Cancer of the Colon and Rectum

Prognostic Factors and Early Detection

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Abstract

Colorectal cancer (CRC) is one of the most common causes of death from malignant disease. Nevertheless, no ideal screening method exists and there is a lack of prognostic and predictive factors to support clinical decisions and to aid the development of a more individualized treatment for patients with CRC. The aim of this thesis was to investigate early detection, prognostic and predictive factors of CRC. In the first paper, a novel method to collect cells for DNA quantification from the rectal mucosa was investigated. The sensitivity and specificity of this test to detect CRC or any pathology in colon and rectum were ultimately too low to be acceptable. In the second paper, the prognostic value of growth differentiation factor 15 (GDF 15) was evaluated in patients curatively operated for colorectal cancer. GDF 15 expression was demonstrated to be associated with a negative prognosis in patients with stages I-III and III disease. In the third paper, the prognostic value of BRAF, PIK3CA KRAS and MSI was evaluated in a cohort of patients with CRC stratified by disease and recurrence. The results indicated that patients with CRC stage III without recurrence have a higher frequency of BRAF mutation compared to stage III patients with recurrence. In the fourth paper, histopathological predictors of pathologic complete response (pCR) as well as the association between pre-treatment carcinoembryonic antigen (CEA) levels and pCR in non-smoking and smoking patients receiving preoperative chemo-radiotherapy for rectal cancer were evaluated. Only in non-smokers was a low CEA level significantly associated with pCR, suggesting that the predictive value of CEA for pCR in rectal cancer in smokers can be limited. In sum, this research has investigated a new method for CRC detection and further evaluated the clinical use of prognostic and predictive markers in CRC.

Keywords: colorectal cancer screening predictive markers prognostic markers

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The best way to predict the future is to invent it.
This thesis is based on the following papers, which are referred to in the text by their Roman numerals given below (I-IV).


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Front cover:
Colon and rectum with cancer in the sigmoid colon
Illustration by Ulrik Wallin
Abbreviations

APC gene  Adenomatous Polyposis Coli gene
APR     Abdominoperineal Resection
CIN     Chromosomal Instability
CRC     Colorectal Cancer
CRT     Chemoradiation Therapy
CSS     Cancer Specific Survival
CTC     Computed Tomographic Colonography
DCBE    Double Contrast Bowel Enema
FAP     Familial Adenomatous Polyposis
FIT     Fecal Immunochemical Test
FSIG    Flexible Sigmoidoscopy
gFOBT   Guaiac Based Fecal Occult Blood Test
GWAS    Genome Wide Association Analyses
Gy      Gray
HNPCC   Hereditary Non-Polyposis Colorectal Cancer
IBD     Inflammatory Bowel Disease
IHC     Immunohistochemistry
LAR     Low Anterior Resection
MCL     Muco Cellular Layer
MMR     Mismatch Repair Gene
MSI     Microsatellite Instability
NegLR   Negative Likelihood Ratio
NPV     Negative Predictive Value
OS      Overall survival
pCR     Pathological Complete Response
PosLR   Positive Likelihood Ratio
PPV     Positive Predictive Value
ROC curve Receiver Operator Characteristic curve
SP-PLA  Solid-phase proximity ligation assay
TMA     Tissue Microarray
TME     Total Mesorectal Excision
TNM     Tumor Nodes Metastases
TTR     Time to recurrence
Introduction

Colorectal cancer (CRC) is one of the most common causes of death from malignant disease (Keighley, 2003). Worldwide there are some variations in the incidence of CRC with the highest occurrences in Western Europe and North America, and the lowest in Asia, Africa and South America. However these variations in incidence are more obvious in colon cancer than in rectal cancer. The risk of developing CRC increases with age, and rectal carcinoma are more common in males whereas colon carcinoma are slightly more common in females (Altekruse et al., 2009). Two thirds of CRC are located in the colon and one third in the rectum. About 50% of the tumors within the colon are located in the sigmoid, 25% in the right colon (caecum and ascending colon) and the remaining 25% are in the transverse colon, splenic flexure descending colon and hepatic flexure.

Etiology

The risk of developing CRC is influenced by both environmental and hereditary factors. The two most known colorectal cancer syndromes are familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). Patients with FAP carry nearly a 100% risk of developing CRC by the age of 40 if untreated. FAP accounts for less than 0.1% of the total cases of CRC and is characterized by a mutation in the APC gene leading to the development of thousands of adenomas. HNPCC, also called Lynch syndrome, accounts for 1-3% of all the incident CRC cases (Aaltonen et al., 1998; Olsson and Lindblom, 2003). Germline mutations in four different DNA mismatch repair genes (hMSH2, hMLH1, hPMS1 and hPMS2) are thought to account for the development of CRC in HNPCC. Apart from these genetic CRC syndromes the risk of developing CRC is increased in the presence of a family history of CRC, especially if a close relative before the age of 55 or multiple relatives were diagnosed with CRC (Strate and Syngal, 2005).

Other risk factors for developing CRC are age, polyps, history of cancer, smoking, and inflammatory bowel disease. The evidence of acquired risk factors, particularly dietary factors, and their influence on the incidence of CRC is controversial. Dietary factors that potentially increase the risk of
CRC include low vegetable, fruit, fiber intake or high red meat or saturated fat consumption and excessive alcohol intake.

The existing data in the literature concerning dietary intake and its effect on CRC have been almost exclusively obtained from observational studies, which are inherently prone to bias and confounding. Additionally, it is difficult to accurately assess dietary exposure because of the reliance on food questionnaires, which are suboptimal due to the high risk of misclassification resulting in a biased result.

Pathology/Staging

Adenocarcinoma is the most common histological type of CRC. Other subtypes like mucinous adenocarcinoma, medullary carcinoma, signet ring cell, squamous cell carcinoma, and adenosquamous carcinoma are less common. Lymphomas, sarcoma and carcinoid tumors can occasionally be seen in colon or rectum. The disease stage based on the tumor, lymph nodes and metastasis (TNM) is the most important factor used for therapy decision and prognostic estimation. The original TNM system has several times been updated and the 7th edition has recently been introduced aiming at more precise staging based on evidence from international datasets (table 1).

Table 1. TNM staging system from AJCC TNM 7th edition (Edge and Compton, 2010)

<table>
<thead>
<tr>
<th>Stage</th>
<th>TNM Classification</th>
<th>Five-Year Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T1-2, N0,M0</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>IIA</td>
<td>T3, N0, M0</td>
<td>60-85%</td>
</tr>
<tr>
<td>IIB</td>
<td>T4, N0, M0</td>
<td></td>
</tr>
<tr>
<td>IIIA</td>
<td>T1-2, N1, N0</td>
<td>15-65%</td>
</tr>
<tr>
<td>IIIB</td>
<td>T3-T4, N1, M0</td>
<td></td>
</tr>
<tr>
<td>IIIC</td>
<td>T (any), N2, M0</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>T (any), N (any), M1</td>
<td>5-7%</td>
</tr>
</tbody>
</table>

Primary tumor

Tx  Primary tumor cannot be assessed
Tis  Carcinoma in situ
T1  Tumor invades submucosa
T2  Tumor invades muscularis propria
T3  Tumor penetrates muscularis propria and invades subserosa
T4  Tumor directly invades other organs or structures or perforates visceral peritoneum
T4a Perforates visceral peritoneum
T4b Directly invades other organ or structures
Nodal status (N)

Nx  Regional lymph nodes cannot be assessed
N0  No metastases in regional lymph nodes
N1a Metastases in one regional lymph nodes
N1b Metastases in 2-3 regional lymph nodes
N1c Satellites in subserosa, without regional lymph nodes
N2  Metastasis in 4 or more regional lymph nodes
N2a 4-6 lymph nodes
N2b 7 or more lymph nodes

Distant metastases (M)

M0  No distant metastases
M1a Distant metastases in one organ
M1b Distant metastases in more than one organ or peritoneum

Other pathologic prognostic factors are tumor grade which describes the level of cell differentiation and is usually divided into four groups; well, moderate, poor and undifferentiated. However, there is considerable inter-observer variation in the interpretation of tumor grade possibly reducing its clinical significance. Of the different tumor grades the only demonstrated independent prognostic factor for CRC is poor differentiation (Compton et al., 2000), which is identified by decrease in tubular formation, severe cell atypia and numerous mitotic figures. However, most of the adenocarcinomas are moderately differentiated. The presence of vascular, lymphatic or neural invasion predicts more advance tumor stage and worse prognosis. More pathologic factors not adapted to clinical routine associated with poor prognosis are presence of tumour budding (Nakamura et al., 2008; Wang et al., 2009) and absence of peritumoral lymphocytic reaction (Ogino et al., 2009a).

Treatment for colorectal cancer

Surgery is the most important treatment for patients with CRC when the treatment has a curative intent. The importance of radiation- and/or chemotherapy in the adjuvant and/or neoadjuvant settings has increased and recently other targeted therapies like monoclonal antibodies has been introduced and has a place in the treatment of advanced CRC cancer. The final choice of treatment modality should always be individualized and based on the stage and location of the disease as well as the patient’s age and general
health condition. In most cases two or more types of treatments are used either concurrently or followed by each other.

Surgery

Surgery with tumor free margins is the most important predictor that influences a patient’s overall survival, and the choice of surgical procedure for CRC is mostly dependent on the extent and location of the disease. For colon cancer, surgery is the main treatment for early stages of the disease, either through conventional open surgery or by using a laparoscopic technique. Attention to the embryological planes and lymphoid and vascular supply of the colon during the surgical dissection is essential to achieve a radical resection and will influence the local recurrence rate and overall survival (Hohenberger et al., 2009). In rectal cancer, the introduction of total mesorectal excision (TME) significantly improved the outcome after rectal cancer surgery (Heald, 1988), and together with radiation therapy is today one of the recommended standard treatments for rectal cancer (CCCG, 2001; Kapiteijn et al., 2001; SRCT, 1997). The TME technique focuses on a sharp dissection outside of the mesorectal fascia which secures the removal of an intact package of the tumor and its main lymphatic drainage and central ligation of the main supplying vessels.

Radiotherapy

Radiotherapy currently has a minimal role in the treatment of colon cancer. However in rectal cancer, pre- or postoperative radiotherapy has been demonstrated, in several randomized clinical trials, to reduce the risk of local recurrence and to prolong survival (Glimelius et al., 2003; van Gijn et al., 2011). In Europe preoperative radiotherapy has been the treatment of choice, whereas in USA postoperative radiotherapy is more commonly used. Preoperative radiotherapy, as compared with postoperative, has a lower risk of local recurrence and fewer adverse effects (CCCG, 2001; Frykholm et al., 1993; Sauer et al., 2004) but relies on an accurate preoperative staging and may therefore over- or undertreat patients. After characterizing the clinical stage and the local extent of the disease rectal tumors can be divided into three main groups; the ‘good’, the ‘bad’ and the ‘ugly’ (Blomqvist and Glimelius, 2008). This group characterization used in Sweden will determine whether pre- or postoperative treatment is recommended (figure 1).
Figure 1. Algorithm for choice of therapy based on MRI-directed pre-operative evaluation. 1) LFR=Local Failure Rate calculated in the group of patients planned for surgery, i.e. irrespective of the surgical outcome. The figures are valid if the surgeon is an experienced rectal cancer surgeon and pre-treatment is given. From L.Blomqvist and B. Glimelius (Blomqvist and Glimelius, 2008)

Early stage or “good” rectal cancers (\(<T_{3a}N_0\)), 30-40% of all rectal cancers, have a low risk of local recurrence and radiotherapy is not routinely recommended. The intermediate or “bad” group, 40-60% of all rectal tumors, consists of \(T_{3b-c}\) tumors with no signs of mesorectal fascia involvement (mrf-), indicating clear circumferential marginal (CRM) after surgery and local lymph node metastasis (\(N_{1-2}\)) and patients with these are offered preoperative radiotherapy since the local recurrence risk is high (Glimelius et al., 2011). Advanced or “ugly” rectal cancers, 10-20% of all rectal cancers, are \(T_4\) tumors or \(T_3\) tumors with lymph node metastases and a potential CRM less than 1 mm. In these “ugly” tumors there is a high risk of a non-radical resection because the surgical planes are usually invaded by the tumor if only a TME is done. The recommended treatment for this group is long radio-chemotherapy with delayed surgery in an effort to achieve a downstaging of the tumor and ultimately a radical resection. More extended surgery than TME may be required.

Chemotherapy
Chemotherapy is given to eradicate tumor cells that have already metastasized, i.e. the primary goal is to achieve systemic control compared to radiotherapy in rectal cancer that serves to obtain local control. In colon cancer stage III the use of postoperative 5-FU in combination with folinic acid (Leucovorin) after curative surgery significantly reduces the recurrence rate by 30% (Moertel et al., 1990). If 5-FU/Leucovorin is combined with oxalip-
latin the recurrence rate is further decreased (Andre et al., 2004; Haller et al., 2011; Twelves et al., 2005). However, these combinations increase the risk of side effects, especially the risk of neuropathy with the combination of oxaliplatin. The use of postoperative chemotherapy in stage II colon cancer is not routinely recommended unless bad prognostic factors are present. In patients with rectal cancer who have received preoperative CRT there are no direct evidence from randomized trials supporting any benefit from adjuvant chemotherapy (Bosset et al., 2006; Collette et al., 2007; Valentini et al., 2002), and therefore chemotherapy is not generally recommended as an adjuvant treatment after curative TME surgery in rectal cancer (Bujko et al., 2010; Glimelius, 2010; Valentini and Glimelius, 2010).

**Pathological complete response**

About 15-25% of all patients with a tumour considered to have intermediate risk of recurrence (the “bad” group) receiving neoadjuvant therapy will have pathological complete response (pCR) i.e. no detectable tumor cells in the resected specimen (Carlomagno et al., 2009; Rosenthal et al., 2008; Shivnani et al., 2007). Pathological complete response is associated with increased disease-free survival in some non-randomized studies (Janjan et al., 2001; Valentini et al., 2002) and it has been suggested that adjuvant chemotherapy could be avoided in this subgroup (Capirci et al., 2008). Nevertheless, when comparing the pCR between different neoadjuvant treatments there is always a concern about confounding factors such as the volume of radiotherapy given, imaging modalities used to define the preoperative stage, and the time interval between neoadjuvant therapy and surgery. In radiotherapy specifically the duration between completion of radiation therapy and surgery has influenced the degree of downstaging of the tumor in one randomized controlled trial (Francois et al., 1999). Hypothetically, patients receiving preoperative radiochemotherapy that develop pCR will not improve overall survival or disease-free survival from a surgical resection. However, this hypothesis is hard to prove since there are several potential misclassifying factors such as the quality of histopathology that independently will have a prognostic value. The biggest challenge is to accurately assess which patients develop pCR that will stay durable. A group in Brazil has been studying operative versus non-operative treatment for distal rectal cancer following radiochemotherapy by using clinical complete response as a surrogate marker for pCR. Their results indicate, in pathological stage 0 rectal cancers (complete clinical response), an excellent long-term results irrespective of treatment strategy (Habr-Gama et al., 2004), however these results have been questioned by other groups (Nyasavajjala et al., 2010).
Colorectal cancer screening

The indications for implementing a screening program for a disease are dependent on several criteria. These have been defined by the World Health Organization (Wilson and Jungner, 1968):

- The disease should be an important health problem for the individual and the community.
- There should be a generally accepted treatment.
- The natural history of the disease should be adequately understood.
- There should be a latent or early symptomatic stage of the disease.
- A suitable screening method must be available.
- The screening method must be accepted by the target population.
- Facilities for diagnosis and treatment should be available.
- The policy for the treatment of the disease must be clear.
- Early treatment of the disease should be of more benefit than treatment started later.
- The cost should be economically balanced in relation to possible expenditure on medical care as a whole.

Colorectal cancer (CRC) constitutes approximately 10% of all malignancies and is one of the leading causes of cancer-related morbidity and mortality in the Western world, making it a major health problem. The majority of CRC is believed to arise from pre-existing benign adenomatous polyps (Bond, 2003; Winawer, 1999) and therefore could potentially be detected before malignant transformation. The five year survival rate for localized CRC that has not spread or grown outside the wall of the bowel is 80-90% and decreases to around 50-70% if the tumor has spread to regional lymph nodes and is further reduced to 10% if distant metastases are present. Consequently, early detection of localized CRC significantly improves survival and the removal of adenomatous polyps can potentially decrease incidence of CRC (Hardcastle et al., 1996; Kronborg et al., 1996; Lindholm et al., 2008; Mandel et al., 2000; Selby et al., 1992). Even though the optimal ages of initiation and cessation of screening for CRC are unknown it appears to be cost-effective with the commonly considered screening methods available compared with no screening (Pignone et al., 2002). Accordingly, CRC fulfills the criteria for being a disease that should be targeted for screening.

The ultimate goal of cancer screening is to reduce mortality through a reduction in the incidence of advanced disease. To achieve this goal a screening test with acceptable validity, reliability, costs, benefit/risk ratio and acceptance in the population is needed. In an average risk population there are several available tools for CRC screening and these can be divided into two broad categories: stool-based tests, including tests for occult fecal blood or
exfoliated DNA; and structural exams, including flexible sigmoidoscopy, colonoscopy, double-contrast barium enema and computed tomographic colonography. Stool-based and structural tests differ in terms of their invasiveness, requirements for colon preparation, cost, and diagnostic yield. Structural tests will primarily identify adenomatous polyps as well as CRC, whereas stool-based tests mostly detect CRC and to a lesser extent premalignant adenomatous polyps (Lieberman and Weiss, 2001). Therefore, the primary goals of stool-based tests are not prevention of CRC although some incidental polyps may be detected. These distinctions underline the importance of clear endpoints when designing screening programs. The use and clinical application of these tests are discussed in detail below.

Stool-based tests

Most of the stool-based tests are designed to detect the presence of occult blood in the stool. Blood in the stool is not only a non-specific finding but also requires that lesions bleed if they are to be detected. Consequently, there is a risk of not detecting CRC and larger polyps that bleed intermittently and small adenomatous polyps which usually do not bleed at all. With this in mind the likelihood for detection of a lesion with intermittent bleeding will increase if the test is repeated. There are two main tests based on the analyte used for the test; guaiac based fecal occult blood test (gFOBT) and fecal immunochemical test (FIT). With all of these tests sensitivity will increase with repeated measures in screening programs. It is important to distinguish between a program sensitivity of a test (achieved by serial testing in a screening program) and the true test sensitivity of a test (achieved by one single test) since the program sensitivity is dependent on compliance within the screening program.

gFOBT

Annual gFOBT tests are the only CRC screening test that has shown a reduction in CRC mortality in randomized controlled trials (Hardcastle et al., 1996; Kronborg et al., 1996; Mandel et al., 2000). These studies demonstrated, over a time period ranging from 8 to 13 years, a 15-33% reduction in CRC mortality, and in the Minnesota study also a 20% incidence reduction of CRC after 18 years of follow up. This incidence reduction is thought to depend on a higher colonoscopy rate of the subjects in the screened group (Mandel et al., 2000). gFOBT can be divided into a low- and high-test sensitivity group. The low-test sensitivity group (rehydrated Hemoccult II) has a onetime sensitivity and specificity of about 36% and 98% and in the high-test sensitivity group (Hemoccult SENSA) a sensitivity and specificity of 64%-79% and 87% for detecting CRC respectively (Allison et al., 1996; Liberman et al., 2001). The limitations of gFOBT are the decreased test sen-
sitivity when the tests are performed by patients at home, the need for food restrictions prior to testing, the requirement of annual testing and the reality that many of those that test positive with gFOBT do not end up having a complete colonoscopy to rule out disease (Nadel et al., 2005).

**Fecal Immunochemical Test**

Fecal immunochemical test (FIT) detects human globin in the heme molecule, making it a more specific test than guaiac based tests. A couple of advantages of FIT are that it doesn’t require any food restrictions before the test and it is more specific for lower gastrointestinal bleeding since the globin molecule is degraded by digestive enzymes in the upper gastrointestinal canal. If FIT is compared to the more sensitive gFOBT (Hemocult SENSA) there are no significant benefits in the overall test performance using FIT. One-time test sensitivity and specificity for the detection of CRC ranges from 66% to 82% and 97% to 98% respectively (Allison et al., 2007; Morikawa et al., 2005; Smith et al., 2006). There is no information of the program sensitivity and specificity of FIT because no trials using FIT as a screening tool have yet been performed. While there are only limited data supporting the relevant retesting interval, some data indicate that two tests may be superior to one (Nakama et al., 1999).

**Exfoliated DNA stool tests**

Based on the adenoma-carcinoma sequence of colorectal carcinogenesis (Vogelstein et al., 1988) a series of DNA mutations ultimately results in malignant tumor growth (figure 2). From the mucous membrane of the colon there is a constant shedding of colonocytes into the lumen, a phenomenon called exfoliation. This exfoliation appears to be increased in CRC (Ahquist et al., 2000; Klaassen et al., 2003). The exfoliated DNA is stable in the stool and therefore can be detected after isolation from the fecal bacterial DNA (Sidransky et al., 1992). Yet there is no known common shared single gene mutation that has been identified in the stool for adenomatous polyps or CRC. Based on the heterogeneity of CRC a multi-target panel of gene mutations seems to be the most reasonable approach to achieve an acceptable sensitivity for DNA based stool tests. The one commercially available multi-target DNA panel test detects a total of 21 point mutations in p53, KRAS, APC, BAT-26 (a marker for detecting microsatellite instability), and a DNA integrity assay (a marker of loss of apoptosis) (Olson et al., 2005; Whitney et al., 2004).
There are several studies evaluating the validity of different panels of DNA mutations found in the stool. The onetime test sensitivity and specificity for CRC detection range from 52%-92% and 93%-97% respectively. When comparing DNA stool tests to Hemoccult II (non-hydrated) and colonoscopy it demonstrated a significantly higher sensitivity for detection of CRC (52% versus 13%) and advanced adenomas (15% versus 11%) but with a similar specificity (94% versus 95%) (Imperiale et al., 2004). The benefits of DNA-based stool tests, apart from their noninvasive nature, are that they do not rely on the intermittent nonspecific presence of blood in the stool, and arguably are not dependent on several stool collections. However, even though the detection of a panel of DNA mutations in stool will detect the majority of CRC, tumors with mutations not included in the multi-marker panel will still be missed. The fast development of proteomics and SNP arrays will hopefully give us a more complete picture of the molecular composition of CRC and adenomatous polyps and improve the yield of DNA-based stool tests in the future. Other limitations of the DNA-based test is the significantly higher unit cost for each test compared with other fecal occult blood tests, and the uncertainty how a positive DNA-based stool test with a negative colonoscopy should be followed up (Woolf, 2004).

**Structural exams**

**Flexible sigmoidoscopy**

Flexible sigmoidoscopy (FSIG) has been proven, in case-control studies, to reduce CRC mortality from 60% to 80% for the segment of the colon that is reached by the sigmoidoscope with its protective effect lasting about 10 years (Newcomb et al., 1992; Selby et al., 1992). One randomized trial and a case control study have confirmed decreased CRC incidence following screening sigmoidoscopy (Newcomb et al., 1992; Thiis-Evensen et al., 1999). A recent randomized multicenter controlled trial from the UK demonstrated a 23% reduction of CRC incidence and 31% reduction of mortality in the FSIG screening group (Atkin et al., 2010). Several studies support
that location of the CRC, proximal or distal, is dependent on gender, age, and race (Francois et al., 2006; Levin et al., 1999; Nelson et al., 1997; Schoenfeld et al., 2005; Theuer et al., 2001). The fact that FSIG only examines the distal 40 cm of the colon together with the different incidence of CRC location in these groups obviously limits the use of FSIG as a population-based screening tool. The American Cancer Society, the U.S. Multi-Society Task Force on Colorectal Cancer and the American College of Radiology (ACS/MSTF/ACR) guidelines recommend the sigmoidoscope to be inserted at least 40 cm (Levin et al., 2008). Nevertheless, insertion beyond this level results in a higher detection rate of advanced neoplasia (Lieberman et al., 2000). If an adenomatous polyp of any size is found on FSIG there is over a two-fold higher risk of synchronous advanced neoplasia, which is why a follow-up colonoscopy is always recommended (Imperiale et al., 2000). FSIG usually only requires limited preparation such as two fleet enemas and under normal circumstances can be performed without sedation. However, from a patient’s perspective, FSIG is not always associated with less discomfort compared to colonoscopy (Zubarik et al., 2002).

**Colonoscopy**

There are no existing data from prospective randomized trials evaluating the effect of screening colonoscopy on the mortality or incidence of CRC. There is an ongoing randomized control trial (NorDICC) in the Nordic countries, the Netherlands and Poland comparing colonoscopy to usual care (no screening). From other randomized control trials evaluating other screening tests, but using colonoscopy as a gold standard performing polypectomy if needed, a 20% to 80% incidence reduction of CRC has been observed (Mandel et al., 2000; Thiis-Evensen et al., 1999). A case control study of a symptomatic patient population colonoscopy demonstrated a 50% reduction in mortality (Muller and Sonnenberg, 1995). There is evidence of reduced incidence rates of CRC after colonoscopy and polypectomy in several other studies (Citarda et al., 2001; Robertson et al., 2005; Winawer et al., 1993). In conclusion, there are only indirect – but substantial – evidence-based data supporting the use of colonoscopy as a screening tool. The major benefit of colonoscopy is that it allows for a complete inspection of the colon and, if necessary, a biopsy and polypectomy at the time of inspection. From a patient’s perspective colonoscopy with sedation is preferred compared to FSIG without sedation (Zubarik et al., 2002), however the more thorough bowel preparation that is required prior to colonoscopy is usually perceived as the most unpleasant part of the colonoscopy. Even though colonoscopy is regarded as the “gold standard” its sensitivity for detection of large adenomas (≥10mm) is 88%-94% (Pickhardt et al., 2004; Rex et al., 1997a) and for cancer 95% (Bressler et al., 2004). The sensitivity of the colonoscopy is dependent on operator experience, bowel preparation, withdrawal time and withdrawal technique (Asano et al., 2007; Barclay et al., 2006; Harewood et
al., 2003; Rex et al., 2002; Simmons et al., 2006). There is also some evidence suggesting that the adenoma detection rate will be higher if the colonoscopy is performed in the morning than in the afternoon (Sanaka et al., 2009). A case-control study from Canada demonstrated that colonoscopy has a protective effect only on left sided CRC but not for CRC in the proximal colon (Baxter et al., 2009). Even though there was no way to ascertain the quality of the colonoscopies in this last study it is an interesting finding that needs to be confirmed in a screening setting performed with high quality colonoscopy. The main complications associated with colonoscopy are bleeding and perforation. Both of these complications are higher if polypectomy is performed, particularly in the proximal colon. Perforation rates also increase with age and concurrent diverticular disease. The overall risk of perforation is 1 of 1,000-2,000 (Gatto et al., 2003; Levin et al., 2006). Cardiopulmonary complications are seen in about half of all adverse events associated with colonoscopy, and are usually related to sedation.

Double contrast barium enema
There are no randomized controlled trials or case-control studies evaluating whether double contrast barium enema (DCBE) as a primary screening tool decreases incidence or mortality from CRC. The only available literature on evaluating the performance of DCBE refers to retrospective studies and is almost exclusively based on asymptomatic average risk populations (de Zwart et al., 2001; Glick, 2000). The sensitivity for detection of CRC by DCBE is 85% to 97% and for polyps 48% (≥10mm) (Fork et al., 1983; Rex et al., 1997b; Winawer et al., 2000). However, these studies are quite heterogeneous and apply different study designs. DCBE gives a full structural picture of the colon and in some circumstances when colonoscopy fails or cannot be performed DCBE can serve as an additional tool. The limitations of DCBE are the low sensitivity together with the inability to remove or biopsy polyps/lesions, which necessitates a second investigation with colonoscopy. Similar to colonoscopy DBCE requires full bowel preparation and is operator dependent but has a lower risk of perforation compared with colonoscopy (1 of 25,000 versus 1 of 1,000 to 2,000).

Computed tomographic colonography
Computed tomographic colonography (CTC) or virtual colonoscopy has increased in popularity since its introduction approximately 15 years ago. The CTC integrates 2D and 3D visualization of the colon facilitating the detection of polyps. Bowel preparation is needed as for DCBE and colonoscopy; even if techniques to avoid a full bowel preparation are available they haven’t yet been evaluated in multicenter screening trials (Iannaccone et al., 2004; Lefere et al., 2005). There are no randomized controlled trials evaluating whether CTC as a primary screening tool decreases incidence or mortality from CRC. Results from two meta-analyses performed on high-risk indi-
individuals as well as average-risk individuals in a screening setting demonstrated pooled sensitivity and specificity for detection of adenomas (≥10mm) to be 85%-93% and 97%, respectively. The pooled sensitivity for CRC was 96% (Halligan et al., 2005; Mulhall et al., 2005). The efficacy of CTC for selecting patients that need therapeutic polypectomy has been evaluated in asymptomatic adults in a study using both CTC and colonoscopy as a primary screening tool in two different arms. The detection rates of advanced neoplasia were almost similar in the two arms, 3.2% in the CTC group versus 3.4% in the colonoscopy group (Kim et al., 2007). The interpretation of CTC is highly operator dependent and the sensitivity and specificity improves with polyp size although more advanced 2D and 3D technology have improved the detection rate especially for flat adenomas. One CTC examination contributes with an estimated organ radiation dose of 7 to 13 mSv. This dose should be evaluated in terms of the organ radiosensitivity, which declines with age and in the context of a screening program. The extra colonic findings produced by CTC average around 15%-69%, with 4.5%-11% being clinically significant findings (Hara et al., 2000; Pickhardt and Taylor, 2006). The consequences of the extra colonic findings must be considered before implementing CTC in a presumptive screening program. It is currently recommended that all patients found to have one or more polyps ≥10mm or 3 or more polyps ≥6mm on CTC should have a colonoscopy with subsequent polypectomy performed. However, it is not clear how to follow up with patients with fewer than three polyps in which the largest polyp is ≤6 mm.

Prognostic and predictive markers of sporadic colorectal cancer

Colorectal cancer still remains the second leading cause of cancer deaths in the Western world despite recent advances in the treatment of advanced-stage as well as early-stage CRC. Several studies have demonstrated an association between the genetic composition and the clinical behavior or response to therapy of a tumor. This might explain why some patients develop toxicity to a drug and why some patients relapse whereas others do not. Factors responsible for tumor susceptibility to treatment and clinical behavior are likely to be dependent on complex interactions within cells and the surrounding stroma. Today the clinical and histopathological stage serve as the reference for preoperative, operative, and post-operative treatment decision making. The recommended staging system, the TNM classification, together with other clinical parameters such as tumor obstruction, perforation, and performance status will determine outcome (Compton et al., 2000; Kemeny and Braun, 1983; Steinberg et al., 1986). Even though the TNM staging
system defines the prognosis it offers no information about the individual treatment response. Looking at other areas such as breast cancer, the molecular characterization of the tumor and identification of prognostic markers are already in clinical practice and are used to individualize treatment. In CRC several potential prognostic and predictive markers have been studied, but the only predictive marker currently used in clinical practice is the presence of KRAS mutation in CRC tumors and its reduced response to EGFR inhibitors (Karapetis et al., 2008). With an eye towards emphasizing their clinical usefulness, the following text reviews the genetics of CRC and some of the most studied markers providing information about patients’ prognosis (prognostic markers), as well as markers providing information regarding response to treatment (predictive markers).

Genetics of CRC
The transition from early adenoma to CRC involves somatic mutations in oncogenes and tumor suppressor genes and was originally described by Fearon and Vogelstein as the adenoma-carcinoma sequence (figure 2) (Fearon and Vogelstein, 1990; Vogelstein et al., 1988). This model has been refined because of the detection of new important factors influencing the tumorigenesis. The understanding of the temporal acquisition of the genetic changes as well as the genetic key principles is now more established and today at least three major pathways in CRC have been described: The Chromosomal Instability Pathway (CIN), the Mutator Phenotype Pathway and the Hypermethylation Pathway. These three pathways are not mutually exclusive since a tumor can exhibit features of multiple pathways at the same time, and these overlaps are not fully defined but seem to have prognostic importance (Jass, 2007).

Chromosomal instability and microsatellite instability
There are two main types of genetic instability in CRC; microsatellite instability (MSI) and chromosomal instability (CIN). CIN is the most common type and is present in 65-70% of all CRC, whereas around 15% of CRCs are MSI. It is well demonstrated that both CIN and MSI have prognostic value in CRC, with a poorer prognosis for patients with CIN+ tumors and better prognosis for patients with MSI+ tumors compared to patients with CIN- and MSI- tumors (Hazard ratio for death 1.45 and 0.65 respectively) (Popat et al., 2005; Walther et al., 2008). MSI results from loss of mismatch repair gene (MMR) function and is associated with microsatellite repeats distributed throughout the genome. Thus, MSI tumors share the near diploid chromosome content as normal cells but with an increased nucleotide mutation rates. In HNPCC there is a germline mutation in the MMR gene leading to
80% lifetime risk of developing CRC along with an increased risk of other cancer such as endometrial, ovarian, gastric and renal carcinoma.

MSI status is believed to influence response to adjuvant therapy with 5-FU in patients with stage II and III disease (Ribic et al., 2003) in that MSI+ tumors had a poor response. This is supported by the plausible biological explanation that the MMR system is a requisite for the cytotoxic effect of 5-FU. However, 5-FU treatment has also been proven effective in patients with MSI+ CRC stage IV (Liang et al., 2002). There is an ongoing clinical trial (E5202) trying to answer these controversies by studying 18q LOH and MSI in patients with stage II disease and their response to chemotherapy.

The APC gene

The initial step in the tumorigenesis is believed to be associated with the loss of the adenomatous polyposis coli (APC) gene. APC promotes degradation of β-catenin and most of the APC mutations result in a truncated APC protein and will accumulate β-catenin inside the nucleus. This modulates the downstream target genes of the Wnt pathway ultimately affecting cell proliferation, migration, differentiation and apoptosis. It is observed that more than 90% of CRC tumors have APC mutations (Thorstensen et al., 2005). The frequent existence of APC mutations in CRC would potentially make it less attractive as a prognostic marker but in CRC as a group several types of specific APC mutations exist that could potentially harbor more information about prognosis and might be clinically useful (Lovig et al., 2002). However, the clinical utility of mutations in β-catenin and APC are not sufficiently validated and therefore currently have no role in clinical practice.

**KRAS**

KRAS mutation is a relatively early step in the adenoma-carcinoma sequence following APC mutation. The most common KRAS mutation is in exon 2 at codon 12 and to a lesser extent at codon 13 (exon 2) and codon 61 (exon 3). The mutations of KRAS result in an EGFR independent activation of intracellular signaling pathways by compromising the ability of GTPase-activating proteins to affect the inactivating hydrolysis of Ras-bound GTP to GDP. This activation results in increased tumor cell proliferation, protection against apoptosis, increased invasion and metastasis, and activation of tumor induced angiogenesis (Ciardiello and Tortora, 2008). KRAS mutations are present in about one third of all CRCs and have been associated with worse prognosis in patients with stage III disease (Andreyev et al., 2001). However, other studies have failed to show any prognostic significance of KRAS mutations in CRC (Ince et al., 2005; Ogino et al., 2009b; Samowitz et al., 2000; Wang et al., 2003), and currently there is no clear evidence suggesting KRAS mutations as an independent prognostic marker.
The predictive role of KRAS mutations was first observed in non-small-cell lung cancer and EGFR inhibitor treatment (Eberhard et al., 2005). More recently KRAS mutations in codon 12 and 13 were found to be a predictor of poor response to treatment with EGFR-specific antibodies in patients with CRC stage IV disease (Amado et al., 2008; Karapetis et al., 2008; Papamichael et al., 2009). Whether other mutations as well as all mutations in codon 13 indicate poor response is not known (ASCO, 2011). Patients with V600 mutations in BRAF (a serine/threonine kinase signaling downstream from KRAS) also showed a poor response to EGFR inhibitors (Di Nicolantonio et al., 2008), although this is not universally agreed upon (Van Cutsem et al., 2011). KRAS fulfills many of the criteria for being an excellent predictive biomarker: the mutation is well defined and easily detectable at a limited part of a gene, the negative predictive value for the response to EGFR inhibition is almost 100%, and the mutations are based on a plausible biological rationale. There is a strong negative association between BRAF and KRAS mutations and they have been proven to be mutually exclusive (Rajagopalan et al., 2002), supporting that they exert equivalent effects in the tumorigenesis.

**TP53**

TP53 is a tumor suppressor gene and is demonstrated to be mutated in approximately 50% of all CRC. Its inactivation is a late event in the adenoma-carcinoma progression and usually coincides with the transition from adenoma to an invasive adenocarcinoma. The results of the prognostic or predictive significance of TP53 in CRC have not been consistent and several studies demonstrate conflicting data. This in part could be a result of insufficient power of the individual studies or the varying study designs with different methods assessing TP53 mutations limiting further comparison between studies (Munro et al., 2005; Russo et al., 2005). However there is some evidence that TP53 mutations have a different incidence depending on the tumor location in the large bowel with a higher frequency of mutations in distal colon and rectal tumors than proximal tumors (Goh et al., 1999; Hamelin et al., 1994).

**Loss of 18q**

Somatic loss of the long arm of chromosome 18 (18q) containing genes associated with CRC (SMAD4 and SMAD2) is linked to poor outcome and response to 5-FU (Bertagnolli et al., 2009; Boulay et al., 2002; Popat et al., 2005; Watanabe et al., 2001). However, the prognostic and predictive value of the loss of 18q has been questioned since some studies have failed to demonstrate a clear correlation between loss of 18q and SMAD4 expression
(Alazzouzi et al., 2005). One explanation of this could be that loss of 18q is rather a result of CIN and not an independent somatic prognostic marker.
Aims of the thesis

The overall aim of this doctoral research was to analyze prognostic and predictive markers in CRC and to investigate a new tool for the detection of CRC. The specific aims were to:

- evaluate a new method for DNA sampling from the rectal mucosa for the detection of CRC or any clinically significant pathology in the colon and rectum (Study I);

- evaluate the prognostic value of GDF15 in patients with CRC (Study II);

- molecularly characterize and evaluate the potential prognostic value of BRAF, PIK3CA, KRAS and MSI status in patients with disease stages II and III CRC patients (Study III), and

- identify the predictive value of pre-treatment carcinoembryogenic antigen (CEA) and other clinical and histopathological factors for pCR in non-smoking and smoking patients receiving preoperative CRT for rectal cancer (Study IV)
Patients

The studies included in this thesis are conducted on four different cohorts of patients (table 2).

Table 2. Patients included in Studies I-IV

<table>
<thead>
<tr>
<th>Study</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study I</td>
<td>Patients scheduled for colonoscopy (n=185) and patients scheduled for colon resection because of a suspected cancer (n=62) at Uppsala University Hospital</td>
</tr>
<tr>
<td>Study II</td>
<td>A population based cohort of 320 patients with CRC from the Central district Hospital in Västerås</td>
</tr>
<tr>
<td>Study III</td>
<td>Patients with stage II and III CRC (n=73) from Uppsala University Hospital and Västerås Central District Hospital</td>
</tr>
<tr>
<td>Study IV</td>
<td>Patients operated for rectal cancer and received neoadjuvant chemoradiation therapy in the Twin Cities Area in Minnesota, USA (n=449)</td>
</tr>
</tbody>
</table>

Study I

Between May 2006 and November 2007 patients referred for colonoscopy (group 1) or scheduled for colon resection because of a suspected cancer (group 2) at Uppsala University Hospital were asked to participate in the study. In group 1, indications for colonoscopy were chiefly gastrointestinal symptoms, history of CRC, or colonoscopic surveillance after CRC resection. In group 2, patients were scheduled for surgery because of a biopsy-verified CRC or a radiological finding suggesting malignancy. Patients with a history of pelvic radiation, chemotherapy within the last month, or any intervention (including bowel enema, colon biopsy or colonoscopy) during the past 15 days or alcohol consumption in the last 48 hours were excluded. Patients with whom we experienced difficulties with the test device were also excluded from the study. Patients in group 1 had a colonoscopy performed within one month after the test procedure. If cecal intubation failed during colonoscopy patients were followed by CTC or DCBE.
Study II

A population based cohort of 320 patients with CRC treated at the Central district Hospital in Västerås between August 2000 and December 2003 were prospectively included in the study. The mean follow-up time in surviving patients was 6 years (8-10). The Clinical Database for Colorectal Cancer at the Regional Oncologic Center in Uppsala/Örebro and surgical and oncology records were used to obtain information about cancer recurrence, cause of death and the use of neo-adjuvant/adjuvant treatment.

Study III

Patients operated for CRC between January 1985 and May 2002 at Uppsala University hospital with fresh frozen tissue samples available (n=300) and patients operated for CRC between August 2000 and December 2003 at Västerås central district hospital (n=320) were identified. Patients with a history of preoperative therapy or with a pathology report suggesting a non-radical resection margin (R1 or R2 resection) were excluded. From this cohort including 620 patients, patients with stage 2 and 3 were selected and stratified in respect to good prognosis (no recurrence during the follow-up time, mean=6.75 years), and bad prognosis (local recurrence or distant metastasis from CRC). In order to qualify, at least 12 lymph nodes should be analyzed, the tumor cell content should be at least 50% in the frozen tissue block and frozen normal tissue should be available. We aimed at a minimum of 20 patients in each stratum, the limit set by the number of recurrences in stage II. Because of these requirements we did not quite fulfill the primary goal of 20 patients in each group and in total only included 73 patients (figure 3).

Figure 3. Stratification of patients with stage II and III CRC in respect to good prognosis (no recurrence during the follow-up time, and bad prognosis (local recurrence or distant metastasis from CRC)).
Study IV

Patients diagnosed between 1998 and 2009 with primary rectal adenocarcinoma without any evidence of distant metastasis and operated with radical total mesorectal excision (TME) were identified (n=1230). Four hundred and forty-nine patients met the criteria for inclusion in the study. Patients with any of the following were excluded: no neoadjuvant CRT (n=720) history of pelvic radiation treatment (n=2), previous transanal endoscopic microsurgery or polypectomy of the primary lesion (n=15), concurrent malignant tumor (n=14), and no information about pre- or post-treatment T-stage in the chart (n=30).
Methods

Study I
DNA collection

A new dedicated device for standardized exfoliated cell collection from the rectal mucosa was used (figure 4). The device consists of one balloon-holding end that is introduced through a proctoscope into the rectum. By inflation of the balloon with a fixed air volume the cell-collecting membrane is brought into contact with the rectal mucosa providing for exfoliated cell collection. The membrane with collected material is retracted into the device following a 10-s long inflation allowing exfoliated cells to adhere to the membrane (figure 5). The device is then removed from the proctoscope. A cell lysis buffer is added in a standardized fashion and collected into a buffer-containing cap for DNA isolation and quantification.

Figure 4. Device for direct cell collection from the surface of rectal mucosa.
Figure 5. (A) Initial state with proctoscope inserted into the rectum; (B) The cell collection device is inserted via the proctoscope; (C) The plunger pushed, elastic nitrile membrane is inflated; (D) The outer surface of the balloon makes direct contact with rectal mucosa (cell collection state); (E) The plunger pulled back; (F) Membrane retracted inside the device. A cap containing a cell lysis buffer is mounted on the device in order to preserve and stabilize the DNA content in the sample.
DNA isolation and quantification

DNA was isolated from 120 µl of cell lysate using QIAamp DNA Mini Kit (QIAGEN, Germany). Quantification was performed using Invitrogen-I T™ PicoGreen® dsDNA assay kit (Invitrogen, USA) and GENios fluorescent microplate reader at wavelengths 485 nm (excitation) and 535 nm (emission) according to the instructions provided with the kit. The final DNA score reflected DNA concentration (µg/ml) recalculated for the original lysate.

Fecal contamination assessment

The fecal contamination in the samples were estimated by measuring optic absorbance of 100 µl of each cell lysate at wavelength 340nm using a GE-Nios fluorescent microplate reader (Tecan, Switzerland). The contamination degree was estimated by values in optical absorbance units as follows: low (≤0.9), moderate (1.0-1.9) and heavy (≥2).

Study II

TMA construction, immunohistochemistry and annotation

Tissue microarrays (TMA) were constructed from H&E stained tumor specimens using a manual arraying device (MTA-1, Beecher Instruments). All the specimens were histopathologically re-evaluated by one pathologist (KJ) and 1.0 mm cores from representative areas of normal mucosa, invasive tumor, and when present, adenomatous tissue and lymph node metastases were extracted.

Immunohistochemistry (IHC) was performed on 4 µm TMA sections using HPA01191 (Atlas Antibodies, Stockholm, Sweden) as the primary antibody to detect GDF15. The GDF15 antibody HPA01191 was generated within the Swedish Human Protein Atlas project (http://www.proteinatlas.org) (Uhlen et al., 2005). Automated IHC was performed on TMAs as previously described (Paavilainen et al., 2010) and subsequently scanned in high-resolution scanners and separated into individual spot images representing different cores in the TMAs. Annotation of spot images were performed estimating the intensity of immunoreactivity for GDF15 (negative [0], weak [1], moderate [2], or strong [3]) and fraction (%) of GDF15 positive cells (<1% [0], 1-24% [2], 25-75% [3]).

Plasma GDF15 analyses

Pre-surgery plasma levels of GDF15 were analyzed by using a modified protocol of solid phase proximity ligation assay (SP-PLA) previously described by Darmanis et al. (Darmanis et al., 2010).
Study III

DNA was extracted from ten µm sections of fresh frozen tumor tissue using QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s recommendation for DNA purification from tissue. The quality and concentration of the extracted DNA was determined using a NanoDrop instrument (Thermo Scientific, Wilmington, DE).

Mutation analyses

Pyrosequencing analysis

PyroMark Q24 BRAF and KRAS v2.0 assays (QIAGEN) was used for Pyrosequencing analysis of BRAF and KRAS mutations. Ten ng genomic DNA were used for each µl PCR reaction of BRAF codon 600, KRAS codons 12/13 and 61; and PIK3CA codons 542/545/546 and 1043/1047. Eight (PIK3CA) or 20 µl (BRAF and KRAS) of each PCR product was subjected to Pyrosequencing analysis using Streptavidin Sepharose High Performance (GE Healthcare, Uppsala, Sweden), PyroMark Gold Q96 reagents, PyroMark Q24 1.0.9 software, and a Q24 instrument (QIAGEN). The existence of identified mutations was confirmed with a second run of analysis.

MSI analysis

MSI analyses were performed with MSI Analysis System, version 1.2 (Promega, Madison, WI). MSI status was defined by the instability in five different mononucleotide repeat markers ((BAT25, BAT26, NR-21, NR-24 and MONO-27). Tumors demonstrating instability in two or more of the five markers were denoted as MSI-High (MSI-H), and MSI-Low (MSI-L) if only one marker showed instability.

Study IV

A retrospective review of charts from patients diagnosed and operated for rectal cancer in the Twin Cities Area was performed. Rectal cancer was defined as a biopsy proven adenocarcinoma with a distal margin at least 15 cm proximal to the anal verge. Initial tumor stage was assessed prior to CRT by endorectal ultrasound (ERUS), MRI, CT, flexible sigmoidoscopy/colonoscopy, chest X-ray and PET-CT. All patients were evaluated with a physical exam and proctoscopy. Tumor height and length were estimated by proctoscopy and the extent of circumferential tumor growth, tumor depth by direct measure with ERUS. If ERUS were not performed or missing then estimates from MRI or colonoscopy were used. CEA levels were determined before initiation of CRT. Smoking status and the number of smoked cigarettes per week were collected from a standardized initial visit form filled in
by the patients prior their initial surgical consultation and also from chart notes taken by the treating oncologist and surgeon at the first visit. If the number of cigarettes per week differed between these sources, an average was calculated and used in the final analysis.

Patients received radiotherapy with concurrent chemotherapy. During the study period two different standardized protocols for the radiation treatment were implemented. For the patients receiving radiotherapy between 1998-2005 (50% of the patients), a four field technique (posterior-anterior and two lateral fields) was used to deliver 15 to 18 megavolt photons at 1.8 Gy/day for five days per week for 5-6 weeks followed by a perineal boost. Patients treated between 2005-2010 (50% of the patients) received intensity modulated radiation therapy utilizing 6 megavolt photons to limit the radiation to surrounding organs and tissues. The delivered target dose for both of these protocols was 45 Gy to the rectal tumor with a boost of 5.4 Gy limited to the mesorectum. A mean radiation dose of 50 Gy (range, 19 to 59.4 Gy) over a mean duration of 5.6 weeks was given. Thirty nine patients (9%) also received an additional boost restricted to the tumor, receiving up to a total of 54 Gy.

Concurrent 5-fluorouracil (5-FU) based therapy was administered either as a continuous intravenous infusion during the initial five weeks of radiotherapy (42%) or as a bolus infusion (48%) or oral 5-FU (10%) throughout the radiotherapy.
Statistical methods

Study I
The test was evaluated for its usefulness in detecting adenocarcinoma alone, or for detecting inflammation, adenoma (>1cm) and adenocarcinoma as a group (i.e. any significant pathology). All the analyses were performed on all the samples regardless of the degree of fecal contamination, and then conducted again after exclusion of samples with heavy contamination. The test specificity, sensitivity, PPV, NPV, pos LR and neg LR were calculated using the DNA cut-off levels 1.5, 2.0 and 2.5 µg/ml. The DNA cut-off levels for a positive result were estimated from previous pilot studies not yet published. Receiver operating characteristic (ROC) curves were constructed. The Mann-Whitney U test was used to compare group 1 and group 2 regarding degree of contamination.

Study II-III
The dependent variables in study II and III were overall survival, time to recurrence (TTR) of CRC and in study III also cancer specific survival (CSS). Overall survival was measured from the time of surgery to the time of death irrespective of cause. TTR was calculated as time to any event related to the same cancer (Punt et al., 2007). Deaths from other cancers, non-cancer related deaths, treatment related deaths and loss to follow-up were censored. CSS was measured from the date of surgery to the date of death in CRC; the observations were censored at death from other causes than CRC and at the end of the study period. Second primary same cancers and other primary cancers were ignored. All observations were censored at the end of the study period in study II 15th April 2010 and in Study III 1th November 2008 (Västerås cohort) and 31st December 2009 (Uppsala cohort).

Study II
The independent variables in this study were intensity and fraction of GDF15 staining in the tumor tissue and GDF15 levels in plasma. The Chi-square test was used to analyze all categorical data, and all the p values were
two-sided and considered statistically significant at p<0.05. When comparing the intensity and fraction of GDF15 staining between normal mucosa and tumor tissue the Wilcoxon Matched Paired Sign test was used. Kaplan-Meier curves were used for survival analyses and the comparison of strata was performed with the Log-Rank test. Independent variables with a p-value less than 0.1 were included in a multivariate Cox-regression analysis.

Because of the skewed distribution of the plasma levels of GDF15 medians were used instead of means. Kruskal-Wallis ANOVA test was used to compare the medians of GDF15 plasma levels between different disease stages and the Mann-Whitney U test to compare the medians of GDF15 plasma levels within each disease stage.

Study III

The independent variables in this study were the existence of MSI status, BRAF, KRAS and PIK3CA mutations in the resected tumor specimen. The Chi-square test was used for comparison between categorical parameters and to test differences in proportions between groups. The Mann-Whitney U test and Kruskal-Wallis test was used for comparisons of non-parametric parameters between two groups or multiple groups respectively.

Study IV

The association between categorical variables was evaluated for significance by chi-square test. Univariate and multivariate analyses were performed using student’s t-test and logistic regression to identify continuous predictors for the endpoints of pCR, tumor downstaging and tumor size reduction. For all analyses a p-value <0.05 was considered significant. Data were analyzed using STATISTICA software (version 7.1, StatSoft Inc, Tulsa, OK, USA).

Sample size calculation

A sample size calculation was performed for both the primary (pCR) and secondary endpoint (tumor downstaging) to detect a difference in CEA (≤5 or >5 ng/mL) with a power of 80% and a two sided α-level of 0.05 respectively. The estimated effect sizes were achieved for pCR and tumor downstaging from a study by Park et al (Park et al., 2006). The statistical software “R” was used for all sample size computations.

Sample size calculation for pCR

No study using CEA cut-off 5ng/mL with the endpoint pCR was found. For that reason, the estimate of the incidence proportion of complete responders in patients with CEA levels ≤5ng/ml and > 5ng/ml were based on a previous
report by Park et al. using the endpoints of “good responders” (complete or near complete response) and “poor responders” (partial or no response). The proportion of patients in each group was assumed to be equal. The estimated total number of patients needed in the study to demonstrate a difference in pCR and tumor downstaging between patients with CEA levels ≤ 5ng/ml and > 5ng/ml were 266 and 78 patients respectively (table 3).

<table>
<thead>
<tr>
<th>Sample size calculation for pCR</th>
<th>Sample size calculation for tumor downstaging</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R-code:</strong></td>
<td><strong>R-code:</strong></td>
</tr>
<tr>
<td><code>power.prop.test(power=.80,p1=.24,p2=.11)</code></td>
<td><code>&gt;power.prop.test(power=.80,p1=.58,p2=.27)</code></td>
</tr>
<tr>
<td>Two-sample comparison of proportions power calculation</td>
<td>Two-sample comparison of proportions power calculation</td>
</tr>
<tr>
<td><strong>n = 132.9193</strong></td>
<td><strong>n = 38.71794</strong></td>
</tr>
<tr>
<td><strong>p1 = 0.24</strong></td>
<td><strong>p1 = 0.58</strong></td>
</tr>
<tr>
<td><strong>p2 = 0.11</strong></td>
<td><strong>p2 = 0.27</strong></td>
</tr>
<tr>
<td><strong>sig.level = 0.05</strong></td>
<td><strong>sig.level = 0.05</strong></td>
</tr>
<tr>
<td><strong>power = 0.8</strong></td>
<td><strong>power = 0.8</strong></td>
</tr>
<tr>
<td><strong>alternative = two.sided</strong></td>
<td><strong>alternative = two.sided</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total number needed 2n=266 patients</th>
<th>Total number needed 2n=78 patients</th>
</tr>
</thead>
</table>

**Table 3. Sample size calculation for pCR and tumor downstaging**
Ethics

Study I
The study was approved by the regional ethical committee (Ref number: 2006:059)

Study II
Ethical approval (Ref number: 00-001) was obtained from the Ethics committee at Uppsala University, Uppsala, Sweden.

Study III
Ethical approval (Ref Number: 00-001) was obtained from the Ethics committee at Uppsala University, Uppsala, Sweden.

Study IV
Ethical approval (IRB Code Number:1011M92896) was obtained from the local Institutional Review Board.
Results

Study I

One hundred and eighty-five patients of 303 in group 1 and 62 of 68 patients in group 2 agreed to participate in the study. After excluding patients because of test problem, alcohol intake the day before the test date, bowel preparation, and preoperative chemotherapy or radiation therapy a total of 153 and 42 patients remained in group 1 and 2 respectively (figure 6).

The distribution of the contamination degree in each group is listed in table 4. The sensitivity, specificity, PPV, NPV, PosLR and NegLR for using the test for the detection of CRC or any pathology are listed in table 5, and also in table 6 with the exclusion of heavily contaminated samples. The detection of CRC in group 1 after exclusion of heavily contaminated samples demonstrated a sensitivity for the DNA cut-off levels 1.5, 2, and 2.5 g/ml of 100%, 80% and 60%, and a specificity of 37%, 46% and 56%, respectively. In group 2, for the same cut-off levels, the sensitivity was 73%, 61% and 55% and the specificity was 67%, 67% and 67%, respectively. ROC curves for group 1 and 2 are presented in figure 7.
### Table 4. The contamination degree in groups 1 and 2

<table>
<thead>
<tr>
<th>Contamination</th>
<th>Low (0-0.99)</th>
<th>Moderate (1.0-1.99)</th>
<th>Heavy (&gt;2.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>103 (67%)</td>
<td>27 (17%)</td>
<td>23 (15%)</td>
</tr>
<tr>
<td>Group 2</td>
<td>27 (64%)</td>
<td>9 (21%)</td>
<td>6 (14%)</td>
</tr>
</tbody>
</table>

### Table 5. Results of using the test instrument to detect any pathology (CRC, adenoma, inflammation) or no pathology (path/no path) or to detect CRC or no CRC (Ca/no ca)

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Cut off value</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
<th>PosLR</th>
<th>NegLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca/no ca</td>
<td>pico DNA 1.5 µg/ml</td>
<td>100%</td>
<td>31%</td>
<td>0.05</td>
<td>1.00</td>
<td>1.45</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.0 µg/ml</td>
<td>80%</td>
<td>39%</td>
<td>0.04</td>
<td>0.98</td>
<td>1.32</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.5 µg/ml</td>
<td>60%</td>
<td>48%</td>
<td>0.04</td>
<td>0.97</td>
<td>1.15</td>
<td>0.83</td>
</tr>
<tr>
<td>Path/no path</td>
<td>pico DNA 1.5 µg/ml</td>
<td>60%</td>
<td>48%</td>
<td>0.04</td>
<td>0.97</td>
<td>1.15</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.0 µg/ml</td>
<td>89%</td>
<td>48%</td>
<td>0.42</td>
<td>0.86</td>
<td>1.60</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.5 µg/ml</td>
<td>78%</td>
<td>56%</td>
<td>0.44</td>
<td>0.84</td>
<td>1.81</td>
<td>0.44</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Cut off value</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
<th>PosLR</th>
<th>NegLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca/no ca</td>
<td>pico DNA 1.5 µg/ml</td>
<td>77%</td>
<td>67%</td>
<td>0.97</td>
<td>0.18</td>
<td>2.31</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.0 µg/ml</td>
<td>67%</td>
<td>67%</td>
<td>0.96</td>
<td>0.13</td>
<td>2.00</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.5 µg/ml</td>
<td>59%</td>
<td>67%</td>
<td>0.96</td>
<td>0.11</td>
<td>1.77</td>
<td>0.62</td>
</tr>
<tr>
<td>Path/no path</td>
<td>pico DNA 1.5 µg/ml</td>
<td>74%</td>
<td>n/a</td>
<td>1.00</td>
<td>0.00</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.0 µg/ml</td>
<td>64%</td>
<td>n/a</td>
<td>1.00</td>
<td>0.00</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.5 µg/ml</td>
<td>57%</td>
<td>n/a</td>
<td>1.00</td>
<td>0.00</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

### Table 6. Results (after exclusion of heavily contaminated samples) of using the test instrument to detect any pathology (CRC, adenoma, inflammation) or no pathology (path/no path) or to detect CRC or no CRC (Ca/no ca)

<table>
<thead>
<tr>
<th>Group 1</th>
<th>Cut off value</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
<th>PosLR</th>
<th>NegLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca/no ca</td>
<td>pico DNA 1.5 µg/ml</td>
<td>100%</td>
<td>37%</td>
<td>0.06</td>
<td>1.00</td>
<td>1.58</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.0 µg/ml</td>
<td>80%</td>
<td>46%</td>
<td>0.06</td>
<td>0.98</td>
<td>1.49</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.5 µg/ml</td>
<td>60%</td>
<td>56%</td>
<td>0.05</td>
<td>0.97</td>
<td>1.36</td>
<td>0.07</td>
</tr>
<tr>
<td>Path/no path</td>
<td>pico DNA 1.5 µg/ml</td>
<td>87%</td>
<td>45%</td>
<td>0.39</td>
<td>0.89</td>
<td>1.57</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.0 µg/ml</td>
<td>79%</td>
<td>55%</td>
<td>0.42</td>
<td>0.86</td>
<td>1.78</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.5 µg/ml</td>
<td>68%</td>
<td>65%</td>
<td>0.45</td>
<td>0.83</td>
<td>2.09</td>
<td>0.48</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2</th>
<th>Cut off value</th>
<th>Sens</th>
<th>Spec</th>
<th>PPV</th>
<th>NPV</th>
<th>PosLR</th>
<th>NegLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca/no ca</td>
<td>pico DNA 1.5 µg/ml</td>
<td>73%</td>
<td>67%</td>
<td>0.96</td>
<td>0.18</td>
<td>2.18</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.0 µg/ml</td>
<td>61%</td>
<td>67%</td>
<td>0.95</td>
<td>0.13</td>
<td>1.82</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.5 µg/ml</td>
<td>55%</td>
<td>67%</td>
<td>0.95</td>
<td>0.12</td>
<td>1.64</td>
<td>0.68</td>
</tr>
<tr>
<td>Path/no path</td>
<td>pico DNA 1.5 µg/ml</td>
<td>69%</td>
<td>n/a</td>
<td>1.00</td>
<td>0.00</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.0 µg/ml</td>
<td>58%</td>
<td>n/a</td>
<td>1.00</td>
<td>0.00</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>pico DNA 2.5 µg/ml</td>
<td>53%</td>
<td>n/a</td>
<td>1.00</td>
<td>0.00</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Study II

The intensity of GDF15 expression was significantly higher in tumor tissue compared to normal mucosa (p<0.001), whereas the fraction of GDF15 expression was not significantly different (p=0.19). Tumors with moderate or high intensity of GDF15 expression were less likely to have vascular inva-
sion (p=0.036), and no other histopathological or clinical parameters were correlated with the intensity or fraction of GDF15 in the tumor.

Patients curatively treated for CRC in stages I-III or in stage III with moderate to high intensity of immunoreactivity for GDF15 had a higher recurrence rate and shorter TTR compared to patients with no or low intensity for GDF15 (figure 8a and 8b).

Plasma levels of GDF15 were available for 57 patients (28 with recurrent disease, 28 without recurrent disease and 8 with stage IV). Patients with high plasma levels of GDF15 had a shorter time to recurrence (p=0.041) and a shorter overall survival (p=0.002) in the univariate analysis, and this remained significant for overall survival in multivariate analysis (HR 2.11; 95% CI 1.04-4.28) (figure 9).

Figure 8a.
Figure 8b.

Figure 8. Time to recurrence according to the intensity of immunoreactivity of GDF15 in the primary invasive tumor tissue in patients curatively operated for CRC stage stages I-III (8a) and III (8b).

Figure 9. Overall survival according to the median GDF15 plasma levels in patients operated for CRC stage stages I-IV (n=57). The multivariate Cox proportional analysis include gender, hereditary for CRC, N-substage and neural invasion.
Study III

The most frequent mutation in the cohort was KRAS mutation (32%) followed by BRAF and PIK3CA mutations. Twenty-two percent was MSI-H (table 7). The occurrence of any of the analyzed mutations (BRAF, KRAS and PIK3CA) was more frequent when the tumor was localized in the colon (98%) versus the rectum (2%) (p=0.0149). MSI-H was associated with female gender (p=0.0376), larger tumor size (>5cm) (p=0.014) and BRAF mutation (p<0.0001). Mutations in BRAF and KRAS were mutually exclusive (figure 10). MSS tumors revealed a higher grade of differentiation (well-moderate versus poor differentiation (p=0.002) (table 8). BRAF and KRAS mutations were mutually exclusive and 63% of all MSI-H tumors were BRAF mutated.

Table 7. The frequency of mutations in the whole cohort

<table>
<thead>
<tr>
<th>Mutation</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRAS mut</td>
<td>23 (32%)</td>
</tr>
<tr>
<td>BRAF mut</td>
<td>17 (23%)</td>
</tr>
<tr>
<td>MSI-H</td>
<td>16 (22%)</td>
</tr>
<tr>
<td>PIK3CA mut</td>
<td>7 (10%)</td>
</tr>
</tbody>
</table>

Figure 10. Number of mutations of KRAS, BRAF, PIK3CA and MSI-H in the entire cohort.
Table 8. Distribution of MSI mutations

<table>
<thead>
<tr>
<th></th>
<th>MSI-H (n=16)</th>
<th>MSS (n=57)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4 (25%)</td>
<td>31 (54%)</td>
<td>P=0.0376</td>
</tr>
<tr>
<td>Female</td>
<td>12 (75%)</td>
<td>26 (46%)</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5cm</td>
<td>3 (19%)</td>
<td>30 (53%)</td>
<td>p=0.0137</td>
</tr>
<tr>
<td>&gt;5cm</td>
<td>13 (81%)</td>
<td>26 (46%)</td>
<td></td>
</tr>
<tr>
<td>Not available</td>
<td>1 (1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>9 (47%)</td>
<td>7 (10%)</td>
<td>P=0.0021</td>
</tr>
<tr>
<td>Well-Moderate</td>
<td>10 (43%)</td>
<td>46 (63%)</td>
<td></td>
</tr>
<tr>
<td>BRAF status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation</td>
<td>10 (63%)</td>
<td>7 (12%)</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td>Wild type</td>
<td>6 (37%)</td>
<td>50 (88%)</td>
<td></td>
</tr>
<tr>
<td>KRAS status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutation</td>
<td>1 (1%)</td>
<td>22 (30%)</td>
<td>p=0.0139</td>
</tr>
<tr>
<td>Wild type</td>
<td>15 (21%)</td>
<td>35 (48%)</td>
<td></td>
</tr>
</tbody>
</table>

BRAF mutations were more common in patients with stage III disease without recurrence (n=7) compared to in patients with stage III with recurrence (n=2) (p=0.034) (table 9). In stage II BRAF mutated tumors had a higher percent of MSI than in stage III. No difference was observed in the frequency of KRAS, PIK3CA mutations and microsatellite instability between patients with stage II and III or between patients with or without recurrence.

Table 9. Mutation frequency in tumors from patients with CRC stage II and III stratified by recurrence.

<table>
<thead>
<tr>
<th></th>
<th>Stage II n=37</th>
<th>Stage III n=36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No recurrence</td>
<td>Recurrence</td>
</tr>
<tr>
<td></td>
<td>(n=19)</td>
<td>(n=18)</td>
</tr>
<tr>
<td>Total mut</td>
<td>9 (47%)</td>
<td>9 (50%)</td>
</tr>
<tr>
<td></td>
<td>p=0.873</td>
<td>p=0.873</td>
</tr>
<tr>
<td>BRAF mut</td>
<td>3 (16%)</td>
<td>5 (28%)</td>
</tr>
<tr>
<td></td>
<td>p=0.376</td>
<td>p=0.376</td>
</tr>
<tr>
<td>KRAS mut</td>
<td>5 (26%)</td>
<td>4 (22%)</td>
</tr>
<tr>
<td></td>
<td>p=0.772</td>
<td>p=0.772</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>1 (5%)</td>
<td>2 (11%)</td>
</tr>
<tr>
<td></td>
<td>p=0.515</td>
<td>p=0.515</td>
</tr>
<tr>
<td>MSI-H</td>
<td>7 (12%)</td>
<td>4 (22%)</td>
</tr>
<tr>
<td></td>
<td>p=0.331</td>
<td>p=0.331</td>
</tr>
</tbody>
</table>

In the survival analyses patients with CRC in stage III and BRAF mutation had a better CSS compared to patients in stage III without BRAF mutation (p=0.0434) (figure 11), and only a non-significant trend of shorter TTR was observed (p=0.054). This difference was not seen for stage II patients.
Study IV

The mean age in the study population was 59 years old (range, 25-90 years), and there were 296 (66%) men and 153 (34%) women. In 384 (86%) of the patients endorectal ultrasound (ERUS) was the primary staging method. The most common preoperative T- and N-stage was T3 (81% patients) and N1 (55% patients). There were 106 (24%) smokers and 343 (76%) non-smokers.

Of the 449 patients included in the final analysis 91 patients (20%) had pCR, 85 (19%) had only microscopic residual disease in the pathology specimen and 265 patients (60%) had a lower T-stage after CRT. Forty-three patients (10%) had a higher pretreatment compared to post treatment T-stage (table 10).

Figure 11. Cancer specific survival in patients with stage III CRC stratified by BRAF mutation status.
Table 10. Tumor response to CRT defined by the endpoints pathologic complete response, tumor downstaging, and tumor size reduction.

<table>
<thead>
<tr>
<th>Tumor response to CRT</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathologic complete response (n=460)</td>
<td>91  (20%)</td>
</tr>
<tr>
<td>Microscopic residual disease</td>
<td>85  (19%)</td>
</tr>
<tr>
<td>Lower T-stage</td>
<td>265 (60%)</td>
</tr>
<tr>
<td>Higher T-stage</td>
<td>43  (10%)</td>
</tr>
<tr>
<td>Mean pre-treatment tumor size in cm (95% C.I) (n=433)</td>
<td>4.7 (4.5-4.9)</td>
</tr>
<tr>
<td>Mean post-treatment tumor size in cm (95% C.I) (n=406)</td>
<td>1.82 (1.6-2.0)</td>
</tr>
<tr>
<td>Tumor size reduction ≥75% (n=392)</td>
<td>175 (44%)</td>
</tr>
</tbody>
</table>

The percent of people smoking was not significantly different in patients with and without pCR (p=0.64). Patients with and without pCR were also similar in terms of age, gender, duration between CRT and surgery, circular extent of tumor growth, duration between completion of CRT and surgery, pretreatment T-and N-stage. CEA levels were only available for 246 patients (55%) of the patients included in the study. Small pretreatment tumor size 4.2 cm (95% C.I 3.8-4.6 cm) vs. 4.8 cm (95% C.I 4.6-5.0 cm) and a low CEA level 3.4 ng/ml (95% C.I 2.3-4.6 ng/mL) vs. 10.0 ng/mL (95% C.I 7.2-12.5 ng/ml) were significant predictors for pCR. There was a non significant trend of more patients who interrupted or had a break in their chemotherapy in the group who did not develop pCR (p=0.05) (table 11).

Table 11. Predictors of pathological complete response

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Pathologic Complete response [95% C.I]</th>
<th>Not pathologic complete response [95% C.I]</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (n=449)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>61 (66%)</td>
<td>235 (66%)</td>
<td>0.93</td>
</tr>
<tr>
<td>Female</td>
<td>31 (34%)</td>
<td>122 (34%)</td>
<td></td>
</tr>
<tr>
<td>Age (n=449)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>58.4 [56-61]</td>
<td>59.0 [58-60]</td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td>Pretreatment CEA (n=246)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.4 [2.3-4.6]</td>
<td>10.0 [7.5-12.5]</td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>Smoker (n=463)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20 (22%)</td>
<td>86 (24%)</td>
<td>0.64</td>
</tr>
<tr>
<td>No</td>
<td>72 (78%)</td>
<td>271 (76%)</td>
<td></td>
</tr>
<tr>
<td>Pretreatment tumor height (n=445)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.9 [6.3-7.5]</td>
<td>6.6 [6.3-6.9]</td>
<td></td>
<td>0.41</td>
</tr>
<tr>
<td>Pretreatment circumferential tumor extent (n=413)</td>
<td>57.0[52-62]</td>
<td>61.5[59-64]</td>
<td>0.15</td>
</tr>
<tr>
<td>Pretreatment tumor size (n=433)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.2 [3.8-4.6]</td>
<td>4.8 [4.6-5.0]</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Duration CRT-op (n=435)</td>
<td></td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>8.3 [7.3-9.4]</td>
<td>8.8 [8.0-9.7]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
When analyzing CEA as a categorical variable using the cut-off level 5 ng/ml the Odds Ratio (OR) for pCR was 0.85 (95% C.I 0.75-0.97), this was still significant in the multivariate analyses adjusting for smoking status (yes/no) OR 0.87 (95% C.I 0.77-1.0). However, when adjusting for number of cigarettes smoked per week pretreatment levels of CEA were not longer a significant predictor of pCR OR 0.84 (95% C.I 0.61-1.16).

When stratifying for smoking status low pretreatment CEA levels were associated with pCR in non-smokers 2.9 ng/mL (95% C.I 1.96-3.9 ng/mL) vs. 8.7 ng/mL (95% C.I 6.0-11.4 ng/mL) (p=0.018) but not in smokers 7.0 ng/mL (95% C.I 0.7-13.3 ng/mL) vs. 15.1 ng/mL (95% C.I 8.6-21.6 ng/mL) (p=0.35) (Figure 12a and b).
Figure 12a and b. CEA levels in patients with and without complete pathologic response stratified by smoking status. Non-smokers, n=199 (12a). Smokers, n=47 (12b).
Pretreatment T-stage and tumor size was significantly associated with tumor downstaging (\(p<0.001\) and \(p=0.03\)). The total number of patients with available data for the estimation of tumor size reduction \(\geq 75\%\) was 392, and no predictors were demonstrated to be significantly associated with this endpoint.
Discussion

Detection of CRC by DNA sampling of the rectal mucosa (Study I)

In this study we evaluated a novel method for sampling DNA from the rectal mucosa as a tool for detection of CRC or any significant pathology in the colon and rectum. The sensitivity and specificity of this method was demonstrated to be low and not acceptable for a potential screening test for CRC. The results most likely reflected the multifaceted characteristics of the patient group(s), which had a high prevalence of colorectal disease. We included patients already scheduled for colonoscopy, which resulted in a higher number of persons with a history of gastrointestinal symptoms or diseases as compared to those in a ‘normal’ population. Hypothetically, these patients might be different in terms of exfoliation of colonocytes into the lumen. Patients with blood in the stool, diarrhea, history of a colon resection/polypectomy, or even abdominal pain could therefore be different in terms of the DNA content in the rectal mucosa compared to the ‘normal’ population even though they had a negative finding at colonoscopy.

Two other studies have evaluated the same technique and test for detection of CRC and any significant pathology in the colon and rectum (Bajwa, 2008; Loktionov et al., 2009). They presented a sensitivity of 73% and specificity of 64% at a cut-off level of 2.0 μg/ml for the detection of CRC. One possible explanation for the higher specificity in their study could be a more narrow patient population in that they only included patients with colorectal symptoms without any history of colorectal disease. Another possible explanation of the low specificity of the test in our study is the high degree of fecal contamination in the samples. The majority of the DNA found in feces is of bacterial origin and therefore a heavily contaminated sample will give a false-positive result. Since this method of DNA sampling from the rectal mucosa is new there are no reference values, as of yet, for grouping into different degrees of contamination. Compared to the previously mentioned studies, in our study we used different cut-off values for low, moderate and heavy contamination. Exclusion of heavily contaminated samples from the analyses will not only influence the results but also make further comparisons between the studies difficult. If quantification of the collected DNA with real-time PCR is performed human DNA will be separated from bacterial DNA and potentially the problem with contaminated samples will de-
crease. However, real-time PCR was implemented late in our study and therefore not available in our analysis.

Several potential areas for improvement concerning the testing method were identified through our study. One area involves restricting the patient’s diet prior to testing. This will possibly lower the presence of feces in the rectum and minimize the fecal contamination in the test samples. However, such a restriction will reduce the applicability of the test as a screening device. Another factor possibly influencing the DNA sampling of the rectal mucosa is passing of stool in the hours immediately preceding the test, leading to an excessive shedding of potential colonocytes from the mucocellular layer (MCL). In our study we had neither dietary restrictions (apart from alcohol abstinence one day before the test) nor exclusion criteria for patients passing stool in the hours immediately preceding the test procedure. Consequently, this might have led to a higher number of contaminated samples and a decreased sensitivity of the test, respectively.

Prognostic markers in CRC (Study II-III)

Several different mutations and major oncogenic pathways have now been identified for CRC and the development and access to new methods for studying genetic mutations has remarkably increased the molecular knowledge about CRC in the last decennium.

In study II we demonstrated a higher recurrence rate in patients curatively operated for CRC stage I-III with moderate or high intensity of GDF15 expression, compared to tumors with no or low intensity of GDF15 expression. This was also demonstrated separately for patients with stage III disease, but not for patients with stage II disease. An association between immunohistochemical GDF15 expression and development of metastases and advanced stage has previously been demonstrated by Xue et al (Xue et al., 2010). They demonstrated not only an association between up-regulation of GDF15 and development of metastases but also, different from our study, an increased immunohistochemical GDF15 expression in stages III and IV compared to stages I and II. Brown et al (Brown et al., 2003) demonstrated an association between high GDF15 serum levels and a shorter time to recurrence and decreased overall survival. In our study plasma GDF15 levels were not significantly different between patients with or without recurrence in stages I-III, even though we observed a trend of higher plasma levels in patients with recurrence in stage III. However, plasma levels of GDF15 were only available for 57 patients (28 without and 21 with recurrent disease and 8 with stage IV disease) in our study and therefore the absence of plasma levels of GDF15 as a prognostic marker could have been a result of a type II error.

The only histopathological feature associated with GDF15 expression in our study was vascular invasion in the tumor. We observed a negative asso-
ciation between increased GDF15 expression and presence of vascular invasion. This is a somewhat paradoxical association since vascular invasion in the tumor is known to be an independent negative prognostic factor in CRC (Compton et al., 2000). However, divergent mechanisms of GDF15 have been demonstrated by its capability in vitro to mediate invasion and angiogenesis in endothelial cells (Ferrari et al., 2005), and at the same time function as a promoter of tumor progression and metastasis (Abd El-Aziz et al., 2007; Derynck et al., 2001; Levy and Hill, 2006). One hypothetical explanation of the conflicting results regarding the role of GDF15 in tumorigenesis can be attributed to the interaction of the tumor with the microenvironment (Albertoni et al., 2002; Krieg et al., 2010). The current belief is that GDF15 has pleiotropic effects in cancer progression by acting as a tumor suppressor inhibiting tumor growth, inducing apoptosis in early stages, while it promotes proliferation, migration, invasion, and metastasis in more advanced disease stages (Mimeault and Batra, 2010). This belief in a dual and stage-dependent role of GDF15 in tumorigenesis could potentially explain why, in our study, a high intensity of GDF15 expression was associated with a shorter time to recurrence in stage III but not in stage II disease.

In study II we used the intensity and fraction of immunoreactivity of GDF15 as a measure of GDF15 expression in CRC tumors. When interpreting the results there are some limitations related to the method of IHC itself that need to be considered. Variability in the duration and the type of fixation techniques used for the IHC might influence the outcome. In our study we estimated this variability to be low since we used the same pathology department throughout the whole study, and all tissue specimens were therefore handled using the same standardized protocol. Another important factor is the validity of the antibody used in the IHC. The antibody for GDF15 used in our IHC had a high specificity on protein array and a single band corresponding to the predicted size in kDa on the Western blot and therefore we believed that the specificity was less likely to influence our results.

In study III we investigated the frequency of BRAF, KRAS, PIK3CA mutations and MSI status in two well characterized patient groups stratified for stage and cancer recurrence. The original patient group from which we selected our cohort consisted of 620 patients from two separate hospitals in Sweden (Västerås Central District Hospital and Uppsala University Hospital). The stratification resulted in four different groups classified by stage (II or III) and recurrence (with or without recurrence). The intention of this stratification was to avoid examining the whole cohort and limit the number of mutation analyses but still possibly discover a trend in the mutation patterns between patients with and without recurrence in stage II and III. The drawback of this strategy is the decreased power resulting from the low number of patients included in each group. Additionally, we performed multiple comparisons between several variables which per se increased the like-
lihood of a random significant result. We elected not to adjust our p-values with any statistical correction method such as those described by Bonferroni since we were interested in detecting a trend in the frequency of mutations between the different groups. Consequently, when interpreting the results of this study these facts must be taken into consideration.

Our goal was to include tumors from 20 patients in each group that according to the pathology report had more than twelve analyzed lymph nodes, more than 50% tumor cells in the tumor specimen and that had not received neoadjuvant therapy. Because of these requirements we did not quite fulfill the primary goal of 20 patients in each group and in total only included 73 patients (Stage II without recurrence (n=19), stage II with recurrence (n=18), stage III without recurrence (n=17), stage III with recurrence (n=19)) (figure 3).

We demonstrated that BRAF mutations were more common in patients with stage III disease without recurrence compared to stage III patients with recurrence. These patients also had a longer CSS and a trend of increased TTR. When no stratification for recurrence was performed no significant difference in frequency of BRAF mutations were seen between stage II and III. There are, to our knowledge, no previous studies supporting any difference in the frequency of BRAF mutations in colon cancer stage II and III (French et al., 2008; Maestro et al., 2007; Roth et al., 2010) however none of these studies stratify for recurrent disease. The only known predictive value of BRAF status has been observed in MSS tumors, where BRAF mutation has been associated with a high cancer specific mortality and shorter overall survival and poor prognosis (Ogino et al., 2009b; Roth et al., 2010; Samowitz et al., 2005). These studies do not include rectal tumors and present a lower frequency of BRAF mutations in MSI tumors (7-52%) compared to our cohort (63%). We confirmed an association between BRAF and MSI status and the degree of association in MSS tumors seemed to depend on whether the patient had had recurrence or not since BRAF mutations in patients with recurrence compared to patients without a recurrence were lower (9% vs. 16%) (data not shown). MSI status is reported to be associated with better prognosis (Hawkins et al., 2001; Kalady et al., 2009) and the high proportion of MSI tumors with BRAF mutations in our study can potentially explain the disparity of our results regarding the predictive value of BRAF status. It is unlikely that inclusion of rectal tumors in our study explains the positive prognostic value of BRAF status since we observed significantly more mutations in tumors from the colon compared to the rectum and there were very few rectal tumors included (9%).
Predictive factors for complete response after CRT in rectal cancer (Study IV)

In our study low levels of CEA demonstrated to be a predictor of pCR in patients diagnosed with rectal cancer receiving CRT. This result is consistent with previous studies where CEA has been proven, independent of other clinicopathologic features, to be associated with pCR after CRT (Moreno Garcia et al., 2009; Park et al., 2009; Park et al., 2006; Yoon et al., 2007). In a study by Das et al. including 562 patients where the total number of patients with available CEA values were not specified, pretreatment CEA was only significantly associated with pCR in the univariate analysis. In contrast to other studies, we also analyzed the influence of smoking status on the predictive value of CEA and confirmed after stratifying for smoking that CEA still was significantly associated with pCR in patients that stated they did not smoke but not in patients who declared they were smokers. This result might reflect a true difference of the predictive value of CEA between smokers and non-smokers but could also be a consequence of a type II error because of the small number of smokers included in our analysis. When adjusting for number of cigarettes consumed per week as a continuous variable, instead of smoking status (yes/no), CEA was no longer significant. This finding may reflect that smoking has a dose-dependent influence on CEA as a predictor for pCR in rectal cancer patients receiving CRT. Another study by Perez et al. (Perez et al., 2009) did account for smoking in their analyses of CEA as a predictor of response to CRT and demonstrated a correlation between post-chemotherapy CEA levels and complete response but not for pre-treatment CEA levels. This study included patients with pathological complete response as well as clinical complete response and given the relatively short follow up time (median, 38 months), late recurrences could have been missed leading to an overestimation of the rate of pathological complete response in the group with clinical complete response.

A smaller pretreatment tumor size was in our study a predictor for pCR in the univariate analysis, but when adjusting for pretreatment CEA this was no longer significant. However, the clinical significance of this finding is questionable since the difference in mean tumor size between patients with and without complete response was only 0.5 centimeters, which is too small to be useful as a predictive marker in a clinical setting.

When analyzing predictors for tumor downstaging pretreatment tumor size and T-stage was significantly associated with tumor downstaging. Even though our study was adequately powered for detecting the predictive value of pretreatment CEA for tumor downstaging this was not significant. These results are different from other studies that demonstrate a value of CEA as a predictor for tumor downstaging (Das et al., 2007; Yoon et al., 2007). Arguably, tumor downstaging is not an accurate method for measuring tumor shrinkage as response to CRT since it basically describes the change of the...
location of tumor cells within the rectal wall which is not necessarily equal to the change of the actual tumor size. We did not find any predictors in our study for reduction in tumor size. There are no studies that specifically use this endpoint in the assessment of predictors for response to CRT in rectal cancer, and there are two obvious disadvantages to using this method: 1) it only measures the tumor in one of three possible dimensions; 2) it is dependent on the accuracy of the pretreatment imaging modality and the pathological assessment of the tumor size which might be difficult to assess after CRT particularly in a retrospective study design. The rationale for including reduction in tumor size as a secondary endpoint was that it gives an estimate of the response to CRT independent of the actual location of the residual tumor in the rectal wall as opposed to tumor downstaging.

One limitation in our study was that in the pathology report tumor regression grade (TRG) was not assessed. TRG is a predictor of disease-free survival after preoperative therapy in patients with rectal cancer (Vecchio et al., 2005), and independent of the preoperative staging method hypothetically provides a more accurate measure of the tumor response to CRT. Other limitations in our study are missing CEA values in a substantial proportion of the patients and the possible misclassification of smoking status or estimated number of cigarettes smoked per week. We were only able to obtain the CEA value from 246 patients of the 449 included in the study potentially introducing a selection bias. However, neither of the endpoints (pCR, down-sizing or downstaging) nor any other of the important preoperative variables (T-stage, tumor size, circumferential growth) were significantly associated with whether CEA was available from the medical record charts or not supporting that patients with missing CEA values are not different from those with available CEA values.

In conclusion we demonstrated an association between low pretreatment CEA levels and pCR in patients treated with neoadjuvant CRT for locally advanced rectal cancer. The predictive value of CEA in smokers can be limited and further studies are needed to evaluate the impact of smoking on the predictive value of CEA for pCR in rectal cancer.
Conclusions

Aim: To evaluate a new method for DNA sampling from the rectal mucosa for the detection of colorectal cancer or any clinically significant pathology in the colon and rectum (study I).

Conclusion: This novel technique is an easy and safe way of collecting DNA from the rectal mucosa. Yet, the sensitivity and specificity of the test in detecting CRC or any pathology in the colon or rectum are too low to be acceptable.

Aim: To evaluate the prognostic value of GDF15 in patients with CRC (Study II).

Conclusion: GDF15 expression has a negative prognostic value in patients curatively operated for CRC stages I-III and III disease.

Aim: To molecularly characterize and evaluate the prognostic value of BRAF, PIK3CA, KRAS and MSI status in disease stages II and III CRC patients (Study III)

Conclusion: The results indicate that patients with CRC stage III without recurrence reveal a higher frequency of BRAF mutation compared to stage III patients with recurrence

Aim: To identify the association between pre-treatment CEA levels and pCR in non-smoking and smoking patients receiving preoperative CRT for rectal cancer (Study IV)

Conclusion: In non-smokers a low CEA level is significantly associated with pCR. The use of CEA as a predictive marker for pCR in smokers treated with CRT can be limited.
Future perspectives

Screening

CRC is one of the most common cancer forms in the world and, according to WHO, CRC fulfills the criteria for being a disease that should be targeted by screening. Even though most data suggest that a screening test is warranted for CRC, the following topics need to be addressed before deciding how CRC screening should be implemented and what the ultimate screening test would be:

1) In an average risk population it is not clear at what age the initiation of screening would be appropriate;
2) It is not clear at what time-intervals the screening test needs to be repeated.
3) Even though adenomatous polyp removal has been proven to reduce CRC incidence it is not clear if this applies to all sizes of polyps and all levels of differentiation, consequently the optimal target lesion for screening is unknown.
4) It is not clear what the optimal frequency of screening is for patients with known inflammatory bowel disease, family history of CRC, or a genetic CRC syndrome.

All of the above topics need to be addressed in the context of the sensitivity and specificity of the used screening method before implementing a screening test with the goal of reducing mortality in CRC.

Ultimately, the efficacy of a screening test is dependent on patient compliance, which influences the program sensitivity of a screening test. More research in this area is required to be able to define optimal methods to improve patient compliance. This subject is closely related to a broader question that in the end will probably have the greatest impact on the risk and mortality of CRC – how can we raise the public and patient awareness of the natural course, risk-factors, presenting symptoms and interventions for screening of CRC?

When using non-invasive stool-based tests for the collection of molecular markers in CRC screening some additional challenges must be addressed. First, the reproducibility and safety of the test in a large population needs to be established. This includes standardization of handling, storage, analysis and the test procedure itself. To facilitate generalization of a test in a population it is important to simplify the test procedure as much as possible to mi-
minimize test variability and minimize misclassification bias. Secondly, CRC is a heterogenic tumor with several different genetic compositions. Hypothetically a battery with multiple markers (“fingerprint of the tumor”) or a combination of tests are required to achieve high enough sensitivity and specificity of a screening test, which ultimately also must be balanced in a cost-efficiency analysis.

Prognostic and predictive factors for colorectal cancer

There is an increasing demand for individualized therapy in almost all fields of medicine. This demand is particularly pronounced in the field of CRC because of the genetic and biologic heterogeneity of the disease. The research for hypothesis-driven prognostic and predictive markers has yielded many interesting results and hypotheses regarding the different steps in the carcinogenesis of CRC. The use of microarray technology and gene expression profiling analyses is currently the fastest expanding and probably the most promising field in identifying possible factors for prognosis and treatment response in CRC. The development of gene expression analysis has not only led to a better understanding of CRC carcinogenesis but has also facilitated the research for predictive and prognostic factors in CRC. The mapping of several thousand genes allows a multi-gene signature of the tumor to be created which can be associated with the clinical behavior of the tumor. Consequently, a shift from hypothesis-driven markers to markers derived from gene expression analyses has occurred. However, the clinical utility of all of these markers are still limited. This in part can be explained by the following factors: (1) lack of fresh tissue samples needed to perform large scale studies, (2) lack of standardized protocols regarding methodological and analytical methods and (3) difficulty to select data useful for the clinical setting. The purpose of genome-wide association analyses (GWAS) is to identify susceptibility loci for a specific trait or disease. As a result, this large-scale multiple testing of genetic markers requires huge data sets to achieve high enough statistical power to avoid a high false positive rate. Another weakness of GWAS is the inability to assess epigenetic modifications that are known to influence gene expression in tumors and by themselves have a particularly important role in CRC tumorigenesis.

In the end, to be able to assess the clinical utility of a biomarker, prospective randomized controlled clinical trials are needed. This study design, compared to observational studies using archived tissue DNA and retrospective data, will give a more informative and unbiased estimate of the clinical usefulness of the biomarkers.

Added to these limitations are the statistical challenges of these new technologies due to an inadequate sample size relative to the performed multiple
comparisons, poorly defined study hypothesis and an overuse of stepwise regression.

The treatment for rectal cancer is different in different parts of the world but patients with node positive rectal cancer or growth adjacent to the mesorectal fascia or into the levators/sphincter complex, i.e. a threatened circumferential margin unless extensive surgery is done, are generally recommended some neoadjuvant treatment before curative resection. One of the biggest challenges today is not only how to accurately identify the correct stage before CRT but also how to be able to assess tumor regression and distinguish between viable tumor cells and fibrosis/necrosis after CRT. Different centers use different staging methods, but the two most common used modalities are ERUS and MRI. Routinely, the post-treatment tumor response is not always assessed but the incentive for this might be greater along with improved imaging and a more accurate staging of the tumor.

The primary treatment for rectal cancer is surgery, however of those patients receiving CRT the rate of pCR is 15-25%. Will the primary treatment for rectal carcinoma like anal squamous carcinoma eventually also be CRT? With the development of more effective chemotherapies and radiation technique in rectal cancer tumor regression can be expected to improve. Hypothetically, we could observe an increase in the rate of pCR if patients with lower disease stages were treated with neoadjuvant CRT as well. Are we selecting the wrong patients for CRT? Should we also give neoadjuvant CRT to patients with stage II disease? These questions are hard to address in that a randomized controlled trial would be difficult to perform considering the large differences between surgery and CRT for patients both regarding morbidity and the social implications of a permanent stoma, but nevertheless it is an important question.
Summary in Swedish (Sammanfattning på svenska)

Inledning

Cancer i tjocktarm och ändtarm är den tredje vanligaste cancerformen i världen och utgör hos män den näst vanligaste och hos kvinnor den tredje vanligaste orsaken till död i cancer följt av lung- och bröstcancer. Den årliga incidensen av kolorektal cancer varierar mycket mellan olika länder med högst incidens i USA, Europa, Australien med ca 50 nya fall per 100 000 invånare jämfört med ca 1 insjuknad per 100 000 invånare i Afrika och Asien. Två tredjedelar av all kolorektal cancer är lokaliserad i tjocktarmen samt en tredjedel i rektum.

De kända riskfaktorerna för kolorektal cancer är hög ålder, polyper, hereditet, rökning, inflammatorisk tarmsjukdom samt tidigare tjock- eller ändtarms cancer. De två mest kända kolorektal cancer syndromen är familial adenomatous polyposis (FAP) och hereditary non-polyposis kolorektal cancer (HNPCC). FAP orsakas av en genmutation (APC) och patienter har vid 40 års ålder en 100% risk att utveckla kolorektal cancer. Patienter med HNPCC (Lynch syndrom) har en mutation i DNA reparations enzym (DNA missmatch repair genes), vilket medför utvecklande av flertalet polyper som i ett senare skede kan utvecklas till cancer.

Merparten av all tjock och ändtarmscancer utgår från körtelcellerna i tarmslumhinnan s.k. adenocarinom. En av de viktigaste prognostiska faktorerna för överlevnad efter operation av tjock och ändtarmscancer är tumörstadiet (TNM-stadiet) vilket bestäms av tumörens penetration i tarmslumhinnan (T-stadiet), antal körtlar med cancerväxt (N-stadiet), samt om spridning till något annat organ ägt rum (M-stadiet).

Behandling

Kirurgisk resektion utgör den viktigaste behandlingen för patienter med potentiell botbar kolorektal cancer, och val av operationsmetod beror till stor del på tumörens utbredning samt lokalisation. Kirurgiskt avlägsnande av tumören med tumör fria marginaler är den viktigaste faktorn associerad till patientens överlevnad. Valet av kirurgiskt ingrepp styrs mestadels av var
tumören är lokaliserad i tarmen. Strålbehandling med eller utan kemoterapi givet innan (neoadjuvant) eller efter (adjuvant) operation har tillsammans med införandet av dissektion utmed embryologiska plan (total mesorectal excision) signifikant förbättrat överlevnaden efter rektal cancer kirurgi. Syftet med kemoterapi är att förinta tumörceller som redan har metastaserat samt öka respons på strålbehandling. Efter genomgången operation för tjocktarms cancer med spridning till lokala lymfkörtlar (stadium III) har postoperativ fluorouracil (5-FU) baserad kemoterapi visats minska risken för recidiv, detta samband har ännu ej klart kunnat visas för behandling av rektal cancer.

Behandlings trenden för rektal cancer skiljer sig något mellan olika länder och i USA ges ofta kombinations behandling med strålning och kemoterapi medan det i Sverige ofta ges strålbehandling utan kemoterapi. Flera stora randomiserade studier har visat att strålbehandling minskar risken för lokalrecidiv och förlänger överlevnaden vid rektal cancer. Denna överlevnads vinst och minskad risk för lokal recidiv verkar vara mer uttalad om strålning ges neoadjuvant jämfört med adjuvant.

Ungefär 15-25% av alla patienter med rektal cancer som genomgår (neo-adjuvant) strålbehandling och kemoterapi innan operation svarar så bra på behandlingen så att inga tumör celler finns kvar i rektum, s.k. patologisk komplett respons (pCR). Den rekommenderade behandlingen för patienter som genomgått preoperativ strålbehandling samt kemoterapi är kirurgisk resektion, detta oavsett hur patientens respons till genomgången strålbehandling och kemoterapi är. Det finns ingen pålitlig metod för att identifiera de patienter som kommer eller har utvecklat pCR efter neoadjuvant behandling.

Kolorektal cancer screening

Kolorektal cancer är en av de vanligaste förekommande cancerformerna och utgår oftast från ett identifierbart och behandlingsbart pre-malignt tillstånd (adenom), vilket gör att kolorektal cancer rent teoretiskt är en sjukdom där incidensen skulle kunna minksas med hjälp av screening. Flertalet screening test för kolorektal cancer har föreslagits och kan i stort delas in i två kategorier; 1) avförings baserade test samt 2) strukturella test. Bland de avförings baserade testen har bara guaiac based fecal occult blood test (gFOBT) visats i flera stora randomiserade studier reducera mortalitet i kolorektal cancer. De andra avförings baserade testen (FIT, DNA mutations test) är ej lika etablerade metoder men har visats ibland ha en högre sensitivitet samt specificitet jämfört med gFOBT.

Strukturella test såsom sigmoideoskopi, kolonoskopi, kolon röntgen med dubbel kontrast samt CT kolonografi har också utvärderats som potentiella screening test. Sigmodeiskopi är begränsad till att enbart undersöka den sista delen av tarmen och därför också potentiellt missa en cancer som växer i den
icke undersökta delen av tarmen. Studier har dock nyligen visat att inciden-
sen av kolorektal cancer samt mortalitet kan minskas m h a screening sig-
modeiskopi.

Det finns inga randomiserade studier som stödjer att koloskopi är ett bra
screening instrument för kolorektal cancer. Däremot finns det indirekta bevis
på att koloskopi kan vara värdefullt screening instrument då det oftast an-
vänds som referens vid utvärderandet av andra screening test. Koloskopi är
också förenat med en icke försvarbar morbidity, vilket måste ta hänsyn till
då koloskopi utvärderas som ett potentiellt screening instrument.

Colon röntgen med dubbel kontrast har för låg sensitivitet för att kunna
användas för screening. Dessutom finns ej vid undersökningen någon möj-
lighet att åtgärda eventuella polyper, utan ytterligare utredning med kolo-
skopi måste oftast genomföras i efterhand. CT kolonografi har samma be-
gränsning som kolon röntgen med dubbel kontrast men med skillnaden att
den har både en högre sensitivitet och specificitet.

Prognostiska och prediktiva markörer för kolorektal
cancer
Kolorektal cancer är molekylärbiologiskt en mycket heterogen tumör grupp
som uttrycker flertalet olika gener. Utvecklandet av ett adenom till en cancer
beskrivs oftast som en stegvis process och involverar flertalet mutationer i
både tumör supressor gener samt oncogener. I dag så kan kolorektal cancer
molekylärbiologiskt delas in i huvudsak i tre olika grupper beroende på vilka
gener i tumören som är muterade; the Chromosomal Instability Pathway
(CIN), the Mutator Phenotype Pathway and the Hypermethylation Pathway.
Det har visats att tumörens kliniska beteende samt svar på behandling är
associerat till tumörens genetiska uttryck. Detta faktum stödjer att behand-
ling av patienter med kolorektal cancer bör individualiseras. I dag utgör
TNM stadiet tillsammans med andra kliniska parametrar de viktigaste faktor-
erna som avgör om tilläggs behandling med kemoterapi skall ges eller inte.
Även om TNM stadiet ger prognostisk information så ger det ingen prediktiv
information om hur patienten kommer att svara på behandling.

Delarbete I
En ny metod att samla DNA från rektal slemhinna för att upptäcka kolorek-
tal cancer eller annan signifikant patologi i tjock- eller ändtarmen utvärdera-
des i denna studie. Ett instrument med en ballong i ena ändan användes för
att samla upp celler från rektal slemhinna. Instrumentet fördes in i rektum
via ett proktoskop varefter ballongen expanderades och celler som fastnade
på ballongens membran samlades för DNA analys. Patienter som planerats genomgå koloskopi (n=185) samt patienter med misstänkt tjocktarms cancer (n=62) planerade för kirurgisk resektion på Uppsala Akademiska Sjukhus mellan maj 2006 och november 2007 inkluderades i studien. Sensitiviteten för detektion av kolorektal cancer var 100 %, 80 % samt 60 % med en specificitet på 37 %, 46 % samt 56 % för nivåerna 1,5, 2, samt 2,5 mängd DNA mätt i µg/ml i gruppen med misstänkt tjocktarmscancer. I gruppen som planerats för koloskopi var sensitiviteten för detektion av kolorektal cancer för samma DNA nivåer 73 %, 61 % samt 55 % med en specificitet på 67 %, 67 % samt 67 %. Sammanfattningsvis visade sig denna metod inte ha tillräcklig hög sensitivitet samt specificitet för att kunna användas som screening test för kolorektal cancer. En bidragande orsak till den låga sensitiviteten och specificiteten kan förklaras av den heterogena grupp av patienter som inkluderades i studien.

**Delarbete II**


**Delarbete III**

Syftet med detta delarbete var att molekylärt karakterisera tumörer från patienter med stadium II och III kolorektal cancer. Från en väldefinierad ur-
sprungs kohort bestående 620 patienter tidigare kurativt opererade för kolorektal cancer selekterades totalt 73 patienter med stadium II och III stratifierade efter förekomst av recidiv. Mutationsanalyser (pyrosektionsiering) av BRAF, PIK3CA, KRAS samt MSI status genomfördes på fryst tumörvävnad och analyserades avseende eventuell association till tumörlokalisation, histopatologi samt andra kliniska karaktäriska. Tumörer lokaliserade i kolon jämfört med i rektum visade sig ha högre förekomst av de i studien analyserade mutationerna, och tumörer med mikrosatellit instabilitet (MSI) hade hög frekvens av BRAF mutationer och låg förekomst av KRAS mutationer. MSI var associerat med lägre frekvens av fjärrmetastaser och BRAF mutationer var vanligare i patienter med stadium III utan recidiv jämfört med stadium III med recidiv. Patienter med kolorektal cancer stadium III samt BRAF mutation hade bättre cancer specifik överlevnad jämfört med patienter utan BRAF mutationer. Dessa resultat tyder på att BRAF mutation hos patienter med stadium III kolorektal cancer kan vara associerat till lägre recidiv risk.

Delarbete IV
Ungefär 10-30% av alla patienter som erhåller neoadjuvant tilläggsbehandling (strålning och cytostatika) innan operation för rektal cancer har ingen tumörcells kvar i rektum vid operationen, s.k. pathological complete response (pCR). Identifiering av de patienter som utvecklat pCR skulle kunna innebära att kirurgi i denna grupp potentiellt kan undvikas. I dag finns dock ingen metod att säkert identifiera patienter som utvecklar pCR efter strål- och cytostatiska behandling. Syftet med detta arbete var att identifiera kliniska och histopatologiska prediktiva faktorer för utvecklande av pCR samt att undersöka sambandet mellan Carcinoembryonic antigen (CEA) och pCR hos rökande och icke rökande patienter som erhållit strål- och cytostatiska terapi. Fyra hundrafyrtionio patienter inkluderades totalt i studien. Det kliniska tumör stadiet uppskattades med hjälp av endorektalt ultraljud, magnetkamera röntgen, datortomografi, sigmodeiskopi/kolonoskopi, lungröntgen samt PET-CT. Nittioen (20%) patienter utvecklade pCR, och 85 (19%) patienter hade enbart mikroskopisk tumör i rektum vid operation. Patienter med pCR hade storleksmässigt en mindre tumör (4.2 cm jämfört med 4.8 cm) samt lägre CEA nivå (3.4 ng/ml jämfört med 10.0 ng/ml) innan genomgången behandling jämfört med patienter som ej utvecklade pCR. Om patienterna delades in beroende på om de var rökare eller ej var CEA enbart signifikant associerat med pCR i den grupp som var icke rökare, och om antal rökta cigarett kontrollerades för i den multivariata analysen var CEA inte längre signifikant associerat till pCR. Slutsatsen av studien var att CEA är signifikant associerat hos icke rökare med pCR, men hos rökare kan CEA som prediktiv faktor vara av begränsat värde.
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