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MALIGNANT TRANSFORMATION
OF THE COLORECTAL MUCOSA IN
INFLAMMATORY BOWEL DISEASE

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ABSTRACT

Ulcerative colitis (UC) and Crohn’s disease (CD) are disorders of unknown etiology, often referred to as inflammatory bowel diseases (IBD). The increased risk of colorectal cancer (CRC) in patients with longstanding, extensive colonic IBD is an important clinical problem. Colonoscopic surveillance, annually or every second year, with multiple biopsies for histopathological detection of dysplasia, is now used to manage this increased risk at many centers for IBD. Dysplasia is a useful prognostic marker for subsequent cancer development but has limitations including substantial inter- and intra-observer variability among pathologists. Furthermore, concomitant active inflammation makes assessment and interpretation of dysplasia difficult. There is a need for more accurate and more objective markers for neoplastic transformation of the colorectal mucosa in high-risk individuals. Although long-term treatment with 5-ASA compounds seems to lower the risk of CRC in IBD, no studies using primarily preventive drugs have been performed.

The aims of this thesis were to assess the utility of other markers for malignant transformation of the colorectal mucosa and also to explore any potential chemopreventive properties of long-term oral therapy with ursodeoxycholic acid in high-risk patients with IBD.

By using monoclonal antibodies, the expression of the proliferative antigens Ki-67 and PCNA was assessed in biopsy specimens with various degrees of epithelial dysplasia and inflammatory changes. Increased staining with MIB-1 and PCNA correlated with increased severity of dysplasia but also with increased inflammation. In the absence of inflammation, immunostaining with these two antibodies may complement dysplasia, especially in the indefinite changes category.

Alkaline sphingomyelinase (SMase) hydrolyses sphingomyelin (SM) generating ceramide which is important in the regulation of cell growth. The activity of alkaline SMase activity is decreased in CRC and premalignant conditions and was also found to be decreased in IBD-patients. There was also an age-dependent decrease of alkaline SMase both in IBD-patients and controls.

Flow cytometric DNA-analyses of biopsy specimen can detect gross chromosomal aberrations and also have the ability to correctly estimate the number of cells in proliferation (S-phase). In normal colorectal mucosa it was found that the S-phase increased linearly with age and decreased from the right colon over the transverse colon to the left colon. The fraction of G2-cells increased significantly with increased S-phase fraction. No aneuploidy was detected.

In 324 UC-patients undergoing colonoscopic surveillance, the S-phase of aneuploid samples was significantly increased compared to diploid, but significantly lowered in comparison with sporadic CRC. An increase in S-phase fraction in patients with IBD without active inflammation may be an additional marker for neoplastic potential in the colorectal mucosa.

Ursodeoxycholic acid, a natural bile salt, has been associated with regression of experimentally induced neoplasia in rats. In a two year prospective, double blind randomized pilot study in high-risk IBD-patients, none of the ten patients in the treatment group had progression of dysplasia, while two of the nine patients in the placebo group were referred for colectomy due to progression of dysplasia. UDCA may exert chemopreventive action(s) in patients with longstanding IBD in the colon, but
further studies are needed in order to determine optimal selection of patients, UDCA-dose and treatment time.
LIST OF PUBLICATIONS


II  Sjöqvist U, Hertervig E, Nilsson Å, Duan RD, Öst Å, Tribukait B, Löfberg R. Chronic colitis is associated with a reduction of mucosal alkaline sphingomyelinase activity. Inflammatory Bowel Diseases 2002;8:258-263.


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<table>
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<th>Description</th>
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<tr>
<td>Alkaline SMase</td>
<td>Alkaline sphingomyelinase</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal carcinoma</td>
</tr>
<tr>
<td>DNA-FCM</td>
<td>DNA-flow cytometry</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>LGD</td>
<td>Low grade dysplasia</td>
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<td>HGD</td>
<td>High grade dysplasia</td>
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<tr>
<td>PSC</td>
<td>Primary sclerosing cholangitis</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyeline</td>
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<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
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<tr>
<td>UDCA</td>
<td>Ursodeoxycholic acid</td>
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1 GENERAL INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic, relapsing inflammatory disorders of the gastrointestinal tract of unknown etiology and variable pathology. The term IBD constitutes two major entities; ulcerative colitis (UC), where the inflammation is confined to the colorectal mucosa and Crohn’s disease (CD), where the inflammation engages the whole bowel wall, affecting any part of the gastrointestinal tract, but the ileocecal region in particular. A third group of IBD are the microscopic colitides (collagenous colitis and lymphocytic colitis) which will not be discussed any further in this thesis, as they do not appear to be associated with an increased risk for malignancy of the large intestine (1).

The major symptoms of IBD are diarrhea with or without passage of mucus and/or blood in the stools together with abdominal cramps. The symptoms are often insidious and differ between UC and CD depending on the localization and extent of the respective disease. While bloody, loose stools with urgency are characteristic for UC, abdominal cramps and fistulas are typical for CD. General symptoms like fever and malnourishment might occur in severe IBD. In addition, extraintestinal inflammatory manifestations in the eyes, joints, skin and in the liver are common in both disorders and are more abundant in extensive IBD.

Besides the clinical symptoms, the diagnosis of IBD is established with ileocolonoscopy allowing direct visual inspection of the mucosa and mucosa sampling for histological examination. In active UC, a loss of normal vascular pattern with a diffusely erythematous, friable and granular mucosa is a typical endoscopic finding. The rectum is always involved and the inflammation can spread in a continuous fashion up to the ileocecal valve. In CD the mucosal lesions are typically patchy, asymmetrical and heterogeneous. Aphthoid ulcerations surrounded by normal mucosa and longitudinal ulcerations and involvement of the ileum are diagnostic for CD. The distinction between UC and CD with only colorectal involvement may be difficult and the term indeterminate colitis is sometimes used (2). Radiological examinations of the small bowel is sometimes necessary in CD and plain abdominal radiographs are carried out in severe IBD for ruling out perforation or toxic dilatation. Cross-sectional imaging methods such as CT, MR and ultrasound are valuable in imaging abdominal abscesses, fistulas and liver disease. The new video capsule will probably be a first-line option for detecting abnormalities in the small bowel in CD-patients (3). Findings of granulomas at histopathology are pathognomonic for CD while basal plasmacytosis is common in UC.

The incidence of UC is 5 to 13 cases every year per 100,000 inhabitants (4-6) and about 4 to 6 new cases yearly per 100,000 inhabitants for CD (4, 7-9) resulting in a prevalence in Sweden of about 35,000 patients with UC and about 25,000 patients with CD. IBD is common in the Scandinavian countries, Northwestern Europe, USA and Australia. In general, these diseases affect children, adolescents and young adults with a second peak in patients 50-65 years of age (10).
Although, the etiology of IBD is not clear, there is a 10-fold higher risk in first-degree relatives to develop the same disease (CD) as the patient (11), suggesting that genetic factors play a role. Indeed, twin studies have shown a significant concordance of IBD in monozygotic compared to dizygotic twins (12). In CD a mutation in the NOD 2 gene on chromosome 16 has been found in 10-15% of the patients (13). Another interesting finding is the effect of smoking. Non-smokers and persons who have stopped smoking are prone to develop UC while smokers are more prone to develop CD (14, 15). A variety of endogenous and exogenous factors (dietary, infectious agents, drug intake, place of birth) have been considered and explored without clear conclusions. In spite of considerable efforts, the etiology and pathogenesis of IBD are still poorly understood, but a disturbance in the regulation of the mucosal immunological response is evident.

The main drugs used in the treatment of IBD are sulphasalazine and 5-aminosalicylic acid, corticosteroids, immunosuppressives, antibacterial drugs and the relatively new monoclonal antibodies against TNF-α. Sulphasalazine, originally developed by the Swedish internist Nanna Swartz in the 1940’s, is still one of the most frequently used anti-inflammatory drugs for maintenance of remission in UC. Newer compounds without the sulphapyridine molecule, which causes most of the side effects, only contain 5-aminosalicylic acid. Corticosteroids are the main therapy in patients with active disease and the use of steroids was first assessed by Truelove and Witts (16). Other steroids such as budesonide, developed in Sweden, have important advantages, particularly less side effects (17, 18). Immunosuppressants such as azathioprine with its active metabolite 6-mercaptopurine and methotrexate are all effective in chronic active IBD, and have a steroid-sparing effect as well. Antibacterial drugs such as metronidazole are used in CD. Infliximab, a chimeric monoclonal antibody against TNF-α, is the first biological therapy in IBD. Several clinical trials have shown efficacy in moderate to severe CD (19, 20) and also for maintenance of remission in CD (21). Clinical trials with infliximab for treatment of UC are in progress. Anti-adhesion molecules represent another novel class of drugs in active IBD (22) and trials, both for UC and CD, are in progress. All these drugs have a relatively narrow therapeutic range and an accurate diagnosis is therefore important before they are prescribed.

Criteria for emergency colectomy in patients with UC are toxic dilatation of the colon, severe hemorrhage or perforation. Elective surgery is considered if medical treatment fails or if the patient is at risk of colorectal cancer (CRC). The overall colectomy rate is highest during the first year (10%) of the disease and subsequently declines during the following years (23). In CD, the extent of disease at diagnosis, female gender and the presence of fistulas have the greatest impact on the risk of surgery and three out of four patients will undergo an intestinal resection of whom half will have a relapse (24).

Overall, the mortality in IBD has decreased since the middle of the 1970’s, mainly due to better medical care (25). In UC the proportion of patients having pancolitis is decreasing while the proportion of patients having proctitis is increasing, thus shifting the disease to a more benign course (26). Still, there is an increased mortality in both UC and CD compared to the general population (27, 28) and this is mostly due to the disease itself, and complications, such as bleeding, perforation, and postoperative mortality. In addition, patients with longstanding colonic IBD have an increased risk
for mortality due to CRC and in the following text aspects of this increased CRC-risk are discussed.
2 BACKGROUND OF THE PRESENT STUDIES

2.1 INTRODUCTION

The increased risk of intestinal cancer is thus one of the major problems in the long-term management of IBD. The risk of CRC in longstanding UC has been particularly in focus since the recognition of this complication in a series of cases reported by Bargen already in 1928 (29), and management strategies have been controversial ever since. The cancer-risk is predominantly a problem in relatively young patients but there are difficulties in correctly assessing the individual cancer risk and hence, there are also problems in taking appropriate actions to prevent the development of CRC. A large retrospective population-based study of 2509 UC-patients (30) showed that death due to CRC in fact was the main cause of the excess long-term mortality, thus emphasizing the clinical importance of the problem. During the last decade, some large, well-defined population-based studies have provided accurate estimates of the cancer risk in IBD, making it easier to predict individual prognosis. Our knowledge has been improved regarding the identification of certain factors indicating a particularly high cancer risk, and some protective factors have also been elucidated. Furthermore, long-term experience of colonoscopic surveillance programs is now at hands and the pros and cons of this approach to reduce the cancer risk have been scrutinized.

2.1.1 Overall cancer risk

From larger, population based studies performed in Sweden and the UK it appears that the elevated, overall cancer-risk among patients with UC (30-32) and CD (30, 31) is due predominantly to an increase of intestinal cancer. However, in an epidemiological study of malignancies in CD (33), a slight excess risk was found for small bowel carcinoma and urinary bladder carcinoma, but not for CRC. Although the absolute number is small, a subgroup of patients having UC and the hepatobiliary complication primary sclerosing cholangitis (PSC), runs a significantly increased risk of developing carcinoma of the bile ducts (34, 35) and also pancreatic malignancies (36).

2.1.2 Small bowel carcinoma in Crohn’s disease

The incidence of small bowel carcinoma among patients with CD is low, but as it is extremely uncommon in an age-matched population, the relative risk becomes significantly increased, as shown in some population-based studies (33, 37-39). Long duration of CD seems to be the most important determinant for this malignant complication (40, 41).

2.2 COLORECTAL CANCER RISK IN ULCERATIVE COLITIS

The risk of CRC is most appropriately assessed by the use of actuarial, life-table methods, when duration of disease is taken into account and the absolute cumulative risk is calculated (32, 42). Such calculations should also compensate for
proctocolectomy and death from other causes than CRC. Relative risk comparisons, given the rarity of CRC in the younger age-groups of the general population, are of limited value. Several larger population-based studies from different geographical regions have taken those considerations into account and provided consistent, independent results regarding the cumulative CRC-risk in UC. A study of 486 patients with extensive UC (i.e. involvement proximal to the splenic flexure) from three different geographical areas in England and Sweden (32) followed for a minimum of 17 years, estimated the CRC-risk to be 11.6% (CI 6.4-16.8) after 25 years from the onset of disease. This increase is approximately six to ten times higher than expected in the general population. In non-extensive UC the risk was only marginally raised (left-sided and distal UC) and not increased at all in patients with proctitis. Remarkably similar results were obtained from a large epidemiological study from Uppsala, Sweden (42) of 679 patients having extensive disease. After 25 years, the cumulative risk was 12% among patients aged 15-39 years at onset. The cumulative cancer risk in patients with left-sided UC at time of diagnosis was less than 5% after 30 years, except for those patients who got the diagnosis between 15-29 years of age who had a 12% risk. As it is well known that patients with distal and left-sided UC at time of initial diagnosis may often have a subsequent progression to more extensive involvement, it could be anticipated that a large proportion of patients in this latter group had extensive disease at the time of cancer development. In a population study from Israel (43), the cumulative risk was 13.8% in extensive UC after 20 years of duration. A prospective study from Copenhagen (44) could not demonstrate any CRC-risk increase at all for a defined UC-cohort, while another, recent Danish study (45), covering the entire country, has provided CRC-risk estimates for UC more in line with those previously reported. In a recent meta-analysis (46) the estimated CRC-risk in all patients with UC was found to be 2% at 10 years, 8% at 20 years and 18% at 30 years, irrespective of disease extent.

2.3 COLORECTAL CANCER RISK IN CROHN’S DISEASE

There are conflicting results of the magnitude of the risk of CRC in CD. Several reports have failed to demonstrate (33, 37, 47-49) while others have demonstrated (50-54) an increased CRC-risk in these patients. There have been two population-based studies, both from Sweden, addressing the CRC-risk in CD. In 1251 patients with CD, diagnosed between 1955-1984 in Stockholm County, the occurrence of CRC was not increased with a standardized morbidity ratio of 0.89 (CI 0.29-2.07) (33). The other one from Uppsala, (n=1655), demonstrated that CD-patients having substantial/extensive colonic involvement and long duration of the disease had more than five times increase of the relative risk of CRC (53). This report as well as reported from Birmingham, England (54, 55), showed that patients with extensive CD of the colon had an absolute cumulative risk of 8% after 22 years of disease duration, quite similar to the risk level previously found in extensive UC (32). In a recent population-based study from Canada (39), the risk of developing CRC was 2.64 ( 95% CI, 1.69-4.12) for CD-patients, quite similar to that found for patients with UC (2.75; 95% CI 1.91-3.97) in that study. This study was however performed using administrative data and hence, was not chart reviewed. No other population-based studies have assessed the CRC-risk in CD-patients with colonic involvement, but there are reasons to believe, that the problem
with CRC in Crohn’s colitis, may become more of a clinical reality as an increasing proportion of this patient group receives long-term immunosuppressive treatment, avoiding or considerably delaying major surgical intervention (i.e. proctocolectomy) (56). An association between longstanding anorectal CD and distal rectal and anal cancers have been reported from St. Mark’s Hospital (57).

2.4 CLINICAL RISK FACTORS FOR COLORECTAL CANCER IN IBD

Several clinical factors indicating an increased CRC-risk have been identified. From most of the larger population-based studies it is apparent that the two major risk factors for malignant transformation of the colorectal mucosa in UC are extensive disease (maximal involvement at any time during the disease history being proximal to the splenic flexure) of long duration (> 8 years) (32, 42, 43). Although young age at onset of UC is associated with a high cumulative CRC-risk (42, 58), this risk may not be an independent risk factor as such but rather interrelated to an observed long duration of UC (42, 58). In patients with extensive UC the presence of concomitant PSC has been demonstrated to be an independent risk factor also for colorectal neoplasia (34, 59). This association has since also been confirmed in several other reports (60-62), but was not reported in studies from the Mayo-clinic (63, 64). In a recent study familial CRC was associated with a more than 2-fold risk of CRC (RR= 2.5, 95% CI 1.4-4.4) in patients with IBD (65), in line with a previous reported study (66). Notably, patients with a first-degree relative with CRC diagnosed before 50 years of age had an even higher relative risk of 9.2 (95% CI 3.7-23).

2.5 PROTECTIVE FACTORS FOR COLORECTAL CANCER IN IBD

5-amino salicylic acid

Patients in a state of chronic continuous disease activity were found to run a higher risk for CRC in a study from Oxford (67), to some extent being contradicted by a later Swedish case-control study indicating that patients with a high inflammatory activity rather had a decreased risk (68), as had those patients taking sulphasalazine for maintenance treatment for more than three months (RR= 0.38; 95% CI: 0.2-0.7). Results in line with the latter observations were also found in a population-based study from Leicestershire (69) where UC-patients non-compliant with long-term sulphasalazine therapy had a significantly increased absolute cumulative risk of CRC (40% vs. 10% after 20 years of duration). In fact, regular medication with 5-ASA (≥ 2g per day) was found to reduce the CRC-risk by 75% (OR 0.25, 95% CI 0.13-0.48) in a recent match-controlled study of 102 cases of UC-patients with CRC (70). The risk reduction for those patients taking mesalazine was even greater (81% (OR 0.19, 95% CI: 0.06-0.61).

The data from Copenhagen (44), indicating that an aggressive medical and surgical approach reduces CRC-risk, may be in line with those observations.

The protective virtues of sulphasalazine and mesalazine in this respect are unclear. Aspirin and other NSAIDs reduce the risk of sporadic CRC and adenomas (71), as well
as polyp formation in FAP (familial adenomatosis polyposis) (72-75) and it might therefore be tempting to assume that the 5-ASA compound in sulphasalazine and mesalazine might have the same properties (e.g. ASA-like actions by cyclooxygenase-inhibiting). A non-pharmacological explanation might be that patients compliant with 5-ASA medication, also, visit the gastroenterologist when experiencing changes in symptoms, thus lowering the risk of CRC (70).

**Ursodeoxycholic acid/Folic acid**

Ursodeoxycholic acid (UDCA), a natural bile acid, used primarily in the treatment of primary biliary cirrhosis, but also in PSC, has been in focus ever since Earnest et al reported that dietary UDCA decreased the incidence of experimental colonic carcinogenesis in a rat azoxymethane model (76). In a retrospective study, comprising 59 patients with UC and PSC, Tung et al showed that the use of UDCA was associated with a lower risk of colonic dysplasia compared to patients not taking the compound (odds ratio 0.18, P=0.005) (77). This protective effect remained after adjusting for duration of colitis, and age at onset. Surprisingly, no protective effect of sulphasalazine was seen in this study.

UDCA has been demonstrated to harbor several anticarcinogenic properties of which one is the reduction of the colonic concentration of deoxycholic acid, recognized as a strong cancer promoter (78, 79).

Low folate intake has been associated with an increased risk of developing adenomas and CRC in the general population (80, 81). A case-control study examining the effects of folic acid use, in patients with pancolitis followed in a surveillance program, suggested that folate supplementation was associated with a 62% (OR 0.38; 95% CI 0.12-1.2, NS) lower incidence of neoplasia as compared to individuals not receiving supplementation (82). Another case-control study showed that the risk of dysplasia or cancer was significantly decreased by 18% for each 10 ng/mL increase in blood cell folate (83) and another study gives further supporting evidence for the routine use of daily folate supplementation (84).

**Immunomodulating drugs**

Only very few reports have discussed possible chemoprotective properties of immunomodulating drugs (azathioprine, 6-mercaptopurine and glucocorticosteroids). Patients treated with azathioprine or 6-mercaptopurine do not appear to have an increased risk of CRC compared to controls (85). On the other hand no CRC protective properties of these two drugs have been shown in UC (77, 84). For glucocorticosteroids, one study (70) has shown a beneficial effect of systemically administered steroids (OR 0.26; 95% CI 0.01-0.7) while other studies (77, 84) have not shown such an effect.

**2.6 MUCOSAL RISK FACTORS FOR COLORECTAL CANCER IN IBD**

**2.6.1 Dysplasia**
Certain premalignant, polypoid changes in the mucosa associated with CRC in UC were recognized in the late 1950’s by Dawson and Pryse-Davies (86), and later also described in flat mucosa by Morson et al at the St Marks Hospital in London, UK (87). Both cellular atypia and certain structural changes, particularly villous changes of the surface epithelium were appreciated, and those alterations were later referred to as dysplasia. A more detailed definition and grading classification was made by an international group of pathologists in 1983 (88). Dysplasia was then classified as « unequivocal neoplastic changes » of the epithelium and classified as either negative (or indefinite), or definite, low- or high-grade dysplasia (LGD and HGD) based on the severity of the changes. This new classification with minor modifications, has since been adopted by most clinical teams with a special interest in CRC in longstanding IBD. However the implementation of the classification in daily clinical practice has not been entirely successful according to a survey from North America (89). Besides problems in the clinical interpretation of the report resulting from the histopathological assessment of dysplasia, there are also problems with both inter- and intra-observer variability among pathologists. The influence of active inflammation causing misinterpretation of non-neoplastic reactive/regenerative features of the mucosa may be considerable and needs to be minimized. The appearance of macroscopic mucosal lesions such as sessile type polyps or raised plaque-like lesions in connection with dysplasia (referred to as DALM- Dysplasia Associated Lesion or Mass) in UC was found by Blackstone et al (90), to be an additional risk factor for the presence or subsequent demonstration of invasive carcinoma. The concept of widespread dysplastic lesions in the colorectal mucosa preceding the development of invasive carcinoma in UC, forms the mainstay for endoscopic surveillance.

2.6.2 DNA-aneuploidy

Chromosomal abnormalities in UC-patients, with and without CRC, was first described in cytogenetic analysis by Xavier et al in 1974 (91). Gross chromosomal aberrations, characterized by loss or gain of at least two to three chromosomes, i.e. DNA-aneuploidy, was later reported in UC by measurements of the total DNA-content of individual cells by Hammarberg et al in 1984 (92). By using DNA-flow cytometry (DNA-FCM) aneuploid cell lines were detected in both preneoplastic and neoplastic mucosa from eight of 51 patients with longstanding UC. The same authors described in a case report, a patient with DNA-aneuploidy, who one year later developed a CRC in the same anatomical area (93). DNA-aneuploidy in UC has been confirmed by several other groups (94-102) and has also become a complementary routine method, besides histopathology, for detection of premalignancy at colonoscopic surveillance (95, 98, 103).

Changes in nuclear DNA-content, aneuploidy, have been found to be closely related with the development of dysplasia and carcinoma in UC, both in retrospective (104) and prospective (92, 103, 105) studies using DNA-FCM. In patients with longstanding UC the overall prevalence of aneuploidy has been estimated to be 8-26% depending on duration and extent of disease and concomitant PSC (103).
In patients with CD, the occurrence of aneuploidy has also been described. Surgical specimens from 23 patients with 26 CRCs were retrospectively analyzed by DNA-FCM (106). There were 14 aneuploid CRCs (54%) of which two patients had concomitant aneuploidy in flat mucosa. Most interestingly, two of the four patients with previous colonoscopic examinations had aneuploidy three and four years before surgery. In a prospective colonoscopic surveillance program of 24 patients with longstanding Crohn’s colitis (107) three patients displayed aneuploidy (but no one dysplasia), and one of these subsequently developed a carcinoma.

DNA-FCM carries an important advantage as the interpretation of aneuploidy seems to be more objective and associated with less inter-observer variability than the assessment of dysplasia (104). In addition, according to the experience from our center, the presence of DNA-abnormalities is not influenced by active inflammation, which is a well-known difficulty in the interpretation of histological dysplasia. Furthermore, findings of DNA-aneuploidy have been shown to be an earlier marker than dysplasia for the malignant transformation when evaluated within the frame of prospective surveillance programs (95, 97). However, it remains to be determined what to do with patients with findings of DNA-aneuploidy but without dysplasia and firm guidelines regarding the interpretation and utility of DNA-aneuploidy are lacking.

Besides ploidy pattern, analysis of the cell cycle composition can be performed due to the large number of cells (about 40,000) obtained, using FCM. In patients with UC, the results from S-phase measurements have been mentioned in only one report (98) but not been evaluated in the assessment of malignant transformation. As a reference, both normal colorectal mucosa has to be studied, as well as frank malignancies (sporadic CRC).

Improvements in the preparation technique (108) and algorithms for background subtraction (109, 110) have been refined, enabling reliable calculations of both S and G2/M-phases and have been routinely applied since 1994 in our center. In sporadic CRC about 30% of the tumors are diploid and can only be discriminated from non-malignant mucosa by studying the S-phase fraction (111-113). In other malignancies such as bladder cancer and cervix cancers, the percentage of cells in S-phase appears to be an important sign of malignancy and prognostic predictor. (114-116).

2.7 CARCINOGENESIS IN IBD

The molecular events leading to neoplasia are complex and not fully understood. The continuous exposure to some hitherto unknown etiological agent, in combination with a possible genetically predisposed individual, may cause reversible or irreversible cell injury. This could result in three different outcomes as the model in figure 1 shows: 1) if the injury itself is harsh (e.g. a severe attack of colitis) the result will be cell death and eventually colectomy if a sufficient amount of cells die. This outcome could also occur in the setting of a milder insult to the colonocytes such as in chronic active disease not responding to anti-inflammatory medication. 2) The chronic inflammation may lead to programmed cell death, apoptosis, which is the other fundamental pathway of cell destruction. 3) A third pathway in which the chronic cell injury causes chronic
repair and chronic regeneration, thus accelerating cell turnover, may cause DNA-lesions in the colonocytes resulting in mutations with clonal expansion in the colon that spreads to form patchy areas in which histological dysplasia, chromosomal aberrations (e.g. aneuploidy) or other genetic abnormalities may be detected.

Subsequent further genetic changes in one or more of these areas may lead to invasive carcinoma. In other inflammatory conditions in the colon such as NSAID-induced colitis, the cell injury is reversible when the agent (NSAID) is removed and recovery will occur by autophagy and synthesis of new products. It is not known whether the severity of the mucosal injury at the initial attack of colonic IBD or the long-term effects of chronic inflammation are of most importance for the malignant transformation of the colorectal mucosa. Furthermore, unraveled and potentially carcinogenic xenobiotics may also be involved in this process.

**Figure 1.** A model of three different possible outcomes in the setting of chronic inflammation in IBD (modified from Jerker Olsson, Ass. Prof., Div of Pathology, Karolinska Institutet).

Much of our understanding and knowledge of CRC in UC has been derived from studies in the setting of sporadic CRC and in table 1 and figure 2 similarities and differences (clinical presentation and genetic abnormalities) of these two forms of CRC are outlined (117, 118).

Features that are similar between UC-associated CRC and sporadic CRC include that both conditions are believed to arise from a precursor lesion (dysplasia and adenoma respectively) and that the 5 year survival is about 50% for both conditions. Apparent differences include unique pathways in the initiating process of the malignant transformation.
Table 1. Clinical characteristics of UC-associated CRC and sporadic CRC.

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<th>UC-CRC</th>
<th>Sporadic CRC</th>
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<tr>
<td>Precursor lesions</td>
<td>Dysplasia (often flat mucosa)</td>
<td>Adenoma (polyp)</td>
</tr>
<tr>
<td>5-year survival</td>
<td>50%</td>
<td>50%</td>
</tr>
<tr>
<td>Synchronous tumors</td>
<td>Multifocal in about 12%</td>
<td>Rare (&lt;5%)</td>
</tr>
<tr>
<td>Age at CRC</td>
<td>30s</td>
<td>60s</td>
</tr>
<tr>
<td>Anatomical site of CRC</td>
<td>Evenly spread</td>
<td>Left-sided most common</td>
</tr>
<tr>
<td>Anaplastic, mucinous CRC</td>
<td>Common</td>
<td>Rare</td>
</tr>
</tbody>
</table>

In sporadic CRC this is believed to start from an adenomatous polyp (adenoma-carcinoma sequence (119)), while in UC, dysplasia arises either from flat mucosa (dysplasia-carcinoma sequence (88)) or from a Dalm (90). Another feature that distinguishes colitis associated CRC from sporadic CRC is age at onset of CRC. UC-patients are much younger than patients with sporadic CRC when experiencing their CRC (mean age of 30 and 60, respectively). Furthermore, the CRCs in UC are uniformly spread in the colon and rectum (120, 121) and often of more anaplastic histology (left-sided tumors most common in sporadic CRC). Finally, synchronous tumors (122), are more common in UC-patients.

Figure 2. A proposed model of genetic sequences of the three most studied genes, in relation to histological events, in the development of UC-associated CRC and sporadic CRC is described (adopted and modified from Itzkowitz et al (117)).

At the molecular level similarities and differences in genetic alterations are shown in Fig 2. The p53 tumor suppressor gene, located on chromosome 17p, encodes a transcription factor (protein p53) that regulates the cell cycle by growth arrest and apoptosis of damaged cells entering the cell cycle. p53 mutations and loss of
heterozygosity (LOH), can be identified by single-strand conformation polymorphism analysis and by polymerase chain reactions. Overexpression of mutated p53 protein can be detected by using immunohistochemistry. In sporadic CRC p53 alterations is typically a late event while several studies in patients with long-standing UC have shown that p53 mutations and LOH are common and early events in the progression from normal epithelium to CRC (101, 123-128). Especially, immunohistochemistry staining of the mutated p53 protein which has a half-life up to 6 hours, compared to the wild-type gene product with a half-time of a couple of minutes, may help the pathologist to correlate mutations of p53 with histopathological dysplasia.

Mutations of proto-oncogens play an important role in sporadic colorectal cancerogenesis (129) and in particularly mutation of the K-ras gene (130). When mutated, the proto-oncogenes (which regulate the cell cycle) become oncogenes, and the proteins expressed are either in to large of amount or altered, leading to unregulated growth and neoplasia. Mutations of the ras family proto-oncogenes are seen in about 50% of sporadic CRC but in UC-associated neoplasia, conflicting results concerning the prevalence of K-ras mutations have been found. Some studies have demonstrated low frequencies of this mutation and at a later stage in UC compared to CRC (131-133) while other studies have detected frequencies of K-ras mutations similar to that of sporadic CRC both in dysplasias and cancers (127, 134-136).

Other tumor suppressor genes like APC, located on chromosome 5q, are often mutated in sporadic CRC (and also responsible for the inherited disease of familial adenomatosis polyposis), and considered as one of the earliest events in sporadic CRC development. In UC the involvement of this gene in the transformation to malignant mucosa has been shown in HGD and cancers, thus at a relatively late stage in the tumorigenesis (136). Since family history of CRC recently was reported to be an independent risk factor for CRC in IBD (65) studies of patients of Ashkenazi Jewish origin with IBD have not shown an increase of APC gene mutations in these patients (137, 138).

2.8 MANAGEMENT STRATEGIES FOR COLORECTAL CANCER IN IBD

It might be argued that the increased risk of CRC in colonic IBD is not high enough compared to the general population (less than 1% of cases of CRC in the general population (139), in Sweden about 5000 new cases/year) to warrant any specific action from physicians handling the patient. On the other hand, it might be regarded as sufficiently high to necessitate expedient prophylactic proctocolectomy within a certain time frame of 3-10 years of onset. The later option was frequently advocated and performed for extensive UC during the sixties and seventies (23). Indeed, the only large study failing to show an increased risk of CRC in UC used an aggressive surgical approach (e.g. colectomy) where 35% of the patients were treated surgically during the first five years of disease (44). This handling of the patients now receives limited support. High-risk patients with IBD are usually in remission with a satisfactory quality of life and are reluctant to accept surgery as a mean of CRC prevention. It should also be remembered that well over 80% of the patients do not develop CRC and furthermore, the morbidity of colectomy and ileostomy of the ileal-pouch anal
anastomosis (IPAA) type operation must be considered when treatment options are evaluated.
Managing the patients within the frame of “routine clinical care”, including vigilance if clinical symptoms of CRC occur, has also been suggested (140). Such an approach is deemed to achieve, at best, a 50% five-year survival as seen in sporadic CRC, and is thus not very attractive. The standard approach at most IBD centers in order to manage the increased CRC-risk, at least in UC, has become colonoscopic surveillance.

2.8.1 Colonoscopic surveillance in ulcerative colitis

Based on the concept of a dysplasia-to-carcinoma sequence in UC (87, 88) and on the development of the flexible fiber-endoscope, programs for surveillance of high-risk patients were introduced in the early 1970s (105, 141, 142). By performing colonoscopies with multiple biopsies from different parts of the colon and rectum at regular intervals, those programs aimed at detecting mucosal dysplasia, or carcinoma in early stages, for individual selection of UC-patients for prophylactic colectomy. The ultimate goal has been to decrease CRC morbidity and mortality in UC. Some of the larger studies have confirmed the association between colorectal dysplasia and carcinoma in UC (141), as well as the sequential development of lower grades of dysplasia into HGD and, ultimately, to invasive carcinoma (87, 141, 143). The need for total colonoscopies has been underlined by the frequent initial finding of dysplasia or DALM only in the proximal colon. It must also be remembered that dysplasia is not always found in association with a carcinoma. Studies of resected colonic specimens and studies in large surveillance programs (143-145) show that no dysplasia (either adjacent to or distant from the tumor) is detected in up to 25% of the patients. The sensitivity of dysplasia as a marker for CRC-development is thus only 75%, but if the endoscopic examinations are performed at regular intervals the risk of missing a carcinoma before it becomes incurable by surgical resection is small (85, 105, 146).

The St. Mark’s group in London has been the pioneer center in the field of colonoscopic surveillance, and their most recent report (143) has provided us with the largest clinical experience so far. In 322 patients with extensive UC entered between 1971-1992, colonoscopic examinations contributed to the detection of 11 symptomless CRC and 12 further patients underwent colectomy due to findings of dysplasia. However, six symptomatic interval tumors also occurred, partly because of compliance problems. One of the most important findings in this study was the 5-year predictive value of LGD as a marker for cancer or high-grade dysplasia of 54% using the classification by Riddell et al (88). Only one early CRC plus abundant cases with LGD were the somewhat disappointing results in an ambitious study from Leeds (146). However, several patients with advanced CRC among defaulters and among patients not followed within the frame of the surveillance program in that study emphasize the importance of compliance as one of the mainstays for a successful follow-up. Based on the experience from a dedicated prospective surveillance program in Stockholm initiated in 1973 (105), the yield of findings leading to surgical intervention seems to be between 0.5-1% of the colonoscopies carried out per year. This figure is in accordance with the expected one based on recent epidemiological CRC risk assessments. Compliance in this program has been high, and so far, with more than 140 patients with
extensive UC followed for up to 29 years, there are still no deaths due to CRC (P. Karlén, personal comm.).

The fundamental issue when surveillance of UC-patients is considered is the impact on cancer morbidity, and mortality. In many of the hitherto reported surveillance programs, the technical feasibility of the concept has indeed been proven (105, 143, 146-148), and moreover, non-randomized studies have indicated that surveyed UC-patients have a much less risk of dying from CRC compared to non-surveyed patients (147, 149). Some authors (150-152) have criticized surveillance programs for being an ineffective and costly way of managing the increased CRC-risk in UC. Instead, the poorly defined management “routine clinical control”, including colonoscopy only when symptoms suggestive of cancer occurs, has been advocated. As true controlled studies comparing colonoscopic surveillance with the passive handling policy of “routine clinical control” will never be performed due to practical and ethical reasons, another way to address the potential value of surveillance is to perform case-control studies. The result from a joint study from the Stockholm-Uppsala area indicates a frequency-response related, protective effect of colonoscopic surveillance on CRC-mortality in UC (153). Furthermore, theoretical decision-type analysis supports the concept since patients selected for colectomy based on findings of LGD at colonoscopy will have almost the same life expectancy as patients undergoing prophylactic colectomy 10 years after onset (154).

2.8.2 Aims and prerequisites for colonoscopic surveillance

By performing complete colonoscopies with multiple biopsies from the entire colon and rectum at regular intervals, a surveillance program for colonic, longstanding IBD aims at detecting mucosal dysplasia, in order to select the high-risk CRC-prone individuals for prophylactic colectomy. The ultimate goal with colonoscopic surveillance is to decrease CRC morbidity and to eliminate the excess CRC mortality. Surveillance is only effective if all patients running the highest risk of CRC are included (e.g. those with extensive, longstanding UC and colonic CD, and in particular, those with early onset and concomitant PSC).

The patients enrolled must be fully informed about the possible consequences of findings of dysplasia. Furthermore, patient compliance is of uttermost importance in a successful surveillance. It is important to adhere to a strict protocol with predefined criteria for surgery (high-grade dysplasia, a DALM, or repeated low-grade dysplasia in multiple locations and/or at repeated examinations).

The optimal timing for initiating surveillance is around 10 years after first onset of symptoms (141-143, 146, 147), as this is the point when the CRC risk starts to increase (32, 42, 43, 58, 155). However, in patients with PSC, in whom the IBD onset may be hard to estimate as the disease is often quiescent, it may be wise to start surveillance as soon as extensive UC has been diagnosed (156). Patients having less known maximal extent beyond the splenic flexure should be offered a colonoscopy at this point (e.g. 10 years) in order to exclude progression of disease extent.
The intervals between colonoscopies should not exceed two years, and annual examinations is recommended by some experts (157). Biannual investigations between 8-20 years of duration have been adopted in the Swedish studies (105, 158), with annual colonoscopies from that point.

Dysplasia may be patchy and unevenly distributed throughout the colon leading to problems with sampling errors. Even if 20-40 biopsies are taken less than 0.1% of the colorectal mucosa is covered (141). Still, the experience from several prospective colonoscopic programs shows that biopsies taken from 6-10 different sites throughout the colon and rectum is sufficient to detect dysplasia, and thereby the risk of missing an incurable CRC is low (105, 141, 143, 146-149). If macroscopic lesions are detected, special attention is warranted and additional biopsies should be sampled in order to exclude a DALM (90, 105, 141, 146). Pedunculated adenomas found in a dysplasia-free, surrounding mucosa may be handled as in non-colitis patients (e.g. snare-polypectomy) (159), whereas sessile polyps should be regarded as a potential DALM, at least in patients over 40-45 years (158). However, in a recent study (160) of 24 UC-patients with DALMs, the authors proposed the same management as for sporadic polyps (e.g. snare polypectomy) because no patient developed CRC or dysplasia elsewhere in the colon and rectum. Longterm follow-up data from this and other studies (159) using a similar approach are lacking, making it difficult and premature to generally recommend this way of handling DALMs. In patients with substantial amounts of postinflammatory pseudopolyps, a safe surveillance may never be offered and instead, prophylactic colectomy should be discussed.

2.8.3 Colonoscopic surveillance in colonic Crohn’s disease

Only limited experience exists from prospective colonoscopic surveillance of patients with longstanding, extensive colonic CD. In fact, the only study published so far (161) reported on 259 patients with chronic Crohn’s colitis of a duration of 8 years or more who were followed for 18 years by colonoscopic surveillance (mean 2.6 examinations per patient). This screening and surveillance program detected dysplasia or CRC in 16% of the patients (10 with indefinite, 23 with low grade and four high-grade dysplasias and five CRC). Furthermore, age > 45 years at the time of colonoscopy was associated with increased risk of neoplasia. After a negative colonoscopy, the probability of findings of neoplasia by the fourth surveillance examination was 22% and comparable to those reported from UC-patients undergoing surveillance (162, 163). The authors recommended that all patients with extensive Crohn’s colitis of long duration (> 8 years) should undergo colonoscopic surveillance.

The reports on cancer frequency in UC based on histopathological records should be subjected to critical histological re-evaluation as suggested in a retrospective study (164). That study, in fact, showed that CRC in Crohn’s colitis had increased and that colonoscopic surveillance was warranted.

The number of patients having CD involving the large bowel with no major resection or colectomy are likely to increase in the future as an effect of improved and more aggressive medical therapy (azathioprine, 6-mercaptopurine and infliximab).
Furthermore, the incidence of CD is steadily increasing, especially the colonic form, with a reported overall incidence of 4.6 per 100000 inhabitants, and with an age specific peak incidence between 20 and 30 years (7), the prevalence of colorectal CD will inevitably grow rapidly.

It may thus be appropriate to consider surveillance with multiple biopsies for detection of histopathological dysplasia in this subgroup of patients with CD as the CRC risk is obviously increased.

Besides dysplasia as a marker for malignant transformation of the colorectal mucosa in CD (139), the use of FCM-analyses may be of value as a large proportion of CRCs in patients with colonic involvement were DNA-aneuploid in a retrospective study (106). Colonoscopy in CD-patients, with colorectal involvement, may be difficult because of colonic strictures. Thinner caliber pediatric colonoscope may help to improve the percentage of complete examinations. Besides technical difficulties with stricturing, there is up to 12% chance that the stricture is of malignant nature (165).

Ongoing prospective programs are needed to determine guidelines regarding surveillance examinations in CD. Based on the discussion above, it would be wise to apply a UC-based colonoscopic strategy at least in patients with Crohn’s colitis of long duration.

### 2.8.4 Search for other preneoplastic markers

Numerous studies in search for more sensitive and specific markers of pre-malignancy than histopathological dysplasia in UC have been performed. Some of the candidate methods, markers and molecules to complement or replace dysplasia are outlined in table 2.

From non-dysplastic epithelium to CRC, several genetic events in combination with histopathological changes take place in a multi-step procedure. Early events in this cascade include global DNA hypomethylation as a result of folic acid deficiency as well as DNA-aneuploidy, representing gross chromosomal changes in the epithelium as mentioned previously. It is not exactly known what kind of genetic mutations global DNA hypomethylation or DNA-aneuploidy represent.

Chromosomal instability in UC-patients with dysplasia or cancer has recently been related to telomere shortening as detected by quantitative fluorescence in situ hybridization (FISH) (166).

Changes in mucin patterns with increased sialylation of the mucin is known to be associated with malignant transformation in the colon and has been suggested to be of use in the surveillance of patients with longstanding UC (167). Using monoclonal antibodies against the mucin-associated sialosyl-Tn antigen, the shift in mucin production has been demonstrated to precede the occurrence of dysplasia in UC by several years in retrospective studies (168) and might also precede the occurrence of aneuploidy in a prospective study (169).
Table 2. Alternative methods and markers for detection of neoplasia in UC.

<table>
<thead>
<tr>
<th>Neoplastic event</th>
<th>Method</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomal aberrations</td>
<td>DNA cytometry</td>
<td>DNA-aneuyploidy</td>
</tr>
<tr>
<td></td>
<td>FISH</td>
<td>Centromere and telomere aberration, translocations</td>
</tr>
<tr>
<td>Overexpression of mucin-associated carbohydrates</td>
<td>Immunohistochemistry</td>
<td>Sialosyl-Tn antigen</td>
</tr>
<tr>
<td>Mutations in tumor suppressor genes</td>
<td>PCR, Immunohistochemistry</td>
<td>p53, APC, src, Rb, DCC, p16, 8p12</td>
</tr>
<tr>
<td>Mutations in proto-oncogenes</td>
<td>PCR, Immunohistochemistry</td>
<td>K-ras, H-ras, c-myc, c-fos, N-myc</td>
</tr>
<tr>
<td>Microsatellite instability</td>
<td>PCR</td>
<td>DNA-mismatch repair genes</td>
</tr>
<tr>
<td>Cellular proliferation</td>
<td>Immunohistochemistry DNA-FCM</td>
<td>Ki-67, PCNA, Cyclin A expression, S-phase</td>
</tr>
<tr>
<td>APC/beta-catenin expression</td>
<td>Immunohistochemistry</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>Inactivation of p53</td>
<td>Immunohistochemistry</td>
<td>metallothionein</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>PCR</td>
<td>Methylated CDH1</td>
</tr>
<tr>
<td>HPP1-gene</td>
<td>PCR</td>
<td>Methylated HPP1</td>
</tr>
<tr>
<td>p14 (ARF) gene</td>
<td>PCR</td>
<td>Methylated p14</td>
</tr>
</tbody>
</table>

Alterations of the p53 tumor suppressor gene probably represents an early event in the neoplastic transformation in UC. Besides what has been discussed earlier (Fig 2), screening for p53 mutations in whole-gut lavage has been proposed in chronic IBD (170). The Rb suppressor gene with LOH has been found in sporadic reports in UC patients with dysplasia and CRC (124) while other putative tumor suppressor genes, such as DCC (18q), p16 (9p) and 8p12 (commonly found with LOH in sporadic CRC), in combination, may serve as an adjunct in distinguishing polyloid dysplasia from adenomas in UC (171).

Mutation of proto-oncogenes are another important event in the malignant process in UC and is a rather late finding as described earlier. By using PCR techniques, K-ras mutated cells can be detected in the stool in CRC-patients and in high risk individuals with UC (172), indicating a potential role in future surveillance programs. Other activated proto-oncogenes have been described in neoplastic mucosa IBD such as src (173) and others including H-ras, c-myc, c-fos and N-myc (174) but their use in cancer surveillance in IBD are unlikely at present.

Genetic instability resulting in mutations of DNA mismatch repair (MMR) genes, which are responsible for the repair of base pair mismatches which frequently occur
during DNA replication, are called microsatellite instability (MSI). MSI is common in the inherited syndrome of hereditary nonpolyposis colorectal cancer (HNPCC) (175-178). In sporadic CRC 12-15% of the cancers display defective MMR (179) and promoter hypermethylation of the MMR gene hMLH1 is strongly believed to be the cause of MSI and has also been shown in subsets of UC-associated dysplasia and CRC (180).

Epithelial cell proliferation in the colorectal mucosa might represent an early stage in the carcinogenesis of IBD. Disturbances in the regulation of the cell cycle can be detected by an increase in the fractions of cells in S- and G2/M-phases and cancer cell populations usually show high proliferative activity. There are several different methods to estimate increased cellular proliferation; increased 3H-thymidine uptake in colonocytes, assessed by autoradiography, has been shown in patients with UC (181-183), as well as increased uptake of bromodeoxyuridine, assessed by immunohistochemistry (184). Furthermore, immunolabeling of cyclins, which regulate the cell cycle, has also been reported in longstanding UC-patients at high risk of CRC (185). The presence of nuclear proteins regulating the cell cycle, such as PCNA (proliferating cell nuclear antigen) and Ki-67, can be detected by immunohistochemical techniques using monoclonal antibodies. These proteins are only active in the proliferative part of the cell cycle (e.g. S- and G2/M-phases) and thus, can be used as markers for increased proliferation. There have been several reports indicating increased proliferation of these antigens with increased severity of dysplasia in UC (186-189). A problem with these nuclear proteins, is that their expression, is also increased in the setting of active inflammation, making their use uncertain as an adjunct for dysplasia.

Metallothioneins (MTs) are zinc-binding proteins whose overexpression may lead to inactivation of the p53 tumor suppressor gene. By using monoclonal antibodies Bruewer et al (190) showed in 14 patients with UC and CRC that increased MTs staining could be observed in normal tissue adjacent to neighboring CRC, suggesting an early step in the development of CRC.

Malfunctioning of adhesion molecules, such as the E-cadherin, is believed to be involved in the development of malignancy. Azarschab et al (191) recently showed in 26 patients with long-standing UC (156 biopsies) that methylation of the E-cadherin promoter (CDH1) was found in 93% of patients with dysplastic samples, in contrast to only 6% of patients without dysplasia. In UC-associated CRCs about half of the tumors display hypermethylation of the CDH1 region (192).

The HPPI1-gene, silenced by methylation in sporadic CRC, has also been proposed as a biomarker for the early detection of malignancy in UC. Sato et al (193) showed that HPP1-methylation was found in 40% of dysplastic tissue and 50% of CRC in a recent small study of UC-patients. The same author also showed that hypermethylation of the p14 (ARF) gene, which inhibits an oncoprotein that degrades the p53 protein, is present in UC-associated dysplasias and CRC (194).

In summary, despite the efforts to find better markers for pre-malignancy than histopathological dysplasia in UC, none appears to be reliable enough for clinical
decision making. Thus, histopathological dysplasia is still the accepted standard for malignant transformation in IBD (UC).
3 AIMS OF THE PRESENT STUDIES

The aims of this thesis were to contribute to the diagnosis and follow up of patients with inflammatory bowel disease with an increased risk for colorectal cancer by:

1. investigating whether the expression of two proliferative antigens; Ki-67 and PCNA, correlates with various degrees of histopathological dysplasia and inflammation (Paper I)

2. investigating whether the activity of the enzyme alkaline sphingomyelinase is reduced in colorectal biopsies from patients with longstanding IBD, and, if a reduction in this activity correlates with epithelial dysplasia and/or DNA-aneuploidy (Paper II)

3. assessing biopsies from normal colorectal mucosa, using DNA-flow cytometry, for determination of ploidy levels and cell cycle composition (Paper III)

4. characterizing the ploidy levels and cell cycle composition in UC patients undergoing colonoscopic surveillance (Paper IV)

5. exploring the possible preventive or reverting effect of long-term oral ursodeoxycholic acid treatment in patients with colorectal IBD with existing findings of low grade dysplasia and/or DNA-aneuploidy (Paper V)
4 MATERIAL AND METHODS

4.1 PATIENTS AND CONTROLS

Paper I

Multiple colorectal biopsies, from four patients with longstanding UC (median duration of 25 years, range 16-42 years) with a median age of 40.5 years (range 29-49 years) undergoing colonoscopic surveillance at Huddinge University hospital were selected because of known dysplasia and or DNA-aneuploidy. Two UC-patients with active inflammation as well as three patients with normal mucosa were used as controls.

Paper II

The patients in this study were selected from ongoing surveillance programs at Huddinge University (HU) Hospital and Lunds University (LU) Hospital. One group of patients (n=15, 13 UC and 2 CD) from HU had findings of DNA-aneuploidy and/or low grade dysplasia at previous colonoscopies while the other group of patients (n=19 UC) from LU displayed no dysplasia. All patients had extensive colitis and the median duration of disease did not differ between the groups (20 years (range 5-46 years) at HU vs. 21 years (range 6-51 years) at LU), and nor did the median age (42 years (range 27-54 years) at HU vs. 49 years (range 24-81 years) at LU). Eleven patients with a median age of 40 years (range 24-81 years) served as controls. They were referred for colonoscopy due to abdominal complaints but with normal macro- and microscopically findings.

Paper III

Forty-four patients, (20 males) with a median age of 55 years (range 21-80 years) were studied. They were referred for colonoscopy at the endoscopy unit at Söder Hospital or Sophia Hospital, due to abdominal complaints, gastrointestinal bleeding or suspicion of CRC, but had both macro- and microscopically normal mucosa.

Paper IV

Three-hundred and twenty-four patients (172 males) with UC, undergoing colonoscopic surveillance at Huddinge University hospital, were analyzed by DNA-flow cytometry for ploidy and cell cycle composition. The median age of the patients at onset of disease was 26 years (range 4-74 years) and the median disease duration at the last analysis for DNA-FCM was 20 years (range 1-58 years). Two hundred and forty five (75.6%) had total or extensive disease and 33 (10.2%) of these patients had concomitant PSC. The results were compared to 79 patients (42 males) with sporadic CRC with a median age of 73 years (range 36-87 years).

Paper V
Nineteen patients (13 UC, 6 CD, median age 43 years, (range 27-73 years)) with longstanding extensive colitis (median duration 21 years (range 8-47 years)) undergoing colonoscopic surveillance were selected from an ongoing surveillance program at Huddinge University Hospital. Findings of LGD/or DNA-aneuploidy had been present in all of these patients at the preceding colonoscopies.

4.2 COLONOSCOPY AND BIOPSY SAMPLING (PAPER I-V)

All patients and controls in this thesis underwent colonoscopy once (controls) or usually several times (patients) and most of the examinations were carried out by either US or RL using Olympus equipment. All of the colonoscopies evaluated in this thesis were complete, i.e., examination of the entire colon and rectum. Preparation before examination included oral lavage with 3-4 liters of polyethylene glycol-electrolyte lavage solution (Laxabon®, TIKA, Sweden) or two doses of 45 ml of sodium phosphate (Phosphoral™, Ferring AB, Sweden). No or only very light sedation was used during the procedures (diazepam 5-10 mg and/ or pethidine 25-50 mg iv).

The macroscopic findings at colonoscopy were important in the evaluation of the extent of UC and CD as well as the normal appearance in the controls. For UC, mucosal changes confined to the rectum with a clear demarcation to normal mucosa proximally, was defined as proctitis. Proctosigmoiditis included mucosal changes confined to the rectum and sigmoid colon. In left-sided colitis the inflammation extended proximal to the transverse colon and in extensive colitis, proximal to the hepatic flexure. Total colitis was defined as mucosal changes from the rectum through the colon beyond the hepatic flexure. For CD, mucosal lesions involving at least one third of the colon, was defined as extensive colitis. The colorectal mucosa of normal controls, showed a normal vascular pattern without any signs of inflammation throughout the colon and rectum.

At each colonoscopy multiple biopsies were sampled from each of nine predetermined segments (1=ecum, 2=ascending colon, 3=hepatic flexure, 4=proximal transverse colon, 5=distal transverse colon, 6=splenic flexure, 7=descending colon, 8=sigmoid colon and 9=rectum) at approximately 10 cm intervals throughout the colon and rectum (Fig 3). If macroscopically nodular lesions were found, additional biopsies from that lesion as well as from flat mucosa were taken to confirm the presence or absence of a DALM.

For histopathology, two biopsies from each segment were taken. All of the biopsies were taken immediately adjacent to each other in all locations (about 1-2 mm:s apart). For DNA-FCM there were two different ways of sampling the biopsies; a) taken from nine different locations, as described above, in case of previous findings of DNA-aneuploidy or b) pooled in three fractions (I-III) as figure 3 shows. Thus, fraction I corresponds to the right colon (location 1-3), fraction II to the mid colon (location 4-6) and fraction III to the left colon and rectum (location 7-9). Samples for histopathology assessment as well as DNA-FCM analyses in normal controls were pooled in three fractions (I-III), as were biopsies for determination of alkaline SMase.
In patients with sporadic CRC, four samples for DNA-FCM were collected from the resected colonic tumor at the operating room and further morphologically assessed at the Department of Pathology, Huddinge University Hospital.

![Diagram of colonic tumor with fractions](image)

**Figure 3.** The biopsies obtained at colonoscopy were taken either separately in nine different fractions (I=cecum, 2=ascending colon, 3=hepatic flexure, 4=proximal transverse colon, 5=distal transverse colon, 6=splenic flexure, 7=descending colon, 8=sigmoid colon and 9=rectum) or pooled in three different fractions (I=1-3, II=4-6 and III=7-9). The figure was kindly provided by Dr Bengt Ödman, South Hospital, Stockholm.

### 4.3 HISTOPATHOLOGICAL ASSESSMENT

All colorectal biopsies obtained at colonoscopy for histopathological evaluation were fixed in neutral buffered (10%) formalin and embedded in paraffin for routine hematoxylin and eosin (H&E) staining. The H&E sections were consecutively evaluated (and reevaluated when appropriate) in paper I, II, III and V in a blinded manner with regard to dysplasia by the same pathologist (ÅO). In paper IV about one fifth of the biopsies were assessed by ÅO, while the other samples were assessed in the setting of clinical routine at the department of pathology, Huddinge University Hospital.

The grading and classification of dysplasia were performed in accordance with the guidelines presented by the Inflammatory Bowel Disease-Dysplasia Morphology Study Group in 1983 (88). Histological neoplastic changes were subsequently classified as not present (NP), indefinite, probably negative, probably reactive (IPR), indefinite unknown (IU), indefinite probably positive, probably dysplastic (IPP), or definite, low grade dysplasia (LGD) or high grade dysplasia (HGD). Definition of a dysplasia associated lesion or mass (DALM), was assessed on the basis of macro- and microscopical findings. Dysplasia was only recorded when the mucosa showed no active inflammation (defined as no more than infiltration of lymphocytes and plasma
cells in the lamina propria (195)) to avoid misinterpretation of reactive, inflammatory changes.

Inflammation was classified as mild, moderate or severe. Mild inflammation included infiltration of neutrophils in the lamina propria and in moderate inflammation there was also infiltration in the surface and/or in the crypt epithelium. Severe inflammation included findings of crypt destruction, erosions, ulcerations and/or granulation tissue.

For control patients with normal histopathology the criteria for the definition of normal colonic mucosa was used as previously described (196) and for patients with sporadic CRC (paper IV), the Dukes’ and TNM-classifications were applied.

4.4 DNA FLOW CYTOMETRY ANALYSIS (PAPER II-V)

Fresh biopsies obtained at colonoscopy were fixed in buffered formalin. The formalin solution was then sucked off with a Pasteur pipette and replaced by 3 ml 95% ethanol in order to remove the formalin. After about 1 h at room temperature, the ethanol was removed and the biopsies were rehydrated in 2 ml water for about 1 h (at room temperature). The water was replaced by 0.2 ml subtilisin Carlsberg solution (0.1% (w/v) Sigma protease XXIV (Sigma P8038, St Louise, MO), 0.1 M tris, 0.07 M NaCl (Merck), pH 7.2) and the samples were incubated for 2 h at 45 °C. The residual pieces of connective tissue were removed and a droplet of the resulting cell suspensions was stained with DAPI-Sulforhodamine and examined microscopically for assessment of representativeness (e.g. epithelial cells and absence of cytoplasm). The incubation was continued if cytoplasm linked to cell nuclei was still seen. If bare nuclei without any residual cytoplasm were present, then the whole suspension of nuclei was stained by directly adding 0.2 ml DAPI-SR101 solution (8µM DAPI (Sigma D9542), 50µM Sulforhodamine 101 (SR101) (Sigma S7635), 0.1M tris, 0.07M NaCl, pH 7.5). After a staining time of at least 30 min, the samples were analyzed using a PAS II flow cytometer (Partec, Munster, Germany) equipped with a mercury arc lamp HBO100. The suspended cell nuclei showed minimal damage, and by avoiding any centrifugation steps, showed extremely low frequencies of aggregates and background level.

For cell cycle analysis, the sliced nuclei option for background subtraction of the Multicycle program (Version 3.0, Phoenix Flow System, San Diego, CA, USA) was used. For calibration of the DNA flow cytometer and standardization of the evaluation of the histograms, formalin fixed hen and trout erythrocytes were added to the suspension of the cell nuclei. Thus, diploid normal cells such as human lymphocytes were always found at a certain channel number (70) of the flow cytometer. The ploidy level of cells at this position was used as reference and the coefficient of variation was less than 3%. At a rule, at least 20,000 cell nuclei were analyzed. The histograms were classified as diploid (DNA-index 1.0±2c) if only a single peak at the reference channel was found. Aneuploid cell populations were assumed to be present when an additional peak was found. Cells with aneuploidy were classified depending on their ploidy levels (position of the aneuploid G1 peak in relation to that of the diploid G1 peak) Tetraploidy (DNA-index 2.0±4c) was assumed to be present if more than 8% of the cells were positioned at G2 and corresponding octaploid G2-peaks were found.

Before 1994, the cell material was mechanically disrupted, fixed in ethanol, and cell nuclei were released by pepsin treatment (197). This technique enabled reliable ploidy level measurement, but was less suitable for the evaluation of low S-phase fraction, due
to background and clumping. Thus, S-phase fraction determination was only included after April 1994.

4.5 DETECTION OF PROLIFERATING ANTIGENS (PAPER I)

For immunostaining with the monoclonal antibodies MIB-1 against Ki-67 and NCL-PCNA against PCNA (proliferating cell nuclear antigen), sections, cut at 4µM, immediately adjacent to those used for histopathological assessment were used. The paraffin embedded sections were de-paraffinized through xylene and graded alcohols. Endogenous peroxidase activity was then blocked with 0.5% H2O2 and then rinsed in water. The sections were then incubated with 1% bovine serum albumin for 30 minutes. For detection of the Ki-67 antigen, the IgG monoclonal antibody MIB-1 (Immunotech, France) was used in a dilution of 1/150 and for detection of PCNA the NCL-PCNA mouse monoclonal antibody (Novocastra Laboratories, UK) was used in a dilution of 1/60. After incubation and rinsing the sections were incubated a second time with the antibody biotinylated anti-mouse IgG (Vector, USA) diluted 1/200, and then washed and incubated with avidin-biotin-peroxidase complex. The sections were finally rinsed in TBS and incubated with DAB solution (60 cc TBS, 48µl H2O2, 4 and DAB tablets (3.3 diaminobezedine tetrahydrochloride, Sigma, USA) and after washes counterstained with hematoxylin.

For assessing the degree of immunostaining with the two monoclonal antibodies MIB-1 against Ki-67 and NCL-PCNA against PCNA, only positive stained nuclei in completely U-shaped, longitudinally cut crypts were evaluated. The scoring was made in a semi-quantitative manner dividing the crypt in three parts: lower, middle and upper thirds. As controls, biopsies with normal macro- and microscopically findings were stained. The controls showed the following pattern: In the case of NCL-PCNA only the lower third displayed staining while for MIB-1 the lower third and occasionally the lower part of the middle third of the crypts showed staining. Thus, increased proliferation (cut-off levels) was staining above these levels (proliferative compartment).

4.6 DETERMINATION OF ALKALINE SPHINGOMYELINASE ACTIVITY (PAPER II)

The biopsy samples were homogenized in 0.5 ml 0.25 M sucrose buffer containing 5 mM MgCl2, 0.15 M KCl, 50 mM KH2PO4, 1 mM PMSF, 1 mM benzamidine and 10 mM taurocholate, pH 7.4, followed by sonication for 10 seconds. After centrifugation at 10,000 rpm at 4 °C for 15 min, the supernatant was saved for determination of alkaline SMase activity and protein content.

Purified milk SM (purity > 98%) and choline labeled 14 C-SM (56 µCi/mg) were kindly provided by Lena Nyberg, Swedish Dairy Association and by Peter Ström, Astra Draco, Lund. Taurocholate (TC), taurodeoxycholate (TDC), glycocholate (GC), glycochenodeoxycholate (GCD), benzamidine, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Co (St. Louis, MO USA).

The activity of alkaline SMase was determined according to Duan et al (198) which is a modification of an original method for neutral SMase (199). Briefly, samples were
added in 375 μl Tris-EDTA buffer pH 9.0 to a final volume of 0.4 ml, containing 50 mM Tris, 0.15M NaCl, 2 mM EDTA and 3 mM bile salt mixture with a molar ratio TC:TDC:GC:GCDC being 3:2:1:8:1. Such a bile salt mixture had previously shown to have the maximum stimulatory effect on alkaline SMase (200, 201). The supplement of EDTA in the buffer served to inhibit the neutral SMase activity which is Mg2+-dependent with a pH optimum at 7.5 (202). 14C-SM dissolved in ethanol was suspended in 0.9% NaCl containing 3 mM bile salt mixture. The reaction was started by adding 20 μl 14C-SM (8.000 dpm) suspension, incubated at 37 °C for 30 min, and terminated by adding 400 μl chloroform/methanol (2:1). After phase partition and centrifugation, an aliquot of the upper phase containing the cleaved phosphocholine was taken and the radioactivity was counted by liquid scintillation. The activity was calculated and normalized as nmole/h/mg sample protein.
Protein content was assayed by a kit obtained from Bio-Rad Co. (Hercules CA, USA), using bovine serum albumin as a standard.

4.7 STUDY DESIGN OF PAPER V

The study was performed as a single center trial at the IBD-unit at Huddinge University Hospital, Karolinska Institutet, Stockholm and was of randomized, controlled, double-blind design. The patients were randomly allocated to therapy with either UDCA (Ursofalk®, Meda AB, Sweden, 500 mg b.i.d) or placebo and the duration of treatment was two years. Table 1 outlines the study in general terms. Colonoscopy with multiple biopsies for histopathology and DNA-FCM was performed at start and at six months interval during the two-year study and the primary outcome was the need for colectomy due to progression of neoplasia.

Study events
Outpatients visits, including full colonoscopy with multiple biopsies for histopathology and DNA-FCM, were performed at study start and at six months interval during the two-year study period. Blood samples for hematology (B-Hemoglobin (g/l), B-Leukocytes, particle concentration (x10^9 /L) and B-Platelets, particle concentration (x 10^9 /L)) and for clinical chemistry (B-Glucose (mmol/L), S-Sodium (mmol/L), S-Potassium (mmol/L), S- Creatinine (μmol/L), S-Bilirubin, total (μmol/L), S-Alkaline phosphatase (μkat/L), S-Alanine aminotransferase (μkat/L), S-Aspartate aminotransferase (μkat/L), S-GT, γ-Glutamyltransferase, (μkat/L), S-Albumin (g/L), S-C-reactive protein (mg/L), S-Orosmucoid (g/L), B-Erythrocyte sedimentation rate (B-ESR) 1 hour (mm/h), S-Cholesterol (mol/L), and S-Triglycerides (mmol/L)) were obtained at each visit as well as urinalysis (dipslides).
Drug accountability and compliance were recorded at each visit, as were any adverse events. Maintenance drugs such as 5-ASA, SASP, olsalazine, metronidazole or azathioprine were allowed provided the dose was kept constant. Other intercurrent drug therapy necessary for the patient’s well being was recorded. Treatment with antacids containing aluminum hydroxide, cholestyramine or colestipol was not allowed during the course of the study because of risk of interaction with UDCA.

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Composed neoplastic score
In order to evaluate if progression or regression of the neoplastic lesions occurred during the observation period of two years, we recorded the existence of aneuploidy or dysplasia at the entry colonoscopy and at each of the four scheduled colonoscopies at six, 12, 18 and 24 months performed during the course of the study. In order to compare and include all of the biopsies taken at each colonoscopy we developed a scoring system. For the histopathological report, numeric values were obtained by giving biopsies with no dysplasia, indefinite, probably negative and indefinite, unknown no points. Biopsies with indefinite, probably positive (probably dysplastic) findings scored one point. LGD rendered two points and HGD three points. If DALM was observed an additional point was added. The sum was then divided by the total amount of biopsies at each colonoscopy resulting in a numeric mean value.

Table 3. Study design of paper V

<table>
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<tr>
<td>medical history</td>
<td>x</td>
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<td>x</td>
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</tbody>
</table>

* Clinical activity; remission vs. activity

DNA-FCM diploid samples were rated zero. Aneuploidy rendered one point and the sum was then divided by the total number of biopsies at each colonoscopy resulting in a numeric mean value and the mean values from the histopathology report were then
added to the results from DNA-FCM resulting in a total composed score. This system was used throughout the entire study at each colonoscopy.

4.8 STATISTICAL ANALYSES

Paper I

We used the Spearman’s rank correlation test to study the relation between histopathological dysplasia and the expression of MIB-1 against Ki-67 and NCL-PCNA against PCNA. A p-value of <0.05 was considered to be statistically significant.

Paper II-V

Variables with a normal distribution were given as means and standard deviation, whereas data with a non-normal distribution were expressed as median and range. We used the Mann-Whitney U-test and Wilcoxon’s signed rank test for evaluation of unpaired and paired comparison of non-normal data. Comparison of data with a normal distribution were done using Student’s t-test for paired and unpaired comparisons. Nominal data were compared using the Chi-squared test. For evaluation of correlations, linear regression analysis (Pearson’s correlation) with f-ratios were used.

4.9 ETHICAL CONSIDERATIONS

All of the studies in this thesis were performed in accordance with the principles stated in the Declaration of Helsinki and approved by the local ethic’s committee at the Karolinska Institutet, Stockholm, Sweden. The interventional study with ursodeoxycholic acid (paper V) was also approved by the Swedish Medical Product Agency (Läkemedelsverket).
5 RESULTS

Paper I- Immunostaining with proliferation markers

Increased immunostaining with NCL-PCNA against PCNA showed a fairly strong positive correlation up to the level of LGD. In non-dysplastic mucosa six of 46 specimens (13.1%) showed an increased staining pattern, in those with indefinite changes, probably positive for dysplasia 14 of 43 samples (32.6%) and in biopsies with LGD, 10 of 23 (43.5%). In biopsies with HGD only 1 of 6 (16.7%) displayed increased staining pattern. Furthermore, increased staining was also seen in biopsies with active inflammation (Fig 4).

Increased immunostaining with MIB-1 against Ki-67 were found in 4 of 46 (8.7%) samples with nondysplastic epithelium, 10 of 42 (23.8%) with indefinite changes, probably positive for dysplasia, 9 of 19 (47.4%) with LGD and four of six (66.6%) with HGD. There was a significant correlation between increasing degrees of dysplasia and the proportion of specimens with increased MIB-1 immunostaining (P=0.008). Again, increased immunostaining was also seen in biopsies with active inflammation.

Figure 4. The percentage of specimens with increased immunostaining of PCNA and MIB-1 in relation to histopathology.

The normal proliferative compartment of colonic cells is localized in the basal half of the crypts as seen in controls, i.e., immunostaining up to the lower third of the crypts with PCNA and up to the middle third of the crypts with MIB-1. Immunostaining with PCNA showed a good correlation to increased dysplasia up to the level of LGD, but this was not maintained in HGD. The reasons for this was not clear, and attempts to ream these specimens were done but lack of tissue remaining in the blocks made this impossible. For MIB-1 staining a significant correlation (P=0.008) between increasing degree of immunostaining and increasing degree of dysplasia was found. However, it was not possible to discriminate between dysplasia and inflamed mucosa using these immunostaining techniques.
Paper II- Alkaline sphingomyelinase

Alkaline SMase activity overall was significantly reduced in patients with and without dysplasia compared with controls (P=0.006, Fig X). Patients with dysplasia showed lower values than those without dysplasia, but the difference was not statistically significant (P=0.18).

![Figure 5. Alkaline SMase activity (nmol/h/mg protein) of 139 biopsies in controls (5.32±0.6), patients without dysplasia (3.62±0.46) and patients with dysplasia (2.76±0.5), P=0.006. The grand mean of all values was 3.7. Mean values with SE.]

There were no correlations between alkaline SMase activity and the degree of dysplasia or between diploid and aneuploid biopsies.

A significant decrease of the alkaline SMase activity with increasing patient age was observed (P=0.008), both in controls (P=0.006) and in patients with dysplasia (P=0.01) but not in patients without dysplasia (NS). No correlation was found between duration of UC and alkaline SMase activity. For diploid biopsies alkaline SMase activity decreased both with age (P=0.02) and duration of disease (P=0.009) but this was not found in the subgroup of only 13 aneuploid samples.

Paper III- Cell cycle composition in normal colorectal mucosa

Altogether 165 diploid biopsies from 44 patients were analyzed with a mean number of cell nuclei of about 20,000/sample. Mean values for cells in S-phase and G2/M of all subjects were 2.65±1.55% (median 2.35, range 0.1-8.3%) and 1.27±0.84% (median 1.1, range 0.2-5.1%), respectively, and the proliferation index (sum of percentage of cells in S- and G2/M-phases) was 3.94±2.1% (median 3.4, range 0.7-10.4%). There were no differences between males and females. The fraction of G2/M cells increased with the fraction of cells in S-phase and this increase was highly significant (P<0.0001).
An increase of cells in S-phase with higher age was observed (P=0.0003, Fig 6).

Figure 6. Relationship between cells in S-phase fraction (%) and age in 165 biopsies from 44 subjects with normal biopsies from the colorectal mucosa, P=0.0003.

The mean S-phase values for the right, transverse and left colon were 3.01±1.82% (median 2.75, range 0.5-8.3), 2.56±1.41% (median 2.3, range 0.1-6.2) and 2.38±1.35% (median 1.9, range 0.8-6.5%) respectively. The differences between the right and the transverse colon, and between the right and the left colon were significant (P=0.02 and 01). Corresponding values for % cells in G2/M were 1.43±0.81% (median 1.3, range 0.2-3.7), 1.28±0.9% (median 1.1, range 0.3-5.1) and 1.09±0.76% (median 0.9, range 0.3-4.1). The difference between the right and left colon was significant (P=0.001). For the proliferation index (S+G2/M) corresponding values were 4.49±2.33% (median 4.2, range 0.7-10.4), 3.85±2.0% (median 3.3, range 1.2-8.4) and 3.49±1.83% (median 2.8, range 1.4-7.9). The difference between the right and left colon was significant (P=0.001).

In the individual subjects the values of the S-phase fraction from the right colon were highly significantly related to those of the transverse colon (P<0.0001). Similar relationships were found when the transverse colon was compared to the left colon and when the right colon was compared to the left colon. The same relationships were also found when only G2/M-fractions or the proliferation index were compared with each other.

These observations demonstrate characteristic individual levels of cell proliferation of the colonic mucosa, independently of site.

Paper IV-Ploidy levels and cell cycle composition in patients with UC

Aneuploidy was found in 80 patients (25%), in 24% without PSC and in 27% with PSC, of the 324 UC-patients during a mean follow-up of 4.48±3.6 years. Ploidy levels with a DNA-index of 0.8-1.3 and 1.4-2.4 were most common and about the same as in sporadic CRC. The median S-phase fraction of 3196 diploid and 275 aneuploid samples from UC-patients without PSC was 2.3% (0.1-15.5) and 2.4% (0.2-16.9), respectively (P=0.7). In PSC-patients the median S-phase fraction from 522 diploid
samples and 72 aneuploid samples was 2.2% (0.4-9) and 3.05% (0.7-15.9), respectively (P<0.0001). The S-phase fraction of the diploid biopsies from UC-patients did not differ from normal controls, but differed from 96 samples from 28 diploid CRCs, 11.7% (1.2-25.6), (P<0.0001). The S-phase fraction of the aneuploid biopsies from UC-patients differed from that of 196 aneuploid samples from 51 patients with CRC, 18.1% (0.7-45), (P<0.0001). The average reproducibility of aneuploidy was 47% but varied between 0-100% depending of number of examinations, extent of aneuploidy in the colon and rectum and DNA-indices. The proportion of aneuploid samples increased from 7 to 27% over a disease duration of 52 years (P<0.0001). Aneuploidy was found in 9% of biopsies with no dysplasia and in 39% of biopsies with indefinite, probably positive for dysplasia and low-grade dysplasia, respectively (P<0.0001). The increase of severity of dysplasia was associated with an increase of S-phase fraction (P<0.0001).

![Figure 7](image-url)

**Figure 7.** Percentage of cells in S-phase in relation to aneuploid samples of CRCs (n=196, S-phase 18.6±0.18 %), aneuploid samples of UC without PSC (n=275, S-phase 3.01±0.15 %), aneuploid samples of UC with PSC (n=72, S-phase 4.1±0.3 %), diploid samples of CRCs (n=96, S-phase 12±0.26 %) and diploid biopsies of UC-patients (n=3718, S-phase 2.7±0.04 %)., P<0.0001. Mean values with standard error of mean.

**Paper V**

The patients enrolled in this pilot study were randomly allocated to receive capsules containing UDCA (Ursofalk®, Meda AB, Sweden) 500 mg b.i.d or an identical placebo. In all, 10 patients received active treatment and nine placebo. The groups were comparable with respect to sex, age at onset of disease, type of colitis (UC or CD), disease duration, duration of pretreatment colonoscopic surveillance and the presence
of concomitant PSC (Table 4). Furthermore, at the preceding colonoscopy, findings of aneuploidy and low-grade dysplasia did not differ between treated and controls, and nor did maintenance therapy with SASP or 5-ASA differ.

Table 4. Patient characteristics at study start; sex, age at onset, type of IBD, disease duration, duration of pretreatment colonoscopic surveillance, presence of concomitant PSC, aneuploidy and low grade dysplasia, previous colonic surgery and IBD-treatment with SASP or 5-ASA. Median values with range, n= number.

<table>
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<th>UDCA (n=10)</th>
<th>Placebo (n=9)</th>
<th>P-value</th>
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<td>Sex (M/F)</td>
<td>6/4</td>
<td>5/4</td>
<td>P=0.8</td>
</tr>
<tr>
<td>Age at onset of IBD (years)</td>
<td>17 (7-59)</td>
<td>20 (6-31)</td>
<td>P=0.7</td>
</tr>
<tr>
<td>IBD (UC/CD)</td>
<td>7/3</td>
<td>6/3</td>
<td>P=0.9</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>19 (9-47)</td>
<td>24 (8-41)</td>
<td>P=0.8</td>
</tr>
<tr>
<td>Pretreatment surveillance (months)</td>
<td>62 (7-137)</td>
<td>33 (7-178)</td>
<td>P=0.8</td>
</tr>
<tr>
<td>PSC (n of total)</td>
<td>3/10</td>
<td>1/9</td>
<td>P=0.3</td>
</tr>
<tr>
<td>Aneuploidy (n of total)</td>
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<td>6/9</td>
<td>P=0.2</td>
</tr>
<tr>
<td>LGD (n of total)</td>
<td>5/10</td>
<td>7/9</td>
<td>P=0.2</td>
</tr>
<tr>
<td>Previous colonic surgery (n of total)</td>
<td>3/10</td>
<td>0/9</td>
<td>P=0.04</td>
</tr>
<tr>
<td>Sulphasalazine/5-ASA (n of total)</td>
<td>8/10</td>
<td>6/9</td>
<td>P=0.5</td>
</tr>
</tbody>
</table>

By chance, the treatment group came to include three patients operated for DALM (n=1) or colorectal cancer (n=2) 18 and 106 months before study start (P=0.04).

There were no differences in composed score between the two groups at study start. For the placebo group the mean neoplastic composed score was 1.11±0.23 and for the treatment group 0.87±0.22 (P=0.47). During the study, two of the patients in the placebo group had a colectomy due to increased degree and severity of mucosal changes. Patient # 7 developed HGD in one location in the descending colon at the planned one-year assessment colonoscopy. At this time, the patient also had LGD in two locations and IPP in five locations. DNA-FCM showed five samples with aneuploidy including high S-phase fractions in two of those. This patient was subsequently operated upon with colectomy and an ileo anal anastomosis (IPAA) at 17 months. No colorectal cancer was detected in the colectomy specimen. Pat # 17 was operated upon due to sessile dysplastic polyps interpreted as DALMs with LGD in the cecum after 12 months in the study. This patient displayed aneuploidy in the same area at the previous colonoscopy together with LGD. The patient received an ileoanal anastomosis because of CD. No cancer was found in the surgical specimen.

Beside these two patients, no significant differences could be seen in any of the groups during the study. The mean values of the composed score for the treated were 0.87±0.22 at study start and 0.71±0.23 at study end (P=0.63). Corresponding values for the placebo group was 1.11±0.22 and 0.88±0.95 (P=0.51). There were no differences in mean S-phase fraction for diploid and aneuploid samples at study start compared to study end. For diploid biopsies in the treatment group the mean S-phase fraction was
2.7±2.4 % at study start vs. 2.94±1.01 at study end (P=0.47). In the placebo group the values were 3.17±1.97 and 3.01±1.87 respectively (P=0.86). For aneuploid samples in the treatment group the mean S-phase fraction was 2.95±1.9 % at study start compared to 3.44±1.8 % at study end (P=0.3). Corresponding values in the placebo group were 3.18±1.2 and 3.97±1.21% (P=0.28). The mean percentage of aneuploid cells of from each aneuploid cell population analyzed separately as well as the mean percentage aneuploid cells per total number of biopsies were also compared but no differences could be seen in either group throughout the study period (data not shown).

**Compliance and side effects**

All of the patients in the treatment group were followed as per protocol except patient #14 who became non-compliant after 12 months due to diarrhea and thereafter withdrawn. Patient #18 could not be investigated by colonoscopy at 24 months due to personal reasons. In the placebo group patient #8 did not attend colonoscopy at six, 18 and 24 months but was compliant in taking medicine. One of the patients in the treatment group with known PSC had an attack of cholangitis (#19) and was hospitalized and treated with antibiotics for a week. Pat #12 in the placebo group had a flare up of colitis that was treated with oral steroids (Prednisone). Both of these patients recovered well after treatment. Laboratory test throughout the study did not show any abnormalities in either group except for patient #19 who experienced cholangitis and patient #12 when experiencing an acute attack of colitis (high inflammatory parameters).
6 GENERAL DISCUSSION

The patients with extensive ulcerative colitis and Crohn’s colitis represent a group at high risk to develop CRC which is a major cause of excess morbidity and mortality among IBD-patients. Based on clinical data, (extent, duration, onset of disease, concomitant PSC, positive family history of CRC), high-risk patients at many centers are nowadays included in colonoscopic surveillance programs at regular intervals in order to reduce the CRC-risk. Mucosal dysplasia has been used as a marker for those patients with IBD considered to be at highest risk to develop CRC, and its identification is the basis for colonoscopic surveillance programs. The use of dysplasia as a criterion for a positive test in cancer surveillance has obvious shortcomings. Besides problems in the clinical interpretation of the report resulting from histopathological assessment of dysplasia, there are also problems with inter- and with intra-observer variability among pathologists. In addition, the influence of active inflammation may cause mis- and over-interpretation of non-neoplastic reactive/regenerative features of the mucosa that may be considerable and needs to be minimized. Moreover, the discrimination between regenerative changes and true dysplastic changes in the “indefinite” category is often difficult even in the absence of active inflammation, also for experienced pathologists. There is thus a great need for more reliable and earlier markers to diagnose patients at high risk. This thesis addresses risk assessment, early detection and refined surveillance of such patients.

Methods for risk assessment and their utility

In paper I we investigated whether an increased cell proliferation, assessed by immunostaining with the two monoclonal antibodies MIB-1 against Ki-67 and NCLPCNA against PCNA, were correlated with histopathological dysplasia in UC, and thus could be a marker for identifying patients at high risk of CRC. PCNA and Ki-67 are both intranuclear proteins, involved in the regulation of the cell cycle. Their exact functions are unclear but their expression occurs only in proliferating cells (in S- and, G2/M-phases) and is increased in colorectal adenomas and CRC (203, 204). We found that increased immunostaining with these two antibodies correlated well with increased severity of dysplasia (up to HGD with MIB-1 and up to LGD with PCNA). The most important clinical role for using these antibodies would be in a situation where the pathologist experiences difficulties in discriminating between changes having a true neoplastic potential in the indefinite, probably dysplastic category changes and regenerative, non-dysplastic changes. It was impossible to discriminate between dysplasia and active inflammation using these immunohistochemistry techniques. However, they may still have a role as adjuncts in the objective diagnosis of early dysplasia since most of the patients with longstanding UC have no or very limited disease activity at the time when the risk of CRC become most apparent. Evidently, such techniques require particularly careful preparation of biopsies and the read-out process is time consuming.

A search for a new, novel marker was undertaken in paper II by measuring the enzyme alkaline sphingomyelinase (SMase). This enzyme, found throughout the entire...
gastrointestinal tract, is involved in the digestion of dietary sphingomyelin (SM) (205), providing the mucosa with ceramide, a hydrolytic product of SM. Ceramide is a key molecule in programmed cell death and thus involved in the regulation of cell proliferation and differentiation (202, 206, 207). Reduced alkaline SMase activity has previously been found both in clinically manifest malignancies, such as colorectal cancer and FAP, and premalignant changes such as colorectal adenomas and in flat mucosa from patients with FAP (208, 209). These facts indicate a potential involvement of this enzyme at an early stage of malignant transformation also in patients with IBD. The activity of alkaline SMase was significantly reduced in IBD-patients compared to controls (P=0.006). We also found a tendency, although not significant, of lower values in patients with dysplasia compared to patients without dysplasia. Another interesting finding was that the activity of this enzyme decreased with age, both in patients and controls (P=0.008). High age is one of the most important risk factors for CRC and reduced alkaline SMase activity could reflect one fundamental biological process in the senescence of the colorectal mucosa. Furthermore, the process of senescence can be reflected by histopathological atrophy which is accelerated in chronic IBD, and especially in patients with morphological dysplasia. The other aim of this study was to investigate alkaline SMase’s role as a potential marker for malignant transformation. However, the age-dependent decrease irrespective of the presence of colitis or not, together with the inter and intra-individual highly scattered values of alkaline SMase activity makes its use as a potential marker uncertain. This together with a lack of correlation between alkaline SMase activity and morphological dysplasia or aneuploidy in the individual biopsies does not support the assumption of a direct relationship between alkaline SMase activity and preneoplastic changes.

DNA flow cytometric analyses (FCM) allow, besides accurate determination of ploidy pattern (i.e. diploidy vs. aneuploidy), an assessment of the cell cycle composition (i.e. S- and G2/M-phases). In the evaluation of patients with IBD, undergoing colonoscopic surveillance, the cell cycle composition has generally not been taken into consideration. The aim of paper III was to study the cell cycle composition in patients with normal macroscopical and microscopical findings, as a reference to patients with IBD. As expected all of the biopsies were diploid with low S and G2/M-phases. Interestingly, we found a significant increase of the S-phase with age (P=0.001) which could be in accordance with the decrease in alkaline SMase with age. The decreased alkaline SMase activity might result in increased proliferation in senescent mucosa. The finding of increase of S-phase with age could be of particular interest since an increase in proliferation is generally believed to be central in the initiation of carcinogenesis (210). Furthermore, there was a topographical decrease of cellular proliferation from the right colon to the left colon (P<0.02) and this together with the age dependent variation were novel findings and fundamental when interpreting DNA-FCM (S-phase) analyses in patients with IBD.

In the large study of 324 patients with UC (paper IV) the value of measuring S-phase fraction as a marker of malignant neoplastic transformation was investigated. Patients with UC displayed low cell proliferation (both in diploid and aneuploid biopsies) and did not differ from normal controls. The main difference between frank malignancies and patients with UC was the high S-phase values found in CRCs (both in diploid and aneuploid tumors). In this study only one UC-associated CRC was found, and
displayed, besides aneuploidy, also a very high S-phase (19.6±4.29%). Therefore, findings of high S-phase values, both in diploid and aneuploid samples, in UC patients, could be an indicator of invasive potential.

Means to minimize the CRC-risk in IBD-patients

Are there any preventive strategies to minimize the CRC risk in patients with IBD? As mentioned earlier, compliance with sulphasalazine therapy seems to be associated with a decreased risk of CRC (44, 68, 69) and folic acid supplementation (82-84) may also offer benefits in this respect. Ursodeoxycholic acid (UDCA), which is the 7β-epimer of chenodeoxycholic acid, is used in the treatment of PSC. In a recent retrospective study, Tung et al, showed that UDCA use was associated with a lower risk of colonic dysplasia compared to patients not taking the drug (77).

In paper V we investigated for the first time, in a prospective, double blind, randomized pilot study, UDCA treatment in high-risk patients. All of the patients had longstanding, extensive colitis of long duration with previously known LGD and/or aneuploidy. Furthermore, three of the patients had been operated with an ileorectal anastomosis (2 due to manifest CRC) and one patient with a colonic resection due to a DALM with LGD. In addition, four of the patients had concomitant PSC. The primary aim was to study the impact of UDCA therapy on the need for colectomy due to progression of dysplasia and also to explore the possible preventive/reverting effects of this therapy on dysplasia and/or aneuploidy in these patients. Although our pilot study was small and probably under powered, two of the patients in the placebo group experienced progression of dysplasia and underwent colectomy, while no one in the treatment group progressed. Although, all standard measurements had been taken (prospective allocation, randomization, blinding etc) in order to recruit comparable study groups, still patients in the UDCA-arm could be considered as dysplasia prone (three patients out of 10 had already experienced CRC (2) or DALM (1)). On the other hand, median duration of disease was five years longer in the placebo group, albeit not statistically significant. Nevertheless, progression to the predetermined end-point for prophylactic surgery adhered to the standard protocol used in our surveillance setting, and should be determined as clinically significant. The results from our pilot study therefore may indicate that there is a potential for UDCA as a long-term adjunct to lower the risk of CRC in those high-risk patients that ultimately will develop this malignancy. Several factors may explain the lack of significant effects seen on the mean dysplasia/aneuploidy scores (composed score). The sample size may have been too small. The time frame may have been too short, although, based on the data from experimental CRC-studies in rats, 24 months appeared to be relatively long. The dose of UDCA was 1 g/day which is rather high by normal standards (e.g. in primary biliary cirrhosis) but may still have been too low in this particularly setting. Nevertheless, our study and the previous retrospective study (77) encourage further prospective investigations of UDCA-therapy as a mean to decrease the CRC-risk in IBD. Obviously, our study raises more questions than answers. Obvious ones are when UDCA-therapy should be initiated; in all patients with extensive IBD of the colon? At diagnosis? After eight years of duration? After the first finding of indefinite, probably dysplastic lesions or after the first finding of aneuploidy (or increased S-phase)?

A combination of enhanced colonoscopic surveillance, using markers that are more sensitive and active anti-neoplastic therapy may be the optimal way to manage the increased CRC risk in patients with longstanding IBD in the future.
Major conclusions:

- Immunostaining of biopsy samples with monoclonal antibodies against proliferation antigens may serve as an adjunct in the assessment of histopathological dysplasia in patients with IBD.

- Impaired regulation of cellular proliferation in IBD might be caused by a decrease in the activity of alkaline sphingomyelinase.

- The cell cycle composition (S+G2/M-phases) of normal colorectal mucosa is age and site dependent.

- Increased S-phase fraction of both diploid and aneuploid biopsies may serve as a new marker for detection of malignancies in the surveillance of patients with IBD.

- Ursodeoxycholic acid may prevent further progression of low-grade dysplasia in patients with IBD but further prospective studies of this possibility are required.
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