectal cancer incidence rates have been increasing by 1.6% per year since 1998 in the USA (American Cancer Society 2011).
2.1.2 Aetiology

The aetiology of CRC is multifactorial, consisting of a variety of environmental and hereditary factors. 95% of CRCs are sporadic, and approximately only 5–6% of the cases are considered to develop from genetic predisposition and hereditary gene mutations. Hereditary forms of CRC include hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome), accounting for 2–5% of all CRC cases, familial adenomatous polyposis (FAP), MUTYH-associated polyposis (MAP), Peutz-Jeghers syndrome (PJS) and juvenile polyposis syndrome (JPS). A substantially increased risk of developing cancer is also associated in hyperplastic polyposis/serrated polyposis (HPP/SPP) (Jasperson et al. 2010). In addition, 20–30% of the sporadic CRC cases have a family history of the disease, likely resulting from gene-environment interactions. It has been estimated that individuals who have a first-degree relative with CRC diagnosed after age 50 have a 2–3 fold increased risk of CRC (Jasperson et al. 2010). Patients with ulcerative colitis (UC) and Crohn’s disease also have an increased risk of developing CRC, accounting for 1–2% of all cases (Eaden et al. 2001, von Roon et al. 2007). Estimated prevalence of CRC in any patient with UC is 3.7%, which
increases to 5.4% for those with pancolitis. The cumulative risk for patients with UC to develop CRC is estimated to be 2% at 10 years, 8% at 20 years, and 18% at 30 years after onset of the disease (Eaden et al. 2001).

Risk factors for CRC include increasing age, male sex, previous colonic polyps or previous colorectal cancer. Exogenous factors increasing the risk of developing CRC are high caloric food rich in animal fat, high consumption of red meat and alcohol, smoking and obesity, inadequate intake of fibre, diabetes mellitus and lack of regular physical activity (Cunningham et al. 2010). Migrants moving from low-risk to high-risk areas have shown a rapid increase in CRC incidence supporting the important role of environmental factors in CRC development (Sandler 1996).

2.1.3 Diagnosis

Symptoms of CRC depend of the location and size of the tumour, which can be asymptomatic in the early stages of the disease. Common symptoms include changes in bowel movement habits, e.g. diarrhoea or constipation, changes in the appearance of stools, such as melena or haematochezia, abdominal pain, anaemia and unexplained weight loss. Diagnosis is usually made by endoscopy, which enables histological diagnosis. Diagnosis is not routinely done using radiological methods such as barium contrast colonography or virtual colonoscopy due to a lack of possibility to remove polyps and take histological samples.

Abnormal endoscopic or radiological findings in the colon and rectum must be further examined histologically. Besides adenocarcinoma, other malignant tumours of the colon and rectum include melanoma, lymphomas, sarcomas, endocrine tumours, gastrointestinal stromal tumours and squamous cell carcinoma of the anal canal.

2.1.4 Screening

An early diagnosis of CRC detection is important to reduce cancer-related CRC mortality as it results in removing tumours at an earlier, more curable stage. Tests that have been considered for population screening include variants of the faecal occult blood test, flexible sigmoidoscopy and colonoscopy. The guaiac-based faecal occult blood test is the most extensively studied, but possibly least sensitive screening method. Screening with faecal occult blood test, if offered every two years, has shown to reduce mortality from CRC about 16% (Hewitson
et al. 2008). Sigmoidoscopy can reduce the incidence of and mortality from left-sided colorectal tumours (Gellad & Provenzale 2010). Colonoscopy-based screening has resulted in earlier detection of cancer and decreased incidence and mortality of CRC. However, 2–6% of prevalent cancers are missed by colonoscopy, and evidence suggests that the colonoscopy miss rate may be higher in proximal colon, possibly reducing the effectiveness of colonoscopy as a screening tool (Gellad & Provenzale 2010). Colonoscopy is also a time-consuming and costly method, and is associated with a higher risk of serious complications than are other methods (Pignone et al. 2002). Carcinoembryonic antigen CEA is a widely used biomarker for CRC follow-up, but its sensitivity and specificity in the detection of CRC is low (Duffy 2001, Sturgeon et al. 2008).

In Finland, an organised colorectal cancer-screening programme was started in 2004. The target population included men and women aged 60–69 years. The primary screening tool has been faecal occult blood test, and positive cases have been referred to colonoscopy. Screening has been optional for municipalities and the programme has expanded gradually. In 2010 almost 68,000 individuals were invited for screening, and 76.6% of them participated in it. Among those screened, 4.1% were found to have blood in the stool, and altogether 45 colorectal cancers were found.

2.1.5 Staging

Dukes’ classification is the original staging system for CRC. It was first published in 1932 and was originally devised for rectal cancer (Dukes 1932). It is based on the extent of primary tumour evaluated by the degree of infiltration through the bowel wall and the presence or absence of lymph node involvement. Thereafter, several modifications of Dukes’ classification have been published (Astler & Coller 1954, Kirklin et al. 1949, Turnbull et al. 1967). Dukes’ classification and its modifications have been widely used staging systems for decades. However, Dukes’ staging and its modifications have been widely replaced by TNM staging classification.

TNM staging maintained by the International Union for Cancer Control (UICC) and American Joint Committee on Cancer (AJCC) is today the most widely used classification system in CRC. TNM staging determines the extent of the cancer invasion through the bowel wall (T), the extent of the regional lymph node invasion (N) and the presence of distant metastases (M). UICC-AJCC TNM staging is continuously updated according to a review of existing data, resulting
in new editions over time. The 6th edition of the TNM staging system had some advantages over the previously reported one; it fully stratified the bowel wall involvement and peritoneal serosa and took into account the number of regional lymph node metastases (Sobin et al. 2009). The latest, 7th revision is presented in Tables 1 and 2 (Stephen et al. 2010). It became operational in January 1, 2010. Histological grading is routine practice in pathologic reporting and is used as a prognostic factor. In the most widely used WHO grading system, adenocarcinomas are graded on the basis of the extent of the glandular formation and are divided into well (over 95% with gland formation), moderately (50–95% with gland formation) and poorly differentiated (under 50% gland formation), adenocarcinomas (Hamilton et al. 2010).

Table 1. TNM classification for CRC.

<table>
<thead>
<tr>
<th>Extent of the tumour</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Local invasion (T)</strong></td>
<td></td>
</tr>
<tr>
<td>Primary tumour cannot be assessed</td>
<td>TX</td>
</tr>
<tr>
<td>No evidence of primary tumour</td>
<td>T0</td>
</tr>
<tr>
<td>Carcinoma in situ *</td>
<td>Tis *</td>
</tr>
<tr>
<td>Invasion to submucosa</td>
<td>T1</td>
</tr>
<tr>
<td>Invasion to muscularis propria</td>
<td>T2</td>
</tr>
<tr>
<td>Invasion to subserosa or into nonperitonealised pericolic or perirectal tissues</td>
<td>T3</td>
</tr>
<tr>
<td>Tumour perforates visceral peritoneum</td>
<td>T4a</td>
</tr>
<tr>
<td>Tumour directly invades other organs or structures **</td>
<td>T4b **</td>
</tr>
<tr>
<td><strong>Regional lymph node metastases (N)</strong></td>
<td></td>
</tr>
<tr>
<td>Cannot be assessed</td>
<td>NX</td>
</tr>
<tr>
<td>No</td>
<td>N0</td>
</tr>
<tr>
<td>One</td>
<td>N1a</td>
</tr>
<tr>
<td>Two to three</td>
<td>N1b</td>
</tr>
<tr>
<td>Tumour deposit ***</td>
<td>N1c ***</td>
</tr>
<tr>
<td>Four to six</td>
<td>N2a</td>
</tr>
<tr>
<td>Seven or more</td>
<td>N2b</td>
</tr>
<tr>
<td><strong>Distant metastases (M)</strong></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>M0</td>
</tr>
<tr>
<td>Confined to one organ</td>
<td>M1a</td>
</tr>
<tr>
<td>Metastases in more than one organ or the peritoneum</td>
<td>M1b</td>
</tr>
</tbody>
</table>

* Intraepithelial or invasion of lamina propria with no extension through the muscularis mucosae into the submucosa, ** Tumour that is adherent to other organs, macroscopically, is classified cT4b. If no tumour is present in the adhesion, microscopically, the classification should be pT1–3, depending on the depth of wall invasion, *** Tumour satellites in the subserosa, or in non-peritonealised pericolic or perirectal soft tissue without regional lymph node metastasis, Stephen et al. 2010
Table 2. Staging for CRS and its relationship to Dukes’ classification.

<table>
<thead>
<tr>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Dukes’</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
<td>A</td>
</tr>
<tr>
<td>I</td>
<td>T1</td>
<td>N1</td>
<td>M0</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
<td>A</td>
</tr>
<tr>
<td>IIA</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
</tr>
<tr>
<td>IIB</td>
<td>T4a</td>
<td>N0</td>
<td>M0</td>
<td>B</td>
</tr>
<tr>
<td>IIC</td>
<td>T4b</td>
<td>N0</td>
<td>M0</td>
<td>B*</td>
</tr>
<tr>
<td>IIIA</td>
<td>T1-T2</td>
<td>N1/N1c</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>N2a</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td>IIIB</td>
<td>T3-T4a</td>
<td>N1/N1c</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T2-T3</td>
<td>N2a</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T1-T2</td>
<td>N2b</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td>IIIC</td>
<td>T4a</td>
<td>N2a</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T3-T4a</td>
<td>N2b</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>T4b</td>
<td>N1-N2</td>
<td>M0</td>
<td>C</td>
</tr>
<tr>
<td>IVA</td>
<td>Any T</td>
<td>Any N</td>
<td>M1a</td>
<td>D</td>
</tr>
<tr>
<td>IVB</td>
<td>Any T</td>
<td>Any N</td>
<td>M1b</td>
<td>D</td>
</tr>
</tbody>
</table>

* Dukes’ D if not radically operated

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### 2.1.6 Surgical treatment

Surgery provides a possibility of permanent cure in CRC. The key to successful surgery is complete excision of the tumour proximally and distally with systematic removal of lymphatics en bloc (Hohenberger et al. 2003). The extent of the resection depends on the relationship of the tumour site to the vascular supply and the site of the tumour. Distal and proximal margins are recommended to be 5–10 cm (Nelson et al. 2001). En bloc multivisceral removal is required if tumour infiltration to neighbouring organs is suspected. However, infiltration is demonstrable histologically in only about half of the cases while the remainder are caused by adhesions due to inflammatory process (Hohenberger et al. 2003, Zirngibl et al. 1990). As for colon tumours, standard procedures are right hemicolecctomy, transverse colectomy, left hemicolecctomy or sigmoid resection depending on the local of the tumour. The remaining parts of the bowel are anastomosed together to maintain continuity of the colon. A stoma is required when an anastomosis is not possible, which is often the case in emergency operations, for example (Bass et al. 2009).
Standard operations for low and midrectal cancer are anterior resection or abdominoperineal resection of the rectum. Surgical treatment of rectal cancer is more challenging due to anatomy of pelvic floor. The concept of total mesorectal excision (TME) was introduced in 1979 (Heald 1979). TME excision ensures a specimen with intact mesorectal fascia and thus total removal of lymphovascular fatty tissue surrounding the rectum. Since Heald in 1986 published a recurrence rate of 2.7% after curative anterior resection of rectal cancer, and later the same author and another TME pioneer Enker published low rates of recurrences, total mesorectal excision has become the golden standard of surgery for rectal cancer (Enker 1997, Heald & Ryall 1986). As the mesorectum is totally removed, a distal mucosal margin of at least 1 cm has to be reached (Karanjia et al. 1990, Kuvshinoff et al. 2001, Leo et al. 2009).

Rectal cancer is removed by abdominoperineal resection (APR) when sphincter-saving operation with adequate resection margin is not possible, usually due to low location of the tumour. In traditional APR procedure, worse local recurrence and survival rates have been shown compared to rates after anterior resection, caused by increased occurrence of positive circumferential resection margin (Marr et al. 2005, Nagtegaal et al. 2005). The perineal phase of APR is the most difficult part of operation, often done synchronously with the abdominal phase and with the patient in the supine position. Holm described an alternative technique, the extended posterior perineal approach, in which the perineal part of the operation is done with the patient in the prone jack-knife position, and the levator muscle is resected en bloc with the anal canal and lower rectum. This creates a cylindrical specimen with more tissue covering and surrounding the tumour in low rectal cancer (Holm et al. 2007).

In patients with early rectal cancer, the choice of treatment is transanal local excision, which can be used for grade 1 or 2 T1 tumours that are under 3 cm in diameter and limited to less than 40% of the rectal wall circumference (Sengupta & Tjandra 2001). Local excision should be reserved for patients with low-risk cancers who accept an increased risk for recurrence or patients with poor general condition as a palliative procedure (Bentrem et al. 2005).

A malignant polyp is defined as one with cancer invading into the submucosa (T1), and it can be removed by endoscopic polypectomy (NCCN 2012b). No additional surgery is required if the polyp has been completely resected with favourable histological features including grade 1 or 2, without angiolymphatic invasion and with a negative resection margin (Cooper et al. 1995).
2.1.7 Adjuvant treatment

Adjuvant treatment is administered after primary tumour resection with the aim of reducing the risk of relapse and death. Adjuvant chemotherapy is considered following curative resection of CRCs. Radiation therapy is combined to the treatment of rectal cancer if the risk of locoregional relapse is considered relatively high (Stage II with T3–4 or Stage III). Radiation therapy can be combined to cytostatics (chemoradiotherapy) and can be given either before surgery (as neoadjuvant treatment) or postoperatively.

Bolus fluorouracil and leucovorin has been the standard adjuvant therapy in Stage III colon cancer for over 30 years. It has been shown to reduce mortality by as much as 30% compared to surgery alone (Moertel et al. 1990). Recent data have shown that oxaliplatin-based chemotherapy regimens are superior to fluorouracil alone in adjuvant treatment in Stage III patients (Andre et al. 2009, Kuebler et al. 2007).

The role of adjuvant chemotherapy in Stage II colon cancer is controversial. According to NCCN and ESMO guidelines, adjuvant treatment is not routinely recommended in Stage II disease, but can be considered in patients with high risk of relapse (Labianca et al. 2010, NCCN 2012a). High risk patients with stage II present at least one of the following characteristics: lymph nodes sampling < 12; poorly differentiated tumour; vascular or lymphatic or perineural invasion; tumour presentation with obstruction or tumour perforation and pT4 stage. On the other hand, it has been suggested that MSI+ in the tumour is a positive prognostic factor, and fluorouracil chemotherapy has seemed useless or even detrimental in these patients (Sargent et al. 2010).

Treatment of metastatic Stage IV CRC has proceeded notably during the last decade. However, treatment of metastatic CRC, when not radically resectable, is palliative, and primarily aimed at relieving symptoms and improving the quality of life of the patients. Standard treatment of disseminated metastatic CRC is still based on fluorouracil, which is usually combined with irinotecan or oxaliplatin. Recent advances in the treatment include the antiangiogenic VEGF-inhibitor bevacizumab, and EGFR-inhibitors cetuximab and panitumumab for patients whose tumour has been tested negative for activating K-RAS mutations (Labianca et al. 2010).
2.1.8 Prognosis and prognostic factors

Overall 5-year survival rates of colorectal cancer have been reported to be about 65% and they correspond to disease progression. Survival rates in stage I patients are 80–90%, and 70–85%, 44–80% and under 10% in stage II, stage III and stage IV patients, respectively (O'Connell et al. 2004).

The extent of malignancy at the time of operative treatment is the most powerful predictor of prognosis in CRC (Puppa et al. 2010). The pathological features with the greatest prognostic power are depth of tumour invasion and presence of lymph node or distant metastases defined by UICC-AJCC TNM-classification. The 5-year survival rate for patients with CRC is largely dependent on TNM stage (Zlobec & Lugli 2008). The TNM staging system was initially developed to predict prognosis, but its function is also to aid in the planning of treatment (Quirke et al. 2007).

Histological grading is routine practice in pathologic reporting, in addition to being an important prognostic parameter. Its grading system is based on the percentage of gland formation (Compton 2007). Carcinoembryonic antigen (CEA) has also been recommended for estimating prognosis, but it is more valuable in postoperative surveillance than as a prognostic marker in treatment definition (Sturgeon et al. 2008).

Outcomes of patients with the same CRC stage are very different and many different indicators have been described in predicting recurrences and survival.
(Puppa et al. 2010). Examples of negative histopathological predictive factors include involvement of malignant cells in peritoneal surface (pT4) and lympho-vascular as well as neural invasion of malignant cells. Configuration of the invasive margin, the number of examined lymph nodes, circumferential resection margin (especially for rectal cancers) and satellite tumour deposit are also predictive factors in CRC. In addition, clinical features such as obstruction and perforation of the intestinal wall have been shown to predict poorer prognosis of patients (Benson et al. 2004, Puppa et al. 2010). CRCs operated in emergency have shown to have worse prognosis compared to elective cases (Bass et al. 2009). Operating surgeon has also been reported to belong to independent prognostic factors (Hohenberger et al. 2003).

Several molecular markers have been described to have potential in predicting prognosis. Such markers include microsatellite instability, loss of heterozygosity at 18q21 (LOH), \textit{BRAF-} and \textit{KRAS} mutations (George & Kopetz 2011). MSI has been shown to correlate with favourable prognosis in CRC, and patients with MSI do not seem to benefit from 5FU adjuvant therapy (Sargent et al. 2010). LOH has been suggested to predict poor prognosis in CRC (George & Kopetz 2011). Wild type \textit{KRAS} gene has been shown to be an important predictive factor for choosing therapy for metastatic CRC, but it does not seem to have important prognostic value (Karapetis et al. 2008). \textit{BRAF} gene mutations have shown to be a strong prognostic factor in metastatic CRC conferring a poor prognosis (Van Cutsem et al. 2011).

### 2.2 Development of colorectal cancer

The colorectal mucosa consists of crypt and surface epithelium and supporting stroma (lamina propria). Each crypt contains normal stem cells locating at the crypt base. Stem cells divide asymmetrically, generating a new population of cells that migrate up the crypt, proliferate and differentiate into mature epithelial cells until they undergo apoptosis (Pino & Chung 2010).

The process from normal cell to malignant lesion is slow. Cancer develops from genetic alteration in one cell, causing uncontrolled proliferation of the cell clone. Mutated cell population achieves growth advantage over normal cells and develops favourable intracellular conditions for the development of additional mutations.
2.2.1 Molecular carcinogenesis

At least three distinct pathways of genomic instability have been described in colorectal cancer (Pino & Chung 2010). In 1990, Fearon and Vogelstein proposed a multistep genetic model of colorectal carcinogenesis, known as adenoma-carcinoma sequence, which represents the chromosomal instability (CIN) pathway (Fearon & Vogelstein 1990). The second pathway consists of Lynch syndrome cancers, which present with MSI due to germline mutations in at least one of the MMR genes MLH1, MSH2, MSH6 or PMS2. The third pathway represents sporadic cancers which present with DNA hypermethylation, also known as CpG island methylator phenotype (CIMP) and MSI that is related to the inactivation of MLH1 by its promoter hypermethylation (Snover 2011). Different steps of these pathways are not completely defined and the pathways are thus not mutually exclusive. It is believed that features of multiple pathways can be seen in tumours (Migliore et al. 2011).

It has been calculated that as many as 80 mutated genes are found in colorectal tumours, but less than 15 have been considered to be driving mutations in carcinogenesis (Pino & Chung 2010). CIN is observed in 65%–70% of sporadic colorectal cancers (Pino & Chung 2010). An initial step in CIN pathway is inactivation of adenomatous polyposis coli (APC) gene, followed by additional mutations of KRAS and subsequently mutations in PIK3CA and TP53 and loss of heterozygosity at chromosome 18q (Pino & Chung 2010). CIN results in aneuploidy or loss or gain of chromosomal regions. Somatic APC mutations are seen in 5% of precursor lesions, in 30–70% of adenomas and in 72% of sporadic malignant tumours, indicating that inactivation of APC is an early event in tumour initiation (Pino & Chung 2010). Hereditary mutation of APC is responsible for familial adenomatous polyposis syndrome (Jasperson et al. 2010).

MSI pathway represents genomic instability and is linked to familial hereditary non-polyposis colorectal cancer (HNPCC) syndrome, which is caused by germline mutation in genes involving mismatch repair (MMR) system. The MMR system is necessary for maintaining genetic fidelity by correcting DNA mismatches. Mutations in genes MSH2 and MLH1 account for 90% of HNPCC cases, mutations in MSH6 account for approximately 10%, while mutations in PMS2 are rare (Jasperson et al. 2010). MSI is detected in about 15% of all colorectal cancers; 3% of these are associated with HNPCC syndrome and the other 12% are sporadic (Boland & Goel 2010).
In sporadic CRC, the second most common pathway is the CIMP pathway, accounting for approximately 15% of sporadic cases (Worthley & Leggett 2010). CIMP is often associated with the hypermethylation of the promoter region of the \textit{MLH1} gene and thereby MSI is the common feature in CIMP (Worthley & Leggett 2010). CIMP also associates with the so-called serrated pathway; other early genetic alterations in this pathway include \textit{BRAF} and \textit{KRAS} mutations (Makinen 2007).

\subsection*{2.2.2 Invasive front}

Invasive front of the tumour is a tumour-host interaction area where tumour progression and tumour cell dissemination ensue (Zlobec & Lugli 2009). In malignant tumour spread, several important molecular events, like gain and loss of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation, and initiation of angiogenesis occur at the invasive front (Bryne \textit{et al.} 1998). For example, secretion of matrix metalloproteases (MMPs) facilitates degradation of basement membrane and extracellular matrix, which is a key step in tumour invasion (Rydlova \textit{et al.} 2008). In colorectal cancer, transition of cancer cells at the invasive margin enables them to detach from the main bulk of the tumour and invade the host tissue (Gabbert \textit{et al.} 1985). It has been suggested that molecular and morphological variations of the tumours at the invasive front may reflect prognosis better than variations of cells in the central part of the tumour (Bryne \textit{et al.} 1998). Also histological grade alters between the central part and the invasive front of the tumour, where cells seem to be less differentiated (Puppa \textit{et al.} 2010). It has also been suggested that pro-tumouric (\textit{i.e.}, tumour budding and infiltrating growth pattern) and anti-tumouric (\textit{i.e.}, peritumoural inflammation) factors captured at the invasive front have prognostic value (Zlobec & Lugli 2009).

\subsection*{2.2.3 Tumour budding}

Tumour budding is defined as the presence of isolated cells or small cell clusters scattered into the surrounding host tissue from the invasive margin of the tumour (Ueno \textit{et al.} 2002). The buds appear to drip from the mass of a more differentiated tumour. The buds can be histologically identified by simple haematoxylin-eosin staining (Morodomi \textit{et al.} 1989). In this process cells reduce intercellular and cell-matrix contacts making them capable of invading their surroundings (Prall 2007, 30
The presence of tumour buds has been reported to occur in 20–40% of tumours, occurring predominantly at the invasive front (Zlobec & Lugli 2010). Examples of non-budding and budding margins of the tumour are presented in Figure 3.

Fig. 3. Examples of a CRC with no budding and low-grade peritumoural inflammation (A), peritumoural inflammation (B) and budding margin (C). In (A), the margin of the tumour is smooth, and the number of peritumoural inflammatory cells is very low. In (B), strong peritumoural inflammatory cell infiltrate forms a band-like zone around the tumour, and there is some destruction of cancer tissue, accompanied with tumour infiltrating lymphocytes in this case. In (C), budding can be observed as isolated strands and clusters of cancer cells (arrows) detached from the gland-forming tumour tissue.

Tumour budding and tumour border configuration have generated considerable interest as additional prognostic factors of colorectal cancer (Zlobec & Lugli 2009). It is linked to configuration of tumour invasive edge, a feature which showed prognostic significance in Jass classification (Jass & Morson 1987). Association between tumour budding and lymph node metastases has been demonstrated to correlate with more advanced TNM stage (Zlobec & Lugli 2009). The presence of tumour budding has been linked to poor prognosis in colorectal
carcinoma in clinicopathological studies, and the International Union against Cancer (UICC) recognises tumour budding as an additional prognostic parameter (Compton et al. 2000). Studies assessing the prognostic significance of tumour budding are presented in Table 3.

Table 3. Histopathological studies assessing tumour budding as prognostic factor for CRC. Modified from Prall 2007.

<table>
<thead>
<tr>
<th>Author</th>
<th>N</th>
<th>Site</th>
<th>Stages</th>
<th>Bud strong (%)</th>
<th>Main result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hase et al. 1993b</td>
<td>663</td>
<td>colorectal</td>
<td>I-III</td>
<td>25.6</td>
<td>worse prognosis with bud. strong</td>
</tr>
<tr>
<td>Ueno et al. 2002</td>
<td>638</td>
<td>rectal</td>
<td>I-III</td>
<td>30.1</td>
<td>worse prognosis with bud. strong</td>
</tr>
<tr>
<td>Okuyama et al. 2003a</td>
<td>196</td>
<td>colon</td>
<td>II-III</td>
<td>43.4</td>
<td>worse prognosis with bud. strong</td>
</tr>
<tr>
<td>Prall et al. 2005</td>
<td>186</td>
<td>colorectal</td>
<td>II-III</td>
<td>33.3</td>
<td>worse prognosis with bud. strong</td>
</tr>
<tr>
<td>Nakamura et al. 2005</td>
<td>491</td>
<td>colorectal</td>
<td>I-III</td>
<td>40.1</td>
<td>important for the prediction of metastasis to the lung</td>
</tr>
<tr>
<td>Kanazawa et al. 2008</td>
<td>159</td>
<td>colorectal</td>
<td>II-IV</td>
<td>29</td>
<td>worse prognosis with bud. strong</td>
</tr>
<tr>
<td>Ohtsuki et al. 2008</td>
<td>149</td>
<td>colorectal</td>
<td>II (T2-T4)</td>
<td>12.8</td>
<td>worse prognosis with bud. strong</td>
</tr>
<tr>
<td>Tanaka et al. 2003</td>
<td>138</td>
<td>colon</td>
<td>II (T3)</td>
<td>19.6</td>
<td>worse prognosis with bud. strong</td>
</tr>
</tbody>
</table>

2.3 CRC and inflammation

In 1891 Dr William B Coley, an American surgeon, noticed for the first time that cancer patients healed spontaneously after bacterial infection (Coley 1893). In the late 1950s, Burnet and Thomas introduced the cancer immunosurveillance theory, suggesting that tumour cells provoke an immunological response resulting in a regression of the tumour (Burnet 1957). It was also suggested that the existence of a mechanism to eliminate potentially dangerous mutant cells is evolutionarily necessary, this mechanism being immunological response. It was further suggested that the primary function of immunity is in fact not to promote allograft rejection but rather to protect from neoplastic diseases (Thomas 1959). Today, a large amount of data has been published supporting the original theory of Burnet and Thomas. The immune system has been proved to be capable of recognizing and eliminating tumours, with the lymphocytes and cytokines they produce playing a key role in this process (Dighe et al. 1994, Dunn et al. 2002).
Certain infections and inflammatory diseases increase the risk of developing various types of cancers (Lin & Karin 2007). For example, gastric carcinoma is associated with *Helicobacter pylori* infection, liver cancer with viral chronic hepatitis and cervical cancer with human papilloma virus. For CRC, patients with ulcerative colitis and Crohn’s disease are at higher risk of developing cancer (Lin & Karin 2007). A preventive effect of long-term use of NSAIDs for CRC has been suggested in epidemiological studies (Cooper et al. 2010, Dube et al. 2007, Rothwell et al. 2010) as well as in a randomised placebo-controlled trial in HNPCC (Burn et al. 2011). In addition to infections, individual immunity has a major role in the development of cancers. Follow-up studies have proved that individuals with severe immune deficits have a higher relative risk for the development of cancer (Dunn et al. 2002).

The connection between cancer and inflammation has been suggested to be mediated by two pathways: an intrinsic pathway driven by genetic alterations that cause inflammation and neoplasia, and an extrinsic pathway driven by inflammatory leukocytes and chronic inflammation that increase cancer risk (Erreni et al. 2011). Chronic inflammation, which leads to CRC, is characterised by production of pro-inflammatory cytokines that can induce mutations in oncogenes and tumour suppressor genes and genomic instability with various mechanisms (Terzic et al. 2010). Key features of cancer-related inflammation in CRC are the expression of inflammatory cytokines and chemokines as well as a prominent leukocyte infiltrate and the activation of transcription factors (e.g. NF-κB, STAT3), which all mediate the immune response and oncogenesis (Erreni et al. 2011, Karin 2006).

In cancer, the systemic inflammatory response may reflect a non-specific inflammatory response secondary to tumour hypoxia or necrosis or local tissue damage. The most common measures of systemic inflammatory response are C-reactive protein (CRP), white cell and platelet counts (Roxburgh et al. 2011). Relationships between components of the systemic inflammatory response and cancer-specific survival have been observed, and combinations of such factors have been used to derive inflammation-based prognostic scores. Such scores include the Glasgow Prognostic Score (GPS), originally constructed by Forrest et al. for lung cancer (Forrest et al. 2003), which combines CRP and albumin concentrations. The prognostic value of CRP and GPS has also been adjusted in colorectal cancers, and both have been shown to correlate with survival rates (McMillan et al. 2007, Roxburgh et al. 2011), (Ishizuka et al. 2012). Neutrophil:lymphocyte ratio-based scoring has also been evaluated as a
prognostic marker in CRC. It showed an association with poorer cancer-specific survival, but the score was not independent of tumour stage (Walsh et al. 2005).

### 2.3.1 Tumour-associated leukocytes

Tumour-associated inflammation has been recognised as a manifestation of immune response against cancer cells (Hung et al. 1998). A leukocyte infiltrate is already present in benign adenoma and is markedly increased in CRC (Erreni et al. 2011). The most represented tumour-associated inflammatory cells are macrophages, T-cells, dendritic cells, neutrophils, eosinophils, mast cells and natural killer cells (Erreni et al. 2011). Among these, tumour-associated macrophages (TAM) and T-cells are the most prominent (Balkwill & Mantovani 2001, Mantovani et al. 2002).

Studies that have evaluated the prognostic significance of pronounced generalised inflammatory cell infiltrate in the centre and around the tumour in primary resectable CRCs are presented in Table 4. Thirty-five of these 38 studies reported significant association with improved survival while such association was not seen in only three studies. In 1987, Jass and colleagues published a new classification of rectal cancer based on peritumoural lymphocytic inflammation, number of metastatic lymph nodes, character of invasive margin and local tumour spread (Jass et al. 1987). These features showed an independent prognostic influence and have been subsequently validated by others (Roxburgh & McMillan 2011). So-called Crohn’s-like reaction, described by Graham and Appelman (Graham & Appelman 1990), consisting of discrete lymphoid aggregates at the invasive margin of the tumour, was associated with lymphatic tumour infiltrates, right-sided tumours and improved survival in three studies (Graham & Appelman 1990, Harrison et al. 1994, Adams & Morris 1997).
### Table 4. Studies assessing prognostic value of tumoural inflammatory reaction in primary operable colorectal cancer. Modified from Roxburgh & McMillan 2011.

<table>
<thead>
<tr>
<th>Author</th>
<th>N</th>
<th>Tumours</th>
<th>Assessment</th>
<th>Location</th>
<th>Main result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spratt &amp; Spjut 1967</td>
<td>1137</td>
<td>Stages I–IV</td>
<td>Inflammatory reaction</td>
<td>Peritumoral</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Murray et al. 1975</td>
<td>148</td>
<td>Colonic resections</td>
<td>Inflammatory reaction</td>
<td>Peritumoral</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Zamcheck et al. 1975</td>
<td>40</td>
<td>Colonic resections</td>
<td>Lymphocytic and plasma cell infiltration</td>
<td>Intratumural</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Watt &amp; House 1978</td>
<td>48</td>
<td>Stages II–III</td>
<td>Lymphocyte number</td>
<td>Invasive margin</td>
<td>Higher lymphocyte numbers present in Dukes B compared with Dukes C disease</td>
</tr>
<tr>
<td>House &amp; Watt 1979</td>
<td>107</td>
<td>Stages I–III</td>
<td>Lymphocyte number</td>
<td>Invasive margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Thynne et al. 1980</td>
<td>92</td>
<td>Stages III colon</td>
<td>Immune reaction</td>
<td>Invasive margin</td>
<td>Not associated with survival</td>
</tr>
<tr>
<td>de Mascarel et al. 1981</td>
<td>82</td>
<td>Stages I–III</td>
<td>Lympho-plasmocytic infiltration</td>
<td>Tumour margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Zhou et al. 1983</td>
<td>1226</td>
<td>Stages I–IV</td>
<td>Lymphocytic infiltration</td>
<td>Tumour margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Svennevig et al. 1984</td>
<td>100</td>
<td>Stage II</td>
<td>Mononuclear cells</td>
<td>Peritumoral</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Carlon et al. 1985</td>
<td>124</td>
<td>Stages I–III rectal resections</td>
<td>Lymphocytic infiltration</td>
<td>Tumour margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Jass 1986</td>
<td>447</td>
<td>Rectal resections</td>
<td>Lymphocytic infiltration</td>
<td>Tumour centre</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Jass 1987</td>
<td>379</td>
<td>Rectal resections</td>
<td>Lymphocytic infiltrate</td>
<td>Tumour margin</td>
<td>Stage independent factor in rectal cancer</td>
</tr>
<tr>
<td>Halvorsen &amp; Seim 1987</td>
<td>534</td>
<td>Colorectal resections</td>
<td>Inflammatory cell reaction</td>
<td>Invasive margin</td>
<td>Stage independent predictor of improved survival</td>
</tr>
<tr>
<td>Adachi et al. 1989</td>
<td>117</td>
<td>Colorectal resections</td>
<td>Inflammatory cell reaction</td>
<td>Tumour margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Graham &amp; Appelman 1990</td>
<td>100</td>
<td>Stages I–III</td>
<td>Crohn’s-like reaction</td>
<td>Tumour margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Di Giorgio et al. 1992</td>
<td>361</td>
<td>Stages I–II</td>
<td>Lymphocytic infiltrate</td>
<td>Tumour margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Kubota et al. 1992</td>
<td>100</td>
<td>Stages I–IV (18 stage IV)</td>
<td>Lymphocytic infiltrate</td>
<td>Invasive margin</td>
<td>Stage independent predictor of improved survival</td>
</tr>
<tr>
<td>Harrison et al. 1994</td>
<td>385</td>
<td>Stages I–III rectal cancer</td>
<td>Jass criteria and Crohn’s-like reaction</td>
<td>Invasive margin</td>
<td>Associated with improved survival. Only Crohn’s like reaction independent prognostic factor</td>
</tr>
<tr>
<td>Coca et al. 1997</td>
<td>157</td>
<td>Stages I–III</td>
<td>Jass criteria</td>
<td>Intratumoral</td>
<td>Peritumoral a stage independent prognostic factor</td>
</tr>
<tr>
<td>Cianchi et al. 1997</td>
<td>235</td>
<td>Stages I–III</td>
<td>Jass criteria</td>
<td>Invasive margin</td>
<td>No association with survival</td>
</tr>
<tr>
<td>Author</td>
<td>N</td>
<td>Tumours</td>
<td>Assessment</td>
<td>Location</td>
<td>Main result</td>
</tr>
<tr>
<td>-------------------</td>
<td>----</td>
<td>---------</td>
<td>---------------------------------</td>
<td>---------------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>Adams &amp; Morris 1997</td>
<td>42</td>
<td>Stages I–IV (8 stage IV)</td>
<td>Jass criteria and Crohn’s-like reaction</td>
<td>Invasive margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Ropponen et al. 1997</td>
<td>276</td>
<td>Stages I–IV (67 stage IV)</td>
<td>Tumour infiltrating lymphocytes</td>
<td>Tumour margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Diez et al. 1998</td>
<td>292</td>
<td>Stages II–III</td>
<td>Lymphocytic infiltrate</td>
<td>Tumour margin</td>
<td>Stage independent predictor of improved survival</td>
</tr>
<tr>
<td>Kelly et al. 1999</td>
<td>125</td>
<td>Stages I–IV (22 stage IV)</td>
<td>Jass criteria</td>
<td>Invasive margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Nielsen et al. 1999</td>
<td>584</td>
<td>Stages I–IV (113 stage IV)</td>
<td>Inflammatory cell reaction</td>
<td>Submucosal region</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Murphy et al. 2000</td>
<td>415</td>
<td>Stage II</td>
<td>Jass criteria and Crohn’s-like reaction</td>
<td>Invasive margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Nagtegaal et al. 2001</td>
<td>1416</td>
<td>Stages I–IV rectal cancer</td>
<td>Jass criteria. All cell types in 160 patients</td>
<td>Invasive margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Cianchi et al. 2002</td>
<td>84</td>
<td>Stages i–ii rectal cancer</td>
<td>Lymphocytic infiltrate</td>
<td>Invasive margin</td>
<td>Not associated with survival</td>
</tr>
<tr>
<td>Chiba et al. 2004</td>
<td>371</td>
<td>Stages I–IV (47 stage IV)</td>
<td>Lymphocytic infiltrate.</td>
<td>Invasive margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Buckowicz et al. 2005</td>
<td>120</td>
<td>Stages I–IV HNPPC (43 stage IV)</td>
<td>Lymphoid reaction and Crohn’s-like reaction</td>
<td>Invasive margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Gao et al. 2005</td>
<td>301</td>
<td>Stages I–IV (50 stage IV)</td>
<td>Inflammatory cell reaction</td>
<td>Tumour centre</td>
<td>Associated with improved survival but not in multivariate analysis</td>
</tr>
<tr>
<td>Szyngarewicz et al. 2007</td>
<td>45</td>
<td>Stages II–III rectal cancer</td>
<td>Jass criteria</td>
<td>Invasive margin</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>Forssell et al. 2007</td>
<td>446</td>
<td>Stages I–IV (110 stage IV)</td>
<td>Macrophage and lymphocytic infiltrate</td>
<td>Invasive margin</td>
<td>Macrophage associated with early stage and lymphocytic infiltrate with improved survival</td>
</tr>
<tr>
<td>Roxburgh et al. 2009c</td>
<td>200</td>
<td>Stages I–II</td>
<td>Inflammatory cell reaction and Jass criteria</td>
<td>Invasive margin</td>
<td>Independent prognostic factor even in patients with low risk tumour characteristics</td>
</tr>
<tr>
<td>Roxburgh et al. 2009a</td>
<td>287</td>
<td>Stages I–III</td>
<td>Inflammatory cell reaction and Jass criteria</td>
<td>Invasive margin</td>
<td>Stage independent prognostic factors</td>
</tr>
<tr>
<td>Chang et al. 2009</td>
<td>150</td>
<td>Stages I–IV colon cancer</td>
<td>Infiltrating lymphocytes</td>
<td>Intratumoral</td>
<td>Combination with MSI-H associated with improved survival</td>
</tr>
</tbody>
</table>

Roxburgh & McMillan 2011
Tumour-associated macrophages

TAMs derive from peripheral blood monocytes and are recruited to tumour site by several molecules, such as chemokines, including monocyte chemotactic protein-1 (MCP-1) and RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and colony-stimulating factors (GM-CSF and M-CSF) (Allavena et al. 2008, Mantovani et al. 1992). They differentiate into mature macrophages and have typical biological features; they can polarise into pro-tumorigenic or anti-tumouric macrophages depending on the signals in tumour-microenvironment (Biswas et al. 2008, Gordon 2003, Gordon & Taylor 2005). Macrophages orchestrate the inflammatory cell response and influence tumour cell growth by secreting cytokines and other active mediators. Functioning as anti-tumourigenic factors macrophages are antigen-presenting cells evoking an adaptive immune response. They also have phagocyte function and their presence within tumours has been linked to removal of apoptotic and necrotic tumour cells (Henry et al. 1998, Ohtani et al. 1997). Pro-tumour functions of TAMs include the production of growth factors, angiogenic factors, proteolytic enzymes and cytokines and repression of adaptive immune response (Erreni et al. 2011), (Allavena et al. 2008).

It is generally accepted that inflammatory cell infiltrates in and around the tumour have a positive prognostic influence on colorectal cancer. The evidence is particularly robust for tumour-infiltrating T-cells and TAMs (Roxburgh & McMillan 2011). The prognostic value of TAMs in primary resectable colorectal cancer has been evaluated in 13 studies (Table 5). Clinical studies have also shown a correlation between the numbers of TAMs and adverse tumour behaviour for breast, prostate, ovarian, cervical, endometrial, oesophageal, and bladder cancers (Hanada et al. 2000, Koide et al. 2004, Leek et al. 1999, Lissbrant et al. 2000, Ohno et al. 2004).
Table 5. The prognostic value of tumour-associated macrophages in primary operable colorectal cancer. Modified from Roxburgh & McMillan 2011.

<table>
<thead>
<tr>
<th>Reference</th>
<th>N</th>
<th>Stage</th>
<th>Location 1</th>
<th>Location 2</th>
<th>Main result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagtegaal et al.</td>
<td>160</td>
<td>I-III rectal</td>
<td>Invasive margin.</td>
<td>Intratumoral</td>
<td>Intratumoural macrophages associated with reduced recurrence and improved survival</td>
</tr>
<tr>
<td>2001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oberg et al.</td>
<td>93</td>
<td>III colorectal</td>
<td>Lymph node</td>
<td>metastases</td>
<td>Associated with improved survival. CD68 was not related to survival in patients receiving adjuvant treatment</td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nakayama et al.</td>
<td>30</td>
<td>I-IV</td>
<td>Invasive front.</td>
<td>Tumour stroma</td>
<td>Associated with reduced recurrence at the invasive front</td>
</tr>
<tr>
<td>2002</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Khorana et al.</td>
<td>131</td>
<td>II-III colon</td>
<td>Tumour epithelium.</td>
<td>Tumour stroma</td>
<td>Related to survival but were not independent of other factors on multivariate analysis</td>
</tr>
<tr>
<td>2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Funada et al.</td>
<td>97</td>
<td>I-III colorectal</td>
<td>Invasive margin</td>
<td></td>
<td>Low macrophage infiltration correlated with earlier stage. Patients with both high CD8+ and CD68+ infiltrates had an excellent survival. CD68 alone not related to survival</td>
</tr>
<tr>
<td>2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lackner et al.</td>
<td>70</td>
<td>II-III colorectal</td>
<td>Tumour margin.</td>
<td>Tumour centre</td>
<td>Independently related to survival at the invasive margin, not in the tumour centre</td>
</tr>
<tr>
<td>2004</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baeten et al.</td>
<td>117</td>
<td>I-IV colorectal</td>
<td>Peritumoural.</td>
<td>Tumour stroma</td>
<td>No survival relationships seen in any area</td>
</tr>
<tr>
<td>2006</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoue et al.</td>
<td>22</td>
<td>I-III colorectal</td>
<td>Tumour stroma</td>
<td></td>
<td>No significant relationship between density and survival</td>
</tr>
<tr>
<td>2005</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tan et al.</td>
<td>60</td>
<td>I-IV colorectal</td>
<td>Intratumoural</td>
<td></td>
<td>Low infiltration related to higher T and N stage and distant metastases. Associated with improved survival.</td>
</tr>
<tr>
<td>2005</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forsell et al.</td>
<td>446</td>
<td>I-IV colorectal</td>
<td>Invasive margin</td>
<td></td>
<td>Correlated with early stage and peritumoural lymphocytic infiltrate. An independent prognostic factor</td>
</tr>
<tr>
<td>2007</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nagorsen et al.</td>
<td>40</td>
<td>I-IV colorectal</td>
<td>Tumour stroma.</td>
<td>Tumour epithelium</td>
<td>Stromal CD163 associated with improved survival. CD68 counts not related to survival</td>
</tr>
<tr>
<td>2007</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zhou et al.</td>
<td>160</td>
<td>III-IV colon</td>
<td>Invasive margin</td>
<td></td>
<td>Related to tumour stage and presence of metastases. Independent prognostic factor for improved survival</td>
</tr>
<tr>
<td>2010a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Algars et al.</td>
<td>159</td>
<td>II-IV colorectal</td>
<td>Invasive margin.</td>
<td>Intratumoral</td>
<td>Associated with improved survival</td>
</tr>
<tr>
<td>2011</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Roxburgh & McMillan 2011
2.3.2 Cytokines

Cytokines are immunomodulating proteins which carry signals between cells and participate in cell-cell communication, and thus have an effect on other cells. Their role in immune response is critical and they can mediate both stimulatory and inhibitory signals between cells (Commins et al. 2010). As regulators of growth and differentiation of cells, cytokines can either stimulate or inhibit tumour growth and progression by mediating the interactions between cancer cells and infiltrating inflammatory cells, thus influencing the tumour microenvironment (Mantovani et al. 2008) (fig. 4). It has been suggested that immune response can shift from anti-tumouric to pro-tumouric environment during the development of cancer (Dunn et al. 2004).

![Diagram of cytokine interactions in tumour microenvironment](https://via.placeholder.com/150)

Fig. 4. Cytokines secreted by tumour and inflammatory cells can either promote or suppress tumour growth. The diagram shows two outcomes of interactions between tumour cells and inflammatory cells in the tumour microenvironment.

More than 200 cytokines belong to cytokine families and they can be classified into categories Th1 (T-helper)-, Th2- and Th17 cytokines, pro- and anti-inflammatory cytokines, and on the basis of their general properties into chemokines, growth factors and interferons (Table 6). Cytokines are secreted by cells of the immune system or by tumour cells, and subsequently by additional immune cells recruited to the area of inflammation, thus increasingly activating
the inflammatory response. Cytokines can act on many cell types causing different effects, and the same effect may be induced by multiple different cytokines (Callard et al. 1999).

Table 6. Functions and targets of selected cytokines.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>T helper</th>
<th>Function</th>
<th>The main functional target</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1ra</td>
<td>A</td>
<td>Anti-inflammatory</td>
<td>IL-1 antagonist</td>
</tr>
<tr>
<td>IL-4</td>
<td>Th2</td>
<td>Anti-inflammatory</td>
<td>B-and Th2 cell growth and differentiation</td>
</tr>
<tr>
<td>IL-6</td>
<td>Th2</td>
<td>Pro-inflammatory</td>
<td>B- and T-cell activation</td>
</tr>
<tr>
<td>IL-7</td>
<td></td>
<td>Growth factor</td>
<td>Leucocyte differentiation</td>
</tr>
<tr>
<td>IL-8 (CXCL8)</td>
<td></td>
<td>Chemokine</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>IL-9</td>
<td></td>
<td>Growth factor</td>
<td>Leukocyte differentiation</td>
</tr>
<tr>
<td>IL-12</td>
<td>Th1</td>
<td>Pro-inflammatory</td>
<td>Th1 cells differentiation, IFN-γ production</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Th1</td>
<td>Pro-inflammatory</td>
<td>Macrophage activation, Th2 cell growth suppression</td>
</tr>
<tr>
<td>Eotaxin (CCL11)</td>
<td></td>
<td>Chemokine</td>
<td>Th2 cells, eosinophils</td>
</tr>
<tr>
<td>IP-10 (CXCL10)</td>
<td></td>
<td>Chemokine</td>
<td>Th1- and NK cells</td>
</tr>
<tr>
<td>MCP-1 (CCL2)</td>
<td></td>
<td>Chemokine</td>
<td>T-cells, monocytes</td>
</tr>
<tr>
<td>MIP-1β (CCL4)</td>
<td></td>
<td>Chemokine</td>
<td>Th-1 cells, monocytes</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td></td>
<td>Growth factor</td>
<td>Angiogenic</td>
</tr>
</tbody>
</table>

T-helper cells are classified into Th1-, Th2- and Th17 cells according to the cytokines they produce (Ellyard et al. 2007). Th1 cytokines promote cellular immune reactions and are thought to be essential for response against tumour cells (Zamarron & Chen 2011). Th-2 cytokines are regulators of humoral immunity and they have been suggested to have a role in suppressing tumour-specific immune response (Cui & Florholmen 2008, Ellyard et al. 2007). Alteration of cytokine profile from Th1 to Th2 subtypes is suspected to be associated with tumour progression from adenoma and early cancer stage to advanced cancer stage (Contasta et al. 2003, Pellegrini et al. 1996). Th17 cells produce IL-17 family pro-inflammatory cytokines and chemokines, but it is not clear whether Th17 cells promote or inhibit tumour progression (Ji & Zhang 2010).

Pro-inflammatory cytokines are produced in the early stage of the immune response and are important in being involved in both innate and acquired immunity. Macrophages are the major source of these cytokines, and pro-inflammatory IL-6 has been demonstrated to promote the growth of colon cancer cells, increase invasiveness and to have angiogenic properties (Knupfer & Preiss
Anti-inflammatory cytokines are negative regulators of inflammation, i.e., they inhibit inflammatory reactions.

Chemokines are chemoattractant cytokines that recruit leukocytes from the circulatory system to sites of injury. Chemokines have been regarded as potential regulators of tumour cell transformation, growth, angiogenesis and metastases and they promote inflammatory microenvironment in CRC (Wang et al. 2009a). On the other hand, chemokines can also suppress tumour growth through promoting antitumour immunity. The levels of pro-inflammatory chemokines such as MCP-1 and pro-angiogenic IL-8 have shown to be elevated in colon tumours compared to normal tissue, indicating their role in tumour progression (Baier et al. 2005). Cytokines such as IL-7, IL-9 and PDGF-BB can act as growth factors. Their functions include regulation of growth and differentiation of inflammatory and platelet cells and angiogenesis (Belizon et al. 2009, Knoops & Renauld 2004, Mackall et al. 2011).

The standard of measuring serum cytokines has been enzyme-linked immunosorbent assays (ELISAs), which are the most widely used and best validated methods, but are limited by their ability to measure only a single protein in each sample. Recent developments in serum cytokine quantification technology include multiplex arrays which detect simultaneously large numbers of cytokines in a single sample and provide investigators with opportunities to address the complexity of inflammatory responses (Zhou et al. 2010b).
3 Aims of the study

These studies were focused on inflammatory reaction and the morphology of colorectal cancer tumours and their influence on patients’ survival. We also evaluated serum cytokine levels in colorectal cancer patients, comparing the levels with healthy controls, and evaluated cytokine profile enhancement in different stages of the disease. The specific aims were:

1. To assess the inflammatory cell reaction at the invasive front and the central area of the tumour of colorectal cancer and its influence on survival.
2. To investigate the association of tumour budding with survival of colorectal cancer patients.
3. To evaluate the alterations in the serum cytokine pattern in CRC patients compared to healthy controls.
4 Materials and methods

4.1.1 Case material

The patients in these studies have been treated in Oulu University Hospital. The material of the studies I and II consists of a consecutive series of 466 colorectal cancer patients operated between the years 1986 and 1996. Complete follow-up could be obtained from 386 patients who were included in the studies. The patients were followed up for 60 months or until their death (mean 41 months). Study III was prospectively introduced to all newly diagnosed colorectal cancer patients operated between the years 2006 and 2010 (N = 344), of which a total of 156 patients signed an informed consent to participate. Control serum samples were obtained from age- and sex-matched healthy voluntary blood donors (Finnish Red Cross, Oulu, Finland; N = 36, age < 65 years) and from cataract surgery patients (Oulu University Hospital, N = 50, age ≥ 65 years). Serum samples were collected in the morning. Samples from the patients were collected prior to surgery and after 12-hr fast. All samples were centrifuged and serum stored at -70°C until further analysis. Eight patients diagnosed with other cancer diseases were excluded. Other exclusion criteria for blood donor controls included e.g. acute infections or severe chronic diseases such as asthma and coronary artery disease according to the regulations used in blood donation. To avoid confounding and to model the situation before the diagnosis of the disease, 32 patients who received preoperative radiotherapy were excluded from study III; the total number of cases for study III was 116.

Medical histories and clinical details were reviewed from clinical records and the outcome of the patients from the cancer registry (The Finnish Cancer Registry). All the studies were approved by the Ethical Committee of Oulu University Hospital.

4.1.2 Clinical and histopathological characteristics

Clinical and histopathological characteristics of the patients in the study material are presented in Table 7.
Table 7. Clinicopathological characteristics of the patients.

<table>
<thead>
<tr>
<th>Clinical variables</th>
<th>Study I N = 374</th>
<th>Study II N = 486 (%)</th>
<th>Study III N = 116 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean / SD</td>
<td>67 / 13</td>
<td>67.9 / 11.2</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>179 (47.9)</td>
<td>220 (47.2)</td>
<td>58 (50.0)</td>
</tr>
<tr>
<td>Female</td>
<td>195 (52.1)</td>
<td>246 (52.8)</td>
<td>58 (50.0)</td>
</tr>
<tr>
<td>Tumour location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal colon</td>
<td>119 (31.8)</td>
<td>147 (31.6)</td>
<td>49 (42.2)</td>
</tr>
<tr>
<td>Distal colon</td>
<td>118 (31.6)</td>
<td>133 (28.5)</td>
<td>27 (23.3)</td>
</tr>
<tr>
<td>Rectum</td>
<td>137 (36.6)</td>
<td>186 (39.9)</td>
<td>40 (34.5)</td>
</tr>
<tr>
<td>WHO grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>87 (23.3)</td>
<td>106 (22.7)</td>
<td>21 (14.2)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>217 (58.0)</td>
<td>276 (59.2)</td>
<td>108 (73.0)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>70 (18.7)</td>
<td>84 (18.0)</td>
<td>19 (12.8)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>84 (22.5)</td>
<td>98 (21.0)</td>
<td>27 (18.4)</td>
</tr>
<tr>
<td>Stage II</td>
<td>145 (38.8)</td>
<td>188 (40.3)</td>
<td>55 (37.4)</td>
</tr>
<tr>
<td>Stage III</td>
<td>87 (23.3)</td>
<td>109 (23.4)</td>
<td>46 (31.3)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>58 (15.5)</td>
<td>71 (15.2)</td>
<td>19 (12.9)</td>
</tr>
</tbody>
</table>

Tumours were staged according to Turnbull’s modified Dukes’ system (I-II) and the 6th edition of TNM-staging (III) and were graded as well, moderate and poorly differentiated according to WHO classification criteria. All histological specimens of the tumours were independently re-evaluated by two pathologists for Dukes’ and WHO histological grading; in cases of disagreement, classification was set up after discussion.

4.1.3 Immunohistochemistry (II)

Five-μm thick sections were cut from the paraffin-embedded tissue blocks. After deparaffinisation and rehydration in graded alcohol series, sections were rinsed with phosphate-buffered saline (PBS) and endogenous activity was blocked. Incubation of the anticytokeratin antibody MNF116 (Dako, Copenhagen, Denmark) was carried out at a dilution of 1:100 for one hour at room temperature. After rinses, diaminobenzidine (DAB) chromogen solution and a substrate buffer (1:200) were used for ten minutes to visualise the immunoreaction.
4.1.4 Inflammatory reaction (I)

Inflammatory cell reaction was estimated at the central part and at the invasive margin of the tumour. Invasive margin was defined as an interface between the host tissues and the invading edge area of a tumour. For estimation, areas of deepest invasion were selected. We used a four-degree scale to assess the overall inflammatory reaction and the amount of lymphoid cells, neutrophilic and eosinophilic granulocytes. A score of 0 indicated an absence of reaction, 1 weak, 2 moderate and 3 severe increase of each cell type. A score of 0 was given when there was no increase of inflammatory cells. A score of 1 denoted a mild and patchy increase of inflammatory cells, but no destruction of invading cancer cell islets. A score of 2 was given when inflammatory cells formed a band-like infiltrate with some destruction of cancer cell islets, while score of 3 denoted a very prominent inflammatory reaction, forming a cup-like zone at the invasive margin, with frequent destruction of cancer cell islets.

Macrophage reaction was graded as absent (grade 0) or present (grade 1). Reaction was graded as 1 when collections of foamy macrophages encircling invading tumour islets were observed and 0 when such collections were not observed.

4.1.5 Crohn’s-like reaction (I)

Crohn’s-like reaction was evaluated according to the criteria established by Graham and Appelman (Graham & Appelman 1990). Since we did not find any differences between mild and intense reaction patterns in the preliminary evaluation, we combined these and graded Crohn’s-like reaction as negative or positive.

4.1.6 MSI testing (I)

DNA microsatellitility instability was evaluated from a total of 99 cases with five National Cancer Institute consensus markers. These markers include BAT 25, BAT 26, D2S123, D5S436, and D17S250 (Boland et al. 1998). A tumour was classified as MSI-high when MSI was detected with two or more markers, MSI-L when only one marker showed MSI and MSS if none of the markers showed instability.
4.1.7 Budding (II)

Tumour budding was considered to be present when narrow strands or clusters of cancer cells of one to three cells in width were observed extending beyond the tumour margin and where this finding appeared to be unrelated to glandular disruption associated with inflammatory cell infiltration. The method is derived from the work of Ueno (Ueno et al. 2002), but simplified according to their suggestion of using a two-tiered system. Furthermore, we attempted to take into account the peritumoural inflammatory reaction, which is why cases with isolated cancer cell clusters, resulting from inflammation-induced glandular disruption, were not classified as representing ‘true’ budding. In a randomly selected subset of 53 cases, the presence of cancer cell clusters was also assessed by immunohistochemistry using the pan-cytokeratin antibody MNF116. In each case, all slides containing cancer tissue were evaluated for budding.

4.1.8 Assessment of intra- and interobserver variation

We randomly selected a total of 39 cases in study I and 25 cases in study II to assess the intra- and interobserver variation in scoring of inflammatory reaction and budding. Six observers in study I evaluated the inflammatory reaction and four observers in study II the budding margin independently and without knowing the clinicopathological information.

4.2 Measurement of serum cytokine levels (III)

Serum cytokine concentrations were analysed by Bio-Plex Pro Human pre-manufactured 27-Plex Cytokine Panel (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s instruction. The pre-manufactured cytokine panel included 27 cytokines: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IFN-γ, tumour necrosis factor-alpha (TNF-α), Interferon gamma-induced protein (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted; RANTES), Eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), fibroblast growth factor (FGF) basic, platelet-derived growth factor, subtype BB (PDGF-BB), and vascular endothelial growth factor A (VEGF-A).
Out of the 27 analytes, 13 cytokines with three or fewer missing values were within the assay working ranges and were included in the study. BioPlex Manager Software 4.1 was utilised in calculating the concentrations.

4.3 Statistical analyses

The SPSS program (version 12.0, SPSS, IL, USA) was used in studies I and II and PASW Statistics 18 (IBM, Chicago, IL, USA) in study III for statistical analysis. For categorical data, cross-tabs with Pearson’s Chi-square test, and for the evaluation of survival statistics, Kaplan-Meier and Cox regression models were used. Mann-Whitney U-test or Kruskal-Wallis test were used for discontinuous variables that were not normally distributed. The predictive power of cytokine profile in discriminating patients from the controls was evaluated by receiver operating characteristics (ROC) analysis, and the goodness-of-fit of the model was tested with the Hosmer-Lemeshow statistic. In all tests, a p-value of less than 0.05 was considered to be statistically significant.
5 Results

5.1 General aspects concerning histopathological and serum analyses

In the histological analysis of the sections obtained from tumour samples the amount of inflammation and tumour budding correlated with disease outcome in colorectal cancer (studies I-II). In serum analysis from patients with and without colorectal cancer a specific cytokine pattern predicted the presence of colorectal cancer. The main results of the studies are presented in Table 8.

Table 8. The main results of the studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Study design</th>
<th>Material</th>
<th>Method</th>
<th>Main result</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Retrospective</td>
<td>Paraffin-embedded tissue material from patients with colorectal cancer</td>
<td>Histological analysis of routine light microscopic sections</td>
<td>Low grade inflammation at invasive margin predicts unfavourable outcome</td>
</tr>
<tr>
<td>II</td>
<td>Retrospective</td>
<td>Paraffin-embedded tissue material from patients with colorectal cancer</td>
<td>Histological analysis of routine light microscopic sections</td>
<td>Tumour budding at invasive margin predicts unfavourable outcome</td>
</tr>
<tr>
<td>III</td>
<td>Prospective</td>
<td>Serum samples from patients with colorectal cancer and healthy controls</td>
<td>Analysis of serum samples</td>
<td>High levels of cytokines IL-8 and IL-6 and low levels of cytokines MCP-1, IL-1ra and IP-10 discriminate colorectal cancer patients from healthy controls</td>
</tr>
</tbody>
</table>

5.2 Inflammation and colorectal cancer

5.2.1 Inflammatory cells in tumour bulk and invasive margin

Analyses to determine the prognostic significance of inflammation were focused on stage I and II tumours (N = 229), as cases in stages III and IV represent advanced disease, where the stage is the major determinant of the prognosis.

For the analysis, invading margin was evaluated in 374 out of 386 cases since in 12 cases the invasive margin was not included in the sections and the cases were excluded from further analysis.
The distribution of inflammatory reaction scores and estimates of the amounts of different inflammatory cell types in the central area and at the invasive margin of stage I-II cancers are presented in Table 3 in study I. The overall inflammatory reaction scores and the amounts of neutrophilic granulocytes, lymphocytes and macrophages were higher at the invasive margin than in the central part of the tumour, but this difference was not seen in eosinophilic granulocytes (see Table 3 in study I). The same trend was also seen in stage III and IV tumours (data not shown in the study report). The prevalence of high-grade inflammation did not differ statistically significantly between right-sided and left-sided or between colon and rectal cancers.

5.2.2 Inflammation and MSI

Some of the patients from the material (N = 99) were tested for MSI with five NIH consensus markers (Boland et al. 1998). High-level MSI was seen in 10% (N = 11/99) and low-level MSI in 18.2% (N = 18) of the cases, and 70.7% (N = 70) were microsatellite stable. High-grade inflammation was observed in 50.0% of both MSS and MSI-L cancers and in 72.7% of MSI-H cancers. The higher percentage of high-grade inflammation in MSI-H cancers did not differ statistically from other cancers regardless of whether MSS and MSI-L cancers were analysed separately or together (P = 0.370 and 0.206, respectively). Differences were not seen in the presence of Crohn’s-like reaction in regard to MSI, either. Crohn’s-like reaction was seen in 31.4% of MSS tumours, in 23.5% of MSI-L tumours and in 45.5% of MSI-H tumours (P = 0.507, Fisher’s exact test).

5.2.3 Inflammatory cell reaction and survival

Univariate analysis

In order to make the classification system more reproducible and easier to analyse we reduced the original four-point scale to a two-point scale: absent to mild reaction were combined as low-grade inflammation and moderate to strong inflammation as high-grade inflammation.

The inflammatory score showed an association with the 5-year survival and recurrence-free survival (Fig. 5) High-grade inflammation at the invasive margin
predicted good outcome. A positive correlation was evident for the overall inflammatory, neutrophilic granulocyte and lymphocyte grades and for presence of macrophages (Table 9).

Fig. 5. The relationship between overall inflammatory cell reaction grades at the invasive front and survival and recurrence-free survival in Dukes’ A and B cancers (Kaplan-Meier).
Table 9. Relationship of intensity of inflammatory reaction in the central part or invasive margin and 5-year survival rates in patients with Dukes’ A or B CRC.

<table>
<thead>
<tr>
<th>Inflammatory cell type</th>
<th>Central region</th>
<th>Invasive margin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-year survival</td>
<td>Log-rank</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>N</td>
</tr>
<tr>
<td>Inflammation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>65.9</td>
<td>166</td>
</tr>
<tr>
<td>High grade</td>
<td>77.9</td>
<td>62</td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>66.9</td>
<td>165</td>
</tr>
<tr>
<td>High grade</td>
<td>75.6</td>
<td>63</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>67.9</td>
<td>207</td>
</tr>
<tr>
<td>High grade</td>
<td>81.5</td>
<td>21</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>62.3</td>
<td>152</td>
</tr>
<tr>
<td>High grade</td>
<td>85.0</td>
<td>76</td>
</tr>
<tr>
<td>Macrophages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>70.4</td>
<td>139</td>
</tr>
<tr>
<td>High grade</td>
<td>67.8</td>
<td>89</td>
</tr>
</tbody>
</table>

There was also a trend to positive correlation with the eosinophilic granulocyte grade, but the effect was not statistically significant (P = 0.078). In the central part of the tumour, only high overall inflammation and lymphocyte grade were significantly associated with survival rate, both indicating a good 5-year survival (Table 9).

Since positive macrophage and overall inflammatory reaction had a strong correlation (c = 0.465, Spearman’s correlation, P < 0.0001), we excluded cases with positive macrophage reaction. In macrophage-negative cases, no correlation was seen in survival with other inflammatory cells. When the cases with high neutrophilic granulocyte, eosinophilic granulocyte or lymphocyte grades were excluded one at a time, macrophage reaction invariably demonstrated a prognostic significance.

Crohn’s-like reaction was present in 113 out of 371 cases (30.5%) and it was more prevalent in cases with high-grade inflammation at the invasive margin. Crohn’s-like reaction did not show any prognostic significance when all cases were analysed (stages I-IV). When cases with low-grade and high-grade overall inflammation at the invasive margin were analysed separately, Crohn’s-like
reaction predicted good prognosis in cases with low-grade inflammation (\( P = 0.025 \), Chi-square)

**Multivariate analysis**

To test the independent prognostic significance of inflammatory reaction against known prognostic factors, Cox proportional hazards model with forward selection method was used. High-grade inflammation at the invasive margin was an independent prognostic factor for both 5-year survival and 5-year recurrence-free survival, and it was an even better prognostic factor than Dukes’ stage when stage IV cases were excluded from the analysis (Table 10). WHO histological grade, gender, mucinous subtype or tumour size did not show prognostic significance (data not shown in study report). When the analysis was limited to stage I and stage II cases, high-grade inflammation at the invasive margin showed independent, positive prognostic influence on both survival and recurrence-free survival (Risk ratio 5.6, CI 2.8–10.9, \( P < 0.00005 \)) while Dukes’ stage, WHO grade, mucinous subtype, gender, or tumour location did not show any prognostic significance.

**Table 10. Assessment of independent prognostic significance of inflammatory reaction in curatively operated Dukes’ stage A-C cancers in relation to Dukes’ stage and location of the tumour in terms of 5-year survival.**

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>Risk ratio</th>
<th>95% Confidence interval</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>3.4790</td>
<td>2.0964–5.8333</td>
<td>&lt; 0.00005</td>
</tr>
<tr>
<td>Dukes’ stage</td>
<td></td>
<td></td>
<td>0.0079</td>
</tr>
<tr>
<td>A vs. B *</td>
<td>1.5867</td>
<td>0.8255–3.0498</td>
<td>0.1661</td>
</tr>
<tr>
<td>A vs. C</td>
<td>2.6425</td>
<td>1.3618–5.1279</td>
<td>0.0041</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td>0.0180</td>
</tr>
<tr>
<td>Proximal vs. distal colon *</td>
<td>1.1236</td>
<td>0.5979–2.1116</td>
<td>0.7173</td>
</tr>
<tr>
<td>Proximal colon vs. rectum</td>
<td>1.9662</td>
<td>1.1372–3.3993</td>
<td>0.0155</td>
</tr>
</tbody>
</table>

Explanations: * = no independent prognostic significance.

Cox stepwise regression analysis
5.3 Tumour budding and colorectal cancer

5.3.1 Budding margin and Dukes’ stage

Budding margin was observed in 112 (24%) of the cases and contour of the invasive margin was sharply invasive in 315 (67.6%). Sharply infiltrative margin was more frequent in the more advanced cancers. This was observed in 35.7% of Dukes’ A, 70.7% of Dukes’ B, 76.1% of Dukes’ C and 90.1% of Dukes’ D cancers (P < 0.0001, Chi-square). The occurrence of budding also increased with Dukes’ stage, but with less prevalence. Budding was observed in 9.2% of Dukes’ A cases and 19.1%, 30.3% and 47.9% in Dukes’ stages B, C and D, respectively (P < 0.0001).

5.3.2 Budding margin and pan-cytokeratin staining

In addition to H&E staining, the presence of a budding margin was also analysed with pan-cytokeratin MNF116 staining in 53 cases. Budding was observed in 28.3% of the cases in H&E stained slices and in 45.1% of the cases by MNF116. A clear association was found with the findings of H&E stained slices and pan-cytokeratin immunohistochemistry. 86.1% of the cases with a budding margin had an irregular budding contour on MNF116 staining, and in 72.1% of the cases with a non-budding margin, the margin in MMF116 staining had a pushing border without budding (P = 0.039; McNemar). In 18.9% of the cases, MNF116 staining showed some budding cells not detected by H&E, but the use of MNF116 did not improve survival statistic (data not shown in study report).

5.3.3 Tumour budding and survival

The relationship between 5-year survival and recurrence-free survival statistics and degree of budding was analysed. Tumour budding was a strong indicator of poor prognosis. 5-year survival was 15.4% in tumours with budding and 63.5% in tumours without budding regardless of the stage (Figure 6). Similar results were also found in both colon (15.0% vs. 70.3%; P < 0.00001) and rectal cancers (16.2% vs. 54.1%; P < 0.00005). In stage I cancers, 5-year survival was only 29.2% in the presence of budding, compared to 82.2% without budding (P = 0.009; log-rank). In stage II cancers, 5-year survival was 29.7% and 72.3%, respectively (P < 0.00001). In stage III cancers 5-year survival rates were not
significantly different (17.6% vs. 41.7%, P = 0.096; log-rank), but in stage IV cancers no patients with budding survived to 5 years, whereas 27.6% of the cases without budding survived (P < 0.0002; log-rank). In stage I and stage II tumours, recurrences were more frequent if budding was present (Stage I: 66.7% vs. 15.6%; P = 0.002; Stage II: 55.2% vs. 23.6%; P = 0.001, Fisher’s exact test).

The sharply invasive contour of the tumour was also an indicator of worse prognosis with a 5-year survival rate of 41.5%, whereas patients with a pushing contour of the tumour had a 5-year survival rate of 73.1% (P < 0.00005; log-rank).

In Cox-regression analysis (including TNM stage, WHO grading, gender, budding, contour of the tumour, localisation and size of the tumour and mucin production), tumour stage was the most significant prognostic factor in survival if patients with stage IV cancers were included (see Table 5 in study II, P < 0.0005,
Cox-regression analysis), and tumour budding was also an independent prognostic factor (P < 0.0005). When stage cancers IV were excluded from the analysis, tumour budding was a better prognostic factor than stage (Table 11). In both analyses, WHO grading, localisation of the tumour, size and mucin production of the tumour did not show significant influence in prognosis.

Table 11. Assessment of independent prognostic significance of budding margin in curatively operated Dukes’ stage A-C cancers in relation to Dukes’ stage and location of the tumour in terms of 5-year survival

<table>
<thead>
<tr>
<th>Prognostic factor</th>
<th>Risk ratio</th>
<th>95% Confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Budding</td>
<td>2.593</td>
<td>1.672–4.020</td>
<td>&lt; 0.0005</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Proximal vs. distal colon</td>
<td>1.579</td>
<td>0.867–2.876</td>
<td>0.135</td>
</tr>
<tr>
<td>Proximal colon vs. rectum</td>
<td>2.420</td>
<td>1.437–4.076</td>
<td>0.001</td>
</tr>
<tr>
<td>Dukes’ stage</td>
<td></td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>A vs. B</td>
<td>1.871</td>
<td>1.025–3.416</td>
<td>0.041</td>
</tr>
<tr>
<td>A vs. C</td>
<td>3.549</td>
<td>1.937–6.505</td>
<td>&lt; 0.0005</td>
</tr>
</tbody>
</table>

Cox stepwise regression analysis.

When colon and rectal cancers were analysed separately, tumour budding was an independent prognostic factor in both colon (risk ratio 3.7; P < 0.0005, 95% CI: 2.253–5.980) and rectal cancers (risk ratio 1.8; P = 0.031, 95% CI: 1.056–3.224). When stage IV patients were excluded from the analysis, tumour budding was still an independent prognostic factor in both colon (risk ratio 4.198; P < 0.0005, 95% CI: 2.281–7.726) and rectal cancers (risk ratio 2.098; P = 0.016, 95% CI: 1.149–3.831).

Inflammatory reaction showed to be a better prognostic factor (Risk ratio 4.4, CI 2.7–7.3, P < 0.00001) than budding (Risk ratio 2.6, CI 1.7–4.1, P < 0.00001) in a Cox regression analysis in which the other prognostic factors mentioned above were also included (data not shown).
5.4 Cytokine levels and colorectal cancer

5.4.1 Univariate analysis

**General aspects of analysis**

The preoperative serum cytokine levels of 116 CRC patients and cytokine levels of 86 healthy controls were evaluated. Higher levels of cytokines PDGF-BB ($P = 0.041$), IL-6 ($P = 1.3E-7$), IL-7 ($P = 2.9E-4$) and IL-8 ($P = 7.3E-11$) and lower levels of MCP-1 ($P = 0.002$) were observed in CRC patients compared to controls. The levels of Th1 cytokines IL-12 and IFN-$\gamma$ also showed a tendency towards increased values in CRC, but the difference was not statistically significant.

**Age, gender and clinicopathological parameters**

Alteration in cytokine levels was seen according to age and gender of the patients. In patients younger than 65 years, the levels of PDGF-BB ($P = 0.048$), IL-1ra ($P = 0.021$), IL-7 ($P = 0.032$), IL-8 ($P = 0.029$), IL-9 ($P = 0.049$), IFN- $\gamma$ ($P = 0.013$), and MIP-1 $\beta$ ($P = 0.044$) were higher than in patients aged 65 or older. The levels of IP-10 were higher ($P = 0.027$) in patients aged 65 or older.

Serum levels were similar in females and males (data not shown), except for eotaxin, the levels of which were higher in male compared to female patients ($P = 0.017$). Significant differences were not seen between cytokine levels of patients in different BMI groups (data not shown in study report).

Cytokine levels according to tumour localisation, histological grade, and TNM classification were analysed. In patients with proximal tumours, the serum levels of IP-10 were higher compared to distal or rectal cancers ($P = 0.032$). In patients with WHO grade 3 cancers, levels of IL-1ra ($P = 0.032$), IL-6 ($P = 0.016$) and IL-8 ($P = 0.02$) were higher compared to grade 1 or 2 tumours. Significant alterations were also seen when assessing the effect of TNM stage. Higher levels of IL-1ra ($P = 0.039$) and IL-8 ($P = 6.99E-5$) were seen in stage IV tumours compared to stage I-III tumours. Higher T class associated with increased serum levels of IL-6 ($P = 0.015$), IL-8 ($P = 9.28E-5$), and MCP-1 ($P = 0.023$). The presence of lymph node metastases was associated with higher IL-8 level ($P = 0.017$). The patients with distant metastases had higher serum levels of IL-
1ra (P = 0.008), IL-4 (P = 0.033), IL-6 (P = 0.011), IL-7 (P = 0.008), IL-8 (P = 3.14E-5), MCP-1 (P = 0.016) and PDGF-BB (P = 0.010) compared to patients without distant metastases.

5.4.2 Multivariate analysis

We generated a logistic regression model to evaluate the potential of serum cytokine profile in discriminating the CRC patients from the controls and to assess the mutual relationship of the differences observed in the univariate analyses. Certain cytokine values from three subjects were not available, and the model was based on 115 patients and 84 controls. The final model is presented in Table 8 in study III. It consists of the five most predictive cytokines chosen using the backward stepwise method. High IL-8 and IL-6, as well as low MCP-1, associated with CRC. Also low IL-1ra and IP-10 were associated with CRC, although these two cytokines showed no difference in the univariate analyses. Hosmer-Lemeshow test for goodness-of-fit of the model indicated a good calibration ($\chi^2 = 11.1$, probability value = 0.195). A ROC analysis for the model (Figure 4) yielded an area under the curve (AUC) of 0.890 (95% confidence interval, CI 0.845–0.934), which denotes an excellent discriminatory capability. ROC curves were also obtained separately for all the cytokines included in the regression model, and the AUCs were following: IL-1ra, 0.541 (95% CI 0.461–0.622); IL-6, 0.724 (95% CI 0.654–0.793); IL-8, 0.769 (95% CI 0.703–0.834); IP-10, 0.510 (95% CI 0.429–0.592); and MCP-1, 0.634 (95% CI 0.557–0.711).
We also generated a logistic regression model for discriminating patients with nodal metastases from the patients without nodal metastases (see Table 9 in study III). The model was restricted to the four most predictive cytokines. In this model, low IFN-γ (OR 0.09, 95% CI 0.005–1.65) and IP-10 (OR 0.11, 95% CI 0.015–0.85) and high IL-8 (OR 9.4, 95% CI 1.5–58.3) and IL-1ra (OR 8.3, 95% CI 0.8–84.5) associated with lymph node metastases.

Regression models were also used to adjust potential confounding factors; adjusting for BMI, sex, and the age of the patients did not influence the results of the univariate analyses (data not shown). Tumour grade was also considered to be potentially confounded by tumour stage, but this could not be confirmed due to the small number of grade 3 (n = 14) tumours.
6 Discussion

Outcomes of patients within the same CRC stage are very different and many indicators have been described in predicting outcome (Puppa et al. 2010). Clinical routine is to give adjuvant chemotherapy to patients with stage III disease (lymph node positive). Whether adjuvant chemotherapy should be offered to stage II patients is a controversial issue. Despite a radical surgical resection of the tumour, approximately one third to one fourth of the patients with stage II CRC (lymph node negative) die of the disease. Thus, there appears to be a subgroup of patients within stage II disease who could benefit from adjuvant therapy. Therefore, many additional prognostic markers to TNM staging, including lymph-vascular and neural spread, peritoneal involvement and the number of examined lymph nodes, have been sought to help the assessment of the risk of developing recurrent disease (Puppa et al. 2010).

6.1 Inflammatory cell reaction

We showed that inflammatory cell reaction in and around the tumour has an independent influence on the prognosis of colorectal cancer, comparable to Dukes’ staging in multivariate analysis. The inflammatory cell reaction was even a better predictor than Dukes’ staging when Dukes’ D patients were excluded from the analysis. High-grade inflammation predicted better survival. In lymph node negative cases (Dukes’ A and B patients) the 5-year survival dropped from 87.6% in cases with high-grade inflammation to 47.0% in cases with low-grade inflammation.

However, it is likely that some of the node-negative patients have been understaged in studies I and II, because at the time the patients were operated between the years 1986–1996, staging of the tumours was not focused on the number of the examined lymph nodes. Today it is recommended that a minimum of 12 lymph nodes should be evaluated to reach more accurate staging, and the number of examined lymph nodes has been shown to have an impact on survival (Puppa et al. 2010). For study III, the mean value of examined lymph nodes was over 15, but for studies I and II, it was very rare to have many lymph nodes harvested. In a subset of 72 cases, the mean number of lymph nodes obtained was 2.76 (data not shown). Since the cases were selected, the subset could be biased, indicating that the retrospective material of studies I and II does not meet the current standards for adequate lymph node harvest.
There is substantial evidence that inflammatory cell infiltration in and around the tumour is associated with improved outcome in colorectal cancer. Generally, an increased density of lymphocytic or inflammatory cell infiltrate is associated with improved survival in CRC, which have been reported in almost 40 studies (see Table 4). Roxburgh et al. used our criteria to evaluate inflammatory reaction at the invasive margin in stage II CRC, and found that it was an independent prognostic marker even in patients with low-risk tumour characteristics (Roxburgh et al. 2009c).

We evaluated the overall inflammatory cell reaction and also the amount of each inflammatory cell type. While overall inflammatory cell reaction was the most significant predictor of favourable outcome, macrophages and lymphocytes were the most valuable predictors among individual cell types. At the invasive front, macrophage reaction and high lymphocyte and neutrophilic granulocyte grade indicated a good prognosis, while eosinophilic granulocyte grade did not. In the central part of the tumour only overall inflammatory cell infiltration and lymphocyte grade were significantly valuable predictors. Besides overall inflammation, the macrophage reaction was the only cell type with independent prognostic significance. Roxburgh concluded in his review (Roxburgh & McMillan 2011) that while generalised non-specific tumoural inflammatory response is strongly related to survival, it has also been shown that most individual immune cell types relate to cancer-specific survival. The evidence is particularly strong for T-lymphocytes, macrophages, dendritic cells and neutrophils. However, it has been reported that assessment of all white cells, rather than individual cell types, is the most important determinant (Ogino et al. 2009, Roxburgh et al. 2009c).

In addition to our study, twelve other studies have evaluated the role of TAMs in colorectal cancer outcome (see Table 4). In nine of these studies a strong positive association was observed between macrophage infiltration grade and improved survival, and in five of these, the strongest association was in macrophages located at the invasive front. In four studies (Baeten et al. 2006, Funada et al. 2003, Inoue et al. 2005, Nagorsen et al. 2007), the relationships between macrophages and survival were not seen. Most (63 of the 85) studies of the association between other individual inflammatory cell type response (T-cell subtypes, natural killer cells, B-cells, neutrophils, mast cells, dendritic cells and eosinophils and survival in CRC have reported a significant survival association while no such association was seen in 22 studies (Roxburgh & McMillan 2011). However, the role of macrophages is controversial as macrophages can have both
anti- and pro-tumouric capacity. A leukocyte infiltration is present in most
tumours, and there is evidence that each type of these leukocytes can be involved
in tumour invasion and metastasis (Mantovani et al. 2008). High density of TAMs
associates with poor prognosis in the majority of malignant tumours (Erreni et al.
2011). Pro-tumour functions of TAMs include the production of growth factors,
proteolytic enzymes and repression of adaptive immune response (Erreni et al.
2011). In our study, macrophage reaction showed to have a dominant role in the
defence against the tumour. Macrophages mediate the inflammatory cell response
and influence tumour cell growth by secreting cytokines and other active
mediators. Macrophages are antigen-presenting cells evoking adaptive immune
response and they also have phagocyte function: their presence within tumours
has been linked to removal of apoptotic and necrotic tumour cells (Henry et al.

The location of TAMs has been suggested to be an important aspect when
debating anti-tumour and pro-tumour activity of TAMs. Galon et al. showed that
the type, density and localisation (invasive margin or tumour centre) of the
immune cells is a primary determinant of tumour progression (Galon et al. 2006).
Peritumoural macrophages are suspected to be less influenced by pro-tumour
cytokines and are located in a less hypoxic area, and may thereby differentiate
into an anti-tumour rather than pro-tumour phenotype. Macrophages along the
tumour margin are able to induce apoptosis, and direct macrophage-to-tumour
cell contact is required to manifest the positive prognostic influence of
macrophage infiltration (Forssell et al. 2007, Sugita et al. 2002). Baileys et al.
noted that when macrophages are evaluated not only at the tumour margin but in
all areas within the tumour, including necrotic areas, accumulation of TAMs is not
a good prognostic indicator (Bailey et al. 2007).

In our study, the intensity of inflammatory reaction was higher at the invasive
margin than in the central part of the tumour, and the predictive power was
significantly better when evaluated at the invasive margin. Interaction between
tumour and host at the invasive margin is a critical interface when tumour
progression occurs. Important events in tumour growth including decreased
expression of adhesion molecules, secretion of proteolytic enzymes, increased
cell proliferation and angiogenesis are most dynamic at the tumour-host interface
(Bryne et al. 1995, Bryne et al. 1998). Structural features of the invasive margin
have been observed to have prognostic valuable in various cancers (Bankfalvi &
Piffko 2000, Bryne et al. 1995, Kristensen et al. 1999, Kristensen et al. 1999),
Proximal and distal colorectal cancers are different in terms of their pathogenesis, clinicopathological characteristics as well as behaviour (Jenkins et al. 2007). Proximal CRC is more frequent in female patients, presents more often with MSI and increased numbers of tumour-infiltrating lymphocytes, and a greater proportion of Lynch syndrome-associated CRCs occur in the proximal colon (Jenkins et al. 2007). The Jass criteria were originally described for rectal cancer, but they have later been validated both in rectal and colon cancers (Jass et al. 1987, Roxburgh & McMillan 2011). In Roxburgh’s study, the hazard ratio for inflammatory infiltrate in relation to survival was higher in rectal compared to colonic cancers (Roxburgh et al. 2009b). However, Ogino et al. reported that high-grade lymphocytic infiltrate was a more common feature in colon cancer compared to rectal cancer (Ogino et al. 2009). Our finding did not support this conception, as we did not find any differences in inflammatory reaction grades between colon and rectal cancers.

In our material, MSI status was not associated with high-grade inflammation or Crohn’s-like reaction. Tumours with high-level MSI are recognised to be characterised by abundant intra- or peritumoural lymphocytic infiltrates and Crohn’s-like reaction (Greenson et al. 2009, Jass et al. 1998, Michael-Robinson et al. 2001, Ogino et al. 2009). One recent study concluded that TILs are independent characteristic of MSI status (Roxburgh & McMillan 2011), which is also in line with our finding.

In our study, validated later by others (Roxburgh et al. 2009c), the inflammatory cell infiltration particularly at the invasive margin of the tumour was capable of discriminating stage I-II patients to low-risk and high-risk groups, independently of stage. High-risk patients could therefore be a potential target for adjuvant chemotherapy.

The reproducibility of the evaluation of prognostic markers from tumour specimens is an important issue. It is also important that methods can be easily taken into clinical use without additional costs. The current staging system according to TNM is simple and reproducible and has a significant prognostic value. In comparison, the WHO histological grading has limitations as subjective variations in interpretation decrease its prognostic valuable (Puppa et al. 2010). The tumour inflammatory reaction is not routinely evaluated in pathologists’ reports, which is largely due to subjective assessment resulting in interobserver variation (Deans et al. 1994, Jass et al. 1996). Our method to evaluate
inflammatory reaction was different compared to those previously reported. It is a
structured assessment of all cell types, and we achieved good reproducibility with
low inter-observer variation. Our method has been validated by others (Roxburgh
et al. 2009c). Roxburgh et al. obtained similar results in node negative CRC
patients by using our scoring method, and as the method was found to be
reproducible, they recommended using the method in future studies.

6.2 Budding

We evaluated the contour of the invasive margin and the occurrence of tumour
budding in colorectal cancer. The invasive margin was sharply invasive in 67.6%
of the cases and was more frequent in more advanced cases. Tumour budding was
seen in 24% of the cases and was more frequent in advanced tumours as it was
seen in only 9.2% in Dukes’ A compared to 47.9% in Dukes’ D cases. In the
literature tumour budding has been reported to occur in 20–40% of tumours
(Zlobec & Lugli 2010).

We showed that tumour budding has a significant influence on prognosis of
CRC patients. It is a strong indicator of poor prognosis. The 5-year survival was
only 15.4% in cases with tumour budding, compared to 63.5% without budding,
regardless of stage, the results being similar in both colon and rectal cancers. In
addition, when localised (lymph-node negative) tumours were analysed, the
presence of budding clearly discriminated patients into groups of different
prognosis. In Dukes’ A cases the 5-year prognosis was 29.2% and 82.2%
according to whether budding was present or not, compared to 29.7% and 72.3%
in Dukes’ B cases, respectively. In multivariate analysis, tumour budding was an
independent prognostic marker, and even better than Dukes’ stage when Dukes’ D
cases were excluded from the analysis. However, some understaging might exist
in lymph-node negative patients, as mentioned above.

Evaluation of tumour budding has been recommended as an additional
prognostic marker in pathologist’s report according to the AJCC (Compton et al.
2000). It has been associated with invasion, metastasis, and poor prognosis in
CRC (Jass et al. 2003). Tumour budding has been shown to be linked to lymph
node metastasis (Hase et al. 1993a, Tateishi et al. 2010), distant metastasis
Its prognostic value on survival of CRC patients has been examined in several
studies, and this feature has been shown to be an independent predictor of poor
In the study of Kanazawa et al., it was found that 5-year survival was 39% and 80% in cases with strong and mild budding, respectively, and budding was shown to be an independent prognostic factor in multivariate analysis (Kanazawa et al. 2008). In addition, tumour budding has been shown to be valuable in stratifying patients into different risk groups among patients with stage III rectal disease (Choi et al. 2007, Okuyama et al. 2003b).

In evaluating tumour budding, intra- and interobserver agreement have been found to be relatively high (Jass et al. 2003, Ueno et al. 2002). Also in our study, intraobserver variation was tested and showed a good agreement between all observations.

Although it is not yet possible to predict precisely which particular patients will benefit from adjuvant chemotherapy, stratifying patients within different risk groups can help clinicians decide on treatment (Benson et al. 2004). The assessment of tumour budding and structured inflammatory cell counting can easily be added to routine histopathological analysis without the need of adding new staining methods to routine H&E staining. They can offer useful prognostic information to clinicians as inflammatory reaction or presence of budding predicts prognosis. While both were independent prognostic factors, peritumoural inflammatory reaction was superior of the two in multivariate analysis in our material. Since the presence of peritumoural inflammation and budding have opposite effects, they can both be utilised in determining the prognosis of the patients. Some attempts have already been made to score these parameters (Zlobec et al. 2011).

### 6.3 Cytokines

We evaluated the alterations of the serum cytokine pattern in CRC patients compared to healthy controls. To our knowledge, our study of serum cytokine profile measurement is the most extensive study in CRC so far. We showed that CRC patients had increased serum levels of PDGF-BB, IL-6, IL-7 and IL-8, and decreased levels of MCP-1 compared to healthy controls. Previous studies have shown that CRC patients have higher serum IL-6, IL-8 and BDGF-BB levels than healthy controls (Belizon et al. 2009, Knupfer & Preiss 2010, Ueda et al. 1994). Instead, an increased levels of IL-7 and decreased levels of MCP-1 were novel findings in our material. However, increased levels of MCP-1 in gastric cancers and increased levels of IL-7 in prostate cancers have been reported earlier (Mengus et al. 2011, Tonouchi et al. 2002).
Alterations in cytokine levels according to different TNM stages of the CRC were also seen in our study. Cytokines that were observed to be elevated in CRC patients compared to controls also had a trend of being more elevated in more advanced stages. In addition, while showing no differences between CRC patients and controls, levels of IL-1ra were elevated in more advanced cases, and IL-4 was also elevated in cases with distant metastases. A shift towards Th2 cytokine response has been suggested to occur in the development of CRC ([Cui & Florholmen 2008, Pellegrini et al. 1996]). We could not demonstrate such polarisation from Th1 towards Th2 profile when patients were compared to controls. Instead, an increase of Th2 cytokines IL-4 and IL-6 in the absence of alteration of Th1 cytokines IL-12 and IFN-γ in cases with distant metastasis was observed. Thus, it may be suggested that a cytokine profile shifting towards Th2 may be related to cancer progression rather than being characteristic of CRC. The knowledge of cancer-specific functions of cytokines and chemokines is still limited. IL-6 has been shown to increase the invasiveness of cancer cells and promote angiogenesis in CRC (Knupfer & Preiss 2010). IL-8 has been suggested to be involved in induction and progression of colorectal carcinoma and the development of colorectal liver metastases (Rubie et al. 2007). Chemokine MCP-1 is thought to be responsible for acquiring TAMs (Mantovani et al. 2008).

We generated a logistic regression model that showed an excellent capacity of cytokine profile to discriminate CRC patients from healthy controls. In multivariate analysis, high levels of IL-6 and IL-8, and low levels of MCP-1, IL-1ra and IP-10 were the most predicting cytokines, reaching an AUC of 0.890 in the ROC analysis. This observation supports the potential of cytokine profiling as a mini-invasive diagnostic tool.

One limitation of our study is that we did not investigate the cytokine profile of individuals with inflammatory or infectious diseases. In addition, our method was specifically fitted to our material. Cytokines have complex contribution in inflammatory conditions, obesity and atherosclerosis as well as cancer types other than CRC (Lin & Karin 2007, Lumeng & Saltiel 2011, Papadakis 2004, Sheikine & Hansson 2004), and it is unlikely that measuring individual cytokines would provide a sensible screening or diagnostic method for CRC. Instead, cytokine profile could provide useful additional information when combined with other prognostic parameters. Therefore, in the future it will be necessary to compare the cytokine alterations induced by CRC to alterations induced by other malignancies, inflammatory diseases and other conditions known to be related to alterations in the cytokine levels. Furthermore, it will be interesting to evaluate
the significance of the relationship of local tumour inflammatory reaction and serum cytokine profile in predicting prognosis.

In our study, after adjusting for disease stage, the only significant correlation between cytokine levels and patients’ age were lower IP-10 and higher PDGF-BB levels in younger patients. Higher eotaxin levels in male patients were the only difference between males and females. These findings are in line with previous observations that cytokine levels have only minor association with age and gender in CRC (Kaminska et al. 2005, Sharma et al. 2010). Elevated levels of IP-10 in patients with proximal compared to distal cancer were the only difference regarding tumour location. This was an unexpected result, as proximal and distal CRCs are known to have different clinicopathological characteristics (Jenkins et al. 2007). Association between serum IL-6 and IL-8 levels and poor differentiation has been reported (Kaminska et al. 2005). This finding was supported in our study, as levels of IL-6, IL-8 and IL-1ra were found to be higher in poorly differentiated cases.

Cytokines and chemokines form a complex network of regulatory proteins, and as regulators of growth and differentiation of cells, they contribute to tumour growth and progression by mediating the interactions between cancer cells and infiltrating inflammatory cells (Commins et al. 2010). As cytokines are pleiotrophic, they can act on many cell types causing different effects. As the same effect may be induced by multiple different cytokines, it is more likely that measurement of cytokines as a group would be more useful as a biomarker rather than determination of a single cytokine at a time. The method used in our study offers an opportunity to study relative changes of cytokine levels, allowing analysis of the shifts in the immune balance occurring in patients with colorectal cancer. However, caution is necessary when considering the application of new multiplex technologies in clinical research. Experience with multiplex arrays remains limited. Good correlations between ELISA and multiplex have been reported, but side-by-side comparisons to other technologies are rare (Zhou et al. 2010b). In addition, while concordance between ELISA and multiplex is generally good when using tissue culture supernatant samples, it is less accurate when using serum samples.
7 Conclusion

Assessment of inflammatory cell reaction at the invasive front of the tumour provides independent prognostic information on patients with colorectal cancer. Evaluation of tumour budding is also an independent prognostic marker. The assessment of both features is reproducible, simple to learn, and does not require additional costs. As low-grade inflammation and the presence of tumour budding predict poor prognosis in lymph node negative (stage I-II) cancers, it would be reasonable that information about these features should be included in routine pathological reporting. Further studies are needed to confirm whether patients with low-grade inflammation or tumour budding will benefit from adjuvant chemotherapy.

Measurement of serum cytokine panel, a cytokine profile, showed an excellent capacity in discriminating patients from healthy controls. As cytokine profile was shown to change during cancer progression, a shift in the immune balance of CRC patients may be useful in staging of CRC. Our study highlights the importance of investigating serum cytokine levels as a group in order to observe relative expression level changes. However, the clinical value of these findings needs to be confirmed by subsequent studies, and with large study populations also including subjects with other conditions known to induce alterations in cytokine levels, such as inflammation, obesity and atherosclerosis.
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