New insights into inflammatory bowel disease and colitis-associated neoplasia

Fiona van Schaik

New insights into the development of inflammatory bowel disease and colitis-associated neoplasia
Thesis with summary in Dutch, University of Utrecht

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New insights into inflammatory bowel disease and colitis-associated neoplasia

Nieuwe inzichten in inflammatoire darmziekten en
ontstekingsgerelateerde neoplasie
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op donderdag 15 november 2012 des middags te 4.15 uur

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Fijgje Dirkje Maria van Schaik

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Promotoren: Prof. dr. P.D. Siersema
Prof. dr. F.J.W. ten Kate

Co-promotor: Dr. B. Oldenburg

Examine everything carefully,
and hang on to what is good

1 Thessalonians 5:21

CONTENTS

CHAPTER 1

General introduction and thesis outline

Definition and epidemiology of IBD

Ulcerative colitis (UC) and Crohn's disease (CD) are two subtypes of inflammatory bowel disease (IBD) and are characterized by chronic, recurrent inflammation of the gastrointestinal tract. Whereas inflammation is limited to the colonic mucosa in UC, any part of the gastrointestinal tract from mouth to anus may be involved in CD. Although the incidence and prevalence of IBD have been evaluated in multiple studies covering various geographic regions in the world, accurate data on the IBD population in the Netherlands are sparse. The most recent incidence figures, 6.9 and 10.0 per 100.000 per year for CD and UC respectively, date back to the period between 1991 and 1994 and cover a restricted patient population in the southeast of the Netherlands.[1] One earlier Dutch study performed in the Leiden region between 1978 and 1981, reported incidence rates of 3.9 and 6.6 per 100.000 per year for CD and UC, respectively.[2,3] Like in other parts of the world, the incidence of IBD in the Netherlands seems to have increased during the past decades. We recently estimated the prevalence of IBD in the Netherlands to be 326 per 100,000 persons in 2007. This number was based on IBD-related Diagnosis Treatment Combinations from a Dutch health insurance company that included 1.2 million policyholders from various regions in the Netherlands.[4] Although mortality has decreased and overall life expectancy is not significantly lower in UC patients and only slightly lower in CD patients compared to the background population[5-7], this condition still constitutes a major health problem that is associated with significant morbidity and an increased risk of developing colorectal cancer (CRC).[8]

This thesis

IBD is a complex disorder. Although multiple mechanisms have been reported to be involved, the pathogenesis of IBD and IBD-associated CRC is still incompletely understood. This thesis addresses several novel issues with regard to the development of IBD (PART I) and colitis-associated neoplasia (PART II). The general aim of this thesis is to provide new tools for the prediction, treatment and risk stratification of IBD and colitis-associated neoplasia.

PART I: NEW INSIGHTS INTO INFLAMMATORY BOWEL DISEASE

Aetiology and pathophysiology of IBD

Although the exact mechanism(s) underlying the development of IBD is/are still not fully understood, the predominant assumption is that IBD develops from an inappropriate response of the mucosal immune system to the commensal intestinal flora in genetically susceptible individuals. This may be facilitated by both failure of the intestinal epithelial barrier function and defects in the mucosal immune system.[9]

The first supporting evidence for a genetic basis of IBD was obtained from family studies showing a familial aggregation of IBD, especially in CD. First-degree relatives of IBD

patients have an approximately 4 to 20 times higher risk of developing IBD as compared to the background population. More indicative are findings from studies in twins in which concordance has been reported to be higher in monozygotic twins than in dizygotic twins.[10] New emerging evidence for a genetic basis of IBD has been obtained from genome-wide association studies (GWAS) that enable the detection of single nucleotide polymorphisms (SNPs) associated with either CD or UC. By means of these studies a hundred of candidate genes for IBD have been identified, of which approximately one third are associated with both CD and UC. These are called 'true IBD susceptibility genes' and include many constituents of the IL23 pathway. Other genes have been associated with one specific type of IBD. For UC, several epithelial barrier genes have been linked to this disorder, whereas for CD strong associations have been made with various genes involved in intracellular processing of bacteria, including NOD2, ATG16L1 and IRGM.[11,12]

A novel gene that may be associated with CD is the gene encoding the Farnesoid X Receptor (FXR). FXR is a bile acid nuclear receptor involved in bile acid homeostasis. Based on experiments in FXR knock-out and wild-type mice, FXR activation appears essential for antibacterial defense and for maintaining intestinal barrier function.[13] Patients with Crohn's colitis are characterized by an impaired antibacterial defense and intestinal barrier function, and according to recent data, altered FXR expression in areas of inflamed mucosa.[14] Our group recently showed that administration of a synthetic FXR agonist prevents intestinal inflammation, with improvement of colitis symptoms, inhibition of epithelial permeability and reduced goblet cell loss.[15] In CD patients, absorption of bile salts in the ileum into the enterohepatic circulation is thought to be impaired, either through active ileal inflammation or through faster passage of intestinal contents through the small and large intestinal tract. In **Chapter 2** we investigate the hypothesis that this may lead to impaired activation of intestinal FXR and FXR target genes involved in antibacterial defense.

Due to a possible loss of tolerance, various antibodies have been detected in serum of patients with IBD. Of these, antibodies against mannose epitopes from the yeast Saccharomyces cerevisiae (ASCA) and antibodies against a protein in the nuclear lamina of neutrophils (pANCA) are the best-known. Both ASCA and pANCA are highly prevalent in patients with CD and UC, respectively.[16-18] More recently, other antibodies directed against bacterial antigens, including anti-OmpC and anti-CBir1, were also found to be associated with IBD. It is unclear whether these serological markers are already present before the onset of IBD and therefore might function as predictors of IBD. In **Chapter 3** we investigate whether four serological markers (ASCA, pANCA, anti-OmpC and anti-CBir1) can predict the occurrence of either CD or UC in a large European cohort of apparent healthy individuals.

In addition to the role for the immune system and genetic susceptibility, environmental factors have been reported to be important in the development of IBD. Smoking and appendectomy are the strongest environmental factors for IBD risk, being associated with

an increased risk of CD and decreased risk of UC, respectively.[19,20] A possible explanation for the increasing incidence of IBD in previous low-incidence areas is the contribution of diet. The association between pre-illness diet and development of IBD is therefore nowadays gaining more attention. Recently, a high intake of fats and meat was found to increase IBD risk, whereas a decreased risk of developing CD and UC has been reported in patients with high fruit and vegetable intake.[21,22] Extensive studies into the role of other environmental factors, such as breastfeeding, contraceptives and non-steroidal anti-inflammatory drugs have produced conflicting results.[20,23]

PART II: NEW INSIGHTS INTO COLITIS-ASSOCIATED NEOPLASIA

Inflammation and cancer in IBD

Patients with UC or CD in the colon (Crohn's colitis) are at an increased risk of developing inflammation-associated CRC. This type of CRC is hypothesized to develop according to an inflammation–dysplasia–carcinoma sequence and therefore differs from the adenoma–carcinoma sequence seen in sporadic CRC.[24-27] The inflammation-induced cellular damage results in dysplastic changes which may over time progress to malignancy. In a recent meta-analysis of our group, we reported that the cumulative risk of CRC in IBD patients was 1%, 2% and 5%, after 10, 20 and more than 20 years disease duration, respectively (Lutgens MW et al, accepted for publication in IBD). In comparison, men and women in the general population of the Netherlands have a risk of approximately 5% of developing a colorectal malignancy during their lifetime.[28] Surveillance has been introduced to detect colitis-associated dysplasia early. However, detection of dysplasia can be difficult because of its heterogenous endoscopic appearance and the poor interobserver agreement among pathologists regarding the grade of dysplasia. In **Chapter 4** we discuss the endoscopic and histopathological features of colitis-associated dysplasia and propose a practical algorithm with the aim of reducing the uncertainties in the diagnosis of dysplastic lesions and improving the management of colitis-associated dysplasia.

Colitis-associated dysplasia may look as an aberrant lesion or can be present in flat, normal appearing mucosa. Microscopically, dysplasia is classified as low-grade (LGD), high-grade (HGD) or indefinite dysplasia (IND).[29] HGD is associated with a high risk of synchronous or metachronous CRC[30] and is therefore generally considered an unambiguous indication for colectomy. Due to large differences in progression rates to HGD and CRC reported in previous studies[31-34] the management of patients with LGD and IND in flat mucosa is less straightforward. Poor interobserver agreement among pathologists might have contributed to these differences. In **Chapter 5** we therefore describe the frequency of progression of flat LGD and IND to advanced neoplasia (HGD, CRC) before and after histopathological review of the diagnosis by a panel of expert gastrointestinal pathologists.

In order to facilitate the diagnosis of dysplasia, various immunohistochemical markers have been studied, including p53, CD44, Ki67, AMACR, β-catenin, Cyclin D1, p21/waf1 and ALDH. All these markers have been found to correlate to some degree with dysplasia or CRC at a certain time point, but the true prognostic value with regard to development of HGD and CRC is unknown. In **Chapter 6** we examine the role of a series of immunohistochemical markers for prediction of neoplastic progression in UC patients with flat LGD or IND.
Endoscopically visible dysplastic lesions are heterogenous in endoscopic appearance and are commonly subdivided in 'adenoma-like' and 'non-adenoma-like'.[35] Adenomas in patients with IBD pose a clinical problem since it is largely unclear whether these adenomas are associated with a higher risk of CRC due to the underlying disease, compared to sporadic adenomas. In **Chapter 7** we therefore compare the outcome of a cohort of IBD patients with and without adenomas to a cohort of non-IBD patients with adenomas.

Chemopreventive effect of drugs in IBD

The management of patients with CD or UC is subdivided in two phases, including 1. induction of remission, during which active disease is treated, and 2. maintenance of remission, which is aimed at the prevention of recurrence of active disease. Mesalazine or 5-aminosalicylic acid (5-ASA) compounds are often used for induction and maintenance therapy in UC. In CD patients, 5-ASA drugs are less effective and corticosteroids are mostly used as first-line therapy for active disease. Systemic immunomodulators, such as the thiopurines azathioprine (AZA) and 6-mercaptopurine (6MP), are commonly used for maintenance of remission in both UC and CD.[36] Since the neoplastic potential of the diseased colon is supposed to be the consequence of inflammation-induced genetic mutations[25], treatment of inflammation should reduce CRC risk in IBD patients. Previous studies have indeed indicated that 5-ASA drugs might prevent colitis-associated CRC.[37] The putative chemopreventive effect of thiopurines has, however, not been reported. In **Chapter 9** we investigate the association between thiopurine use and risk of advanced neoplasia (HGD, CRC) in a large IBD population in the Netherlands.

Thiopurines-associated malignancies

Due to the cytotoxic and immunosuppressive properties of thiopurines[38,39], the use of these drugs has often been associated with an increased malignancy risk. In organ transplant patients, use of thiopurines was found to result in an increased risk of non-Hodgkin lymphoma and skin cancer.[40] IBD patients using thiopurines have also been found to be at an increased risk of lymphoma.[41,42] The risk of skin cancer in these patients is less clear. In **Chapter 10** we investigate the association between thiopurine use and the risk of non-melanoma skin cancer (basal cell carcinoma and squamous cell carcinoma) in a large cohort of Dutch patients with IBD.

PART III: DISCUSSION AND SUMMARY

In the last part of this thesis, the results are discussed and suggestions for further research given.

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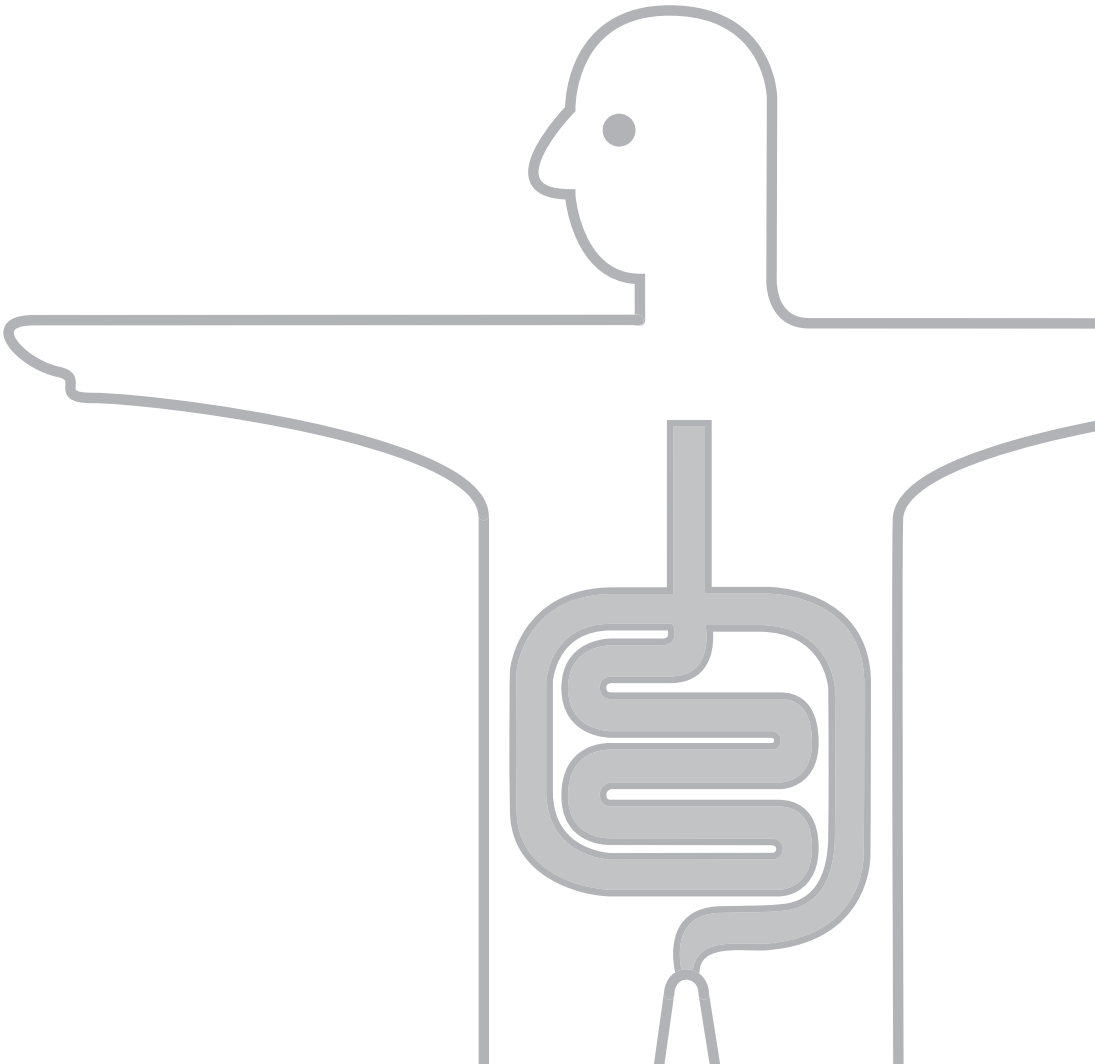
PART I

NEW INSIGHTS INTO INFLAMMATORY BOWEL DISEASE



CHAPTER 2

Pharmacological activation of the bile acid nuclear farnesoid X receptor is feasible in patients with Crohn's colitis

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ABSTRACT

Background and aim

The bile acid-activated nuclear receptor Farnesoid X Receptor (FXR) is critical in maintaining intestinal barrier integrity and preventing bacterial overgrowth. Patients with Crohn's colitis (CC) exhibit reduced ileal FXR target gene expression. FXR agonists have been shown to ameliorate inflammation in murine colitis models. We here explore the feasibility of pharmacological FXR activation in CC.

Methods

Nine patients with quiescent CC and 12 disease controls were treated with the FXR ligand chenodeoxycholic acid (CDCA; 15 mg/kg/day) for 8 days. Ileal FXR activation was assessed in the fasting state during 6 hrs after the first CDCA dose and on day 8, by quantification of serum levels of fibroblast growth factor (FGF) 19. Since FGF19 induces gallbladder (GB) refilling in murine models, we also determined concurrent GB volumes by ultrasound. On day 8 ileal and cecal biopsies were obtained and FXR target gene expression was determined.

Results

At baseline, FGF19 levels were not different between CC and disease controls. After the first CDCA dose, there were progressive increases of FGF19 levels and GB volumes during the next 6 hours in CC patients and disease controls (FGF19: 576 resp. 537 % of basal; GB volumes: 190 resp. 178 % of basal) without differences between both groups, and a further increase at day 8. In comparison with a separate untreated control group, CDCA affected FXR target gene expression in both CC and disease controls, without differences between both groups.

Conclusions

Pharmacological activation of FXR is feasible in patients with CC. These data provide a rationale to explore the anti-inflammatory properties of pharmacological FXR activation in these patients.

INTRODUCTION

The bile acid nuclear Farnesoid X Receptor (FXR) is the master regulator of bile acid homeostasis. FXR is mainly expressed in the ileum and liver, and regulates various genes encoding for bile acid transport proteins, including apical sodium-dependent bile acid transporter (ASBT) and ileal bile acid binding protein (IBABP).[1,2] Expression of the enterokine fibroblast growth factor (FGF)15 (human orthologue FGF19), which induces gallbladder (GB) refilling in the mouse, is also controlled by FXR.[3] It has been hypothesized that FGF15 functions as an "ileal brake" by signaling the end of the postprandial and return to the interdigestive phase. More recent data indicate a role for FXR in the regulation of lipid and glucose metabolism.[4,5]

There is clear evidence that the ileum is a key location where prevention of excessive intestinal inflammation and maintenance of intestinal barrier (both at the level of the small intestine and the colon) are orchestrated. Patients with Crohn's colitis (CC) are known to have an impaired antibacterial defense and impaired intestinal barrier function. For example, endogenous antimicrobial peptides such as α-defensins are produced in the ileum, and their levels are reduced in Crohn's disease, thereby compromising mucosal host defence.[6] In addition, phospholipid concentration and composition in the colonic mucus layer (pivotal in intestinal barrier function) are dependent on bile acid-induced phospholipid secretion in the ileum with subsequent spread to the distal colon by propulsory motility, and these are deficient in patients with inflammatory bowel disease (IBD).[7,8] Finally, FXR has been implicated in maintaining intestinal barrier integrity and in the prevention of intestinal bacterial overgrowth.[9] According to recent data, patients with CC have an altered FXR expression in areas of inflamed mucosa.[10] In two murine models for colitis, we recently showed that the administration of a semi-synthetic FXR agonist ameliorates intestinal inflammation, with improvement of colitis symptoms, preservation of intestinal barrier function, reduced goblet cell loss and inhibition of proinflammatory cytokine expression.[11] The underlying mechanism for these anti-inflammatory effects is thought to be inhibition of NF-κB.[11,12] Furthermore, we recently found reduced FXR target gene expression in the ileum of patients with clinically quiescent CC.[13]

The aim of this study was to investigate whether pharmacological activation of FXR with its endogenous ligand chenodeoxycholic acid (CDCA) is feasible in patients with CC. As a read-out for FXR activation as well as to obtain more insight in the regulation of gallbladder motility in the fasted state, we also measured serum FGF19 levels and determined GB volumes after CDCA ingestion.

PATIENTS AND METHODS

Ethics statement

This study was approved by the Institutional Ethics Committee of the University Medical Center Utrecht, the Netherlands, and the Central Committee on Research involving Human Subjects, the Hague, the Netherlands. Each patient gave written informed consent. The study was monitored by an independent external monitor. The study was registered at the Dutch Trial Register under number NTR2009 (www.trialregister.nl).

Patients and protocol

Patients with clinically quiescent CC (Harvey-Bradshaw Index (HBI) ≤4)[14] and an indication for surveillance colonoscopy and disease controls who underwent colonoscopy for other clinical reasons were included. Disease controls were excluded in case of previous inflammation of the gastrointestinal tract, with the exception of prior infectious gastroenteritis more than 6 months before the study. Additional exclusion criteria for both groups were: stool frequency >4/day; Body Mass Index >30 kg/m^2 (potential interference with ultrasonographic GB volume measurements); C-reactive protein (CRP) > 20 mg/L within three months before the study, aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), gamma glutamyl transpeptidase (GGT) or alkaline phosphatase (ALP) above upper limit of normal within 3 months before the study, abnormal prothrombin time (PT) or activated partial thromboplastin time (APTT); prior surgery of the gastro-intestinal tract (except appendectomy); previous cholecystectomy or papillotomy; GB or bile duct stones; concomitant primary sclerosing cholangitis or other significant hepatic or biliary pathology; any malignancy within 5 years before the study; use of steroids, cyclosporine, methotrexate, anti-TNF compounds, antibiotics, loperamide or codeine, laxatives or other drugs potentially interfering with CDCA (e.g. ursodeoxycholic acid or bile acid sequestrants) within one month before the study, and pregnancy or lactation. Four patients (three controls, one CC) were included in the study despite minimal increases of Alkaline Phosphatase (AF), gamma-glutamyltransferase (GGT) and APTT (resp. 2 U increase of AF in one patient, 2 U increase of GGT in another patient and 1 second increase of APTT in two patients), since these increases were thought not to lead to any safety concerns for these patients. Two included CC patients used oral anticoagulants and therefore APTT and PT were artificially increased during screening.

Seven days before the colonoscopy, body weight, length and Harvey-Bradshaw index were assessed in the morning after an overnight fast. Next, baseline fasting GB volume was determined by ultrasound and blood was collected from a peripheral venous cannula for determination of plasma FGF19 level. After ingestion of CDCA (15 mg/kg body weight; Tramedico, Weesp, the Netherlands; Sigma-tau, Dusseldorf, Germany) GB volume was determined and blood was sampled every hour during 6 hrs. The next six days CDCA (15 mg/

kg) was ingested at bedtime. In case patients experienced side effects of CDCA ingestion, CDCA dosages were reduced with 50%. Dosages were again increased with one capsule (i.e. 250 mg) per day to the original dosage upon disappearance of side effects. Patients collected stools for 24 hrs at day 7. Patients fasted the night before colonoscopy (day 8), and ingested the last dose of CDCA in the early morning, before colonoscopy. Upon arrival at the outpatient clinic, compliance was measured by pill counts. Thereafter, fasting GB volume was determined and blood was collected. After bowel preparation (4L Colofort, Ipsen Farmaceutica, Hoofddorp, the Netherlands), patients underwent colonoscopy in the fasting state, during which biopsies were taken for histological evaluation and analysis of gene expression. For the latter, biopsies obtained from the ileum (n=3) and caecum (n=3) were immediately placed in liquid nitrogen and stored at -80°C until RNA isolation. All ileal biopsies were obtained in the distal ileum, immediately proximal to the ileal Bauhini valve.

FGF19 analysis

FGF19 levels were assessed by a sandwich enzyme-linked immunosorbent assay specific for FGF19 as described elsewhere.[15] The following characteristics of FGF19 dynamics were assessed: baseline fasting FGF19 level prior to first CDCA ingestion ($FGF19_{t0}$); minimal ($FGF19_{min}$) and maximal ($FGF19_{max}$) FGF19 level expressed in ng/mL and as percentage of $FGF19_{t0}$, defined as the minimal resp. maximal FGF19 level during the 6 hrs after the first CDCA ingestion; time (in hrs) to reach $FGF19_{min}$ resp. $FGF19_{max}$ ($Time\ FGF19_{min}$, $Time\ FGF19_{max}$); maximal decrease resp. increase in FGF19 level compared to $FGF19_{t0}$ ($\Delta FGF19_{min,}$ $\Delta FGF19_{max}$) in ng/mL and as percentage of $FGF19_{t0,}$ and the area under the curve (AUC) of the changes in FGF19 level during 360 minutes after the first CDCA ingestion (in ng/mL*360 min.) calculated by the trapezoidal rule[16] as AUC = 30*((X0+aX360)+2*(aX60+aX120+aX180+aX240+aX300)), in which X is the FGF19 level at the specific time point and 'a' is the change in FGF19 level compared to the fasting status (t=0). Likewise, AUCs were calculated as percentages change from baseline (percent*360 min.). In addition, after 8 days of CDCA ingestion we assessed fasting FGF19 level (FGF19_t8) in ng/mL and as percentage of $FGF19_{t0}$, and the increase in FGF19 level compared to $FGF19_{t0}$ ($\Delta FGF19\_t8$) in ng/mL and as percentage of $FGF19_{t0.}$

Gallbladder volume measurement

GB volumes were determined by ultrasonography (5.0 MHz transducer, SDR 1500; Philips Ultrasound Inc., Santa Ana, CA, USA) after an overnight fast. Sagittal and transverse scans were obtained of the GB at its largest dimensions. GB volume was calculated with the sum-of-cylinders method.[17] For each time point, the mean of 3 measurements (at 5 min. intervals) was calculated. Parameters for evaluation of GB dynamics were similar to those assessed for FGF19, as described above.

Fecal bile acid excretion

Bile acids were extracted from faeces with methanol/HCl (95:5 vol/vol) and quantified using an enzymatic assay (Diazyme Laboratories, Poway, USA).

mRNA extraction and qRT-PCR analysis

Biopsies were homogenized (Omni TH tissue homogenizer, Omni International, Kennesaw, USA) and RNA was isolated using RNeasy Micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The quantity, quality and integrity of isolated mRNA were determined by absorption measurement and RNA gel electrophoresis. Subsequently, cDNA was generated from 500 ng of total RNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Roche, Basel, Switzerland). qRT-PCR analysis was carried out using SYBR green PCR master mix (Biorad, Veenendaal, the Netherlands) and a MyIQ real time PCR cycler (Biorad). Values were quantified using the comparative threshold cycle method. Transcript levels were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT). Primers are listed in the Supplementary Table. Transcript levels were compared with a separate group of controls without inflammatory bowel disease who had a colonoscopy but no CDCA ingestion. The fold change in expression levels compared to these controls was assessed for CC and disease control groups on CDCA.

Endpoints

The primary study endpoint was the difference between patients with CC and disease controls in change of fasting FGF19 level after 8 days of CDCA ingestion compared to baseline. Secondary study endpoints included differences between patients with CC and disease controls in: 1. change of fasting plasma FGF19 level after ingestion of the first dose of CDCA; 2. change of fasting GB volumes after ingestion of the first dose of CDCA; 3. expression of FXR and FXR target genes in ileal and caecal biopsies and 4. fecal bile acid excretion.

Sample size calculation

In patients with CC, no data are available regarding the effect of CDCA on plasma FGF19 level, GB volumes or expression of FXR and FXR target genes in the enterocyte. Two previous studies reported mean fasting plasma FGF19 levels of 0.19 – 0.29 ng/mL in healthy individuals.[15,18] One of these studies demonstrated an increase of 250% after ingestion of CDCA in gallstone patients.[18] Assuming that the CDCA-stimulated increase of plasma FGF19 in gallstone patients would equal the increase in our disease controls and that the increase would be reduced with 30% in our patients with CC, 12 patients per group would be required to yield a statistical power of 0.8 (α = 0.05). We decided to end the study with slightly less CC patients (n=9), because we were not able to recruit more patients despite extensive efforts.

Statistical analysis

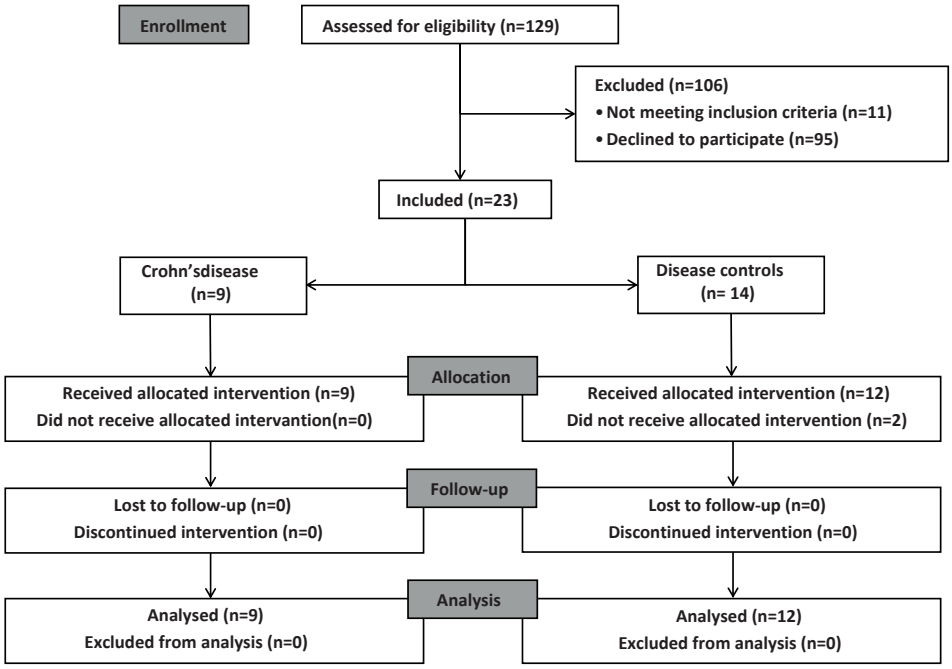
The effects of CDCA ingestion on GB volumes and FGF 19 levels during the first 6 hrs and potential differences between the two patient groups were compared by repeated measures ANOVA with Bonferroni correction. Differences between individual time points within each group were assessed by a paired t-test. Baseline characteristics and various individual parameters for GB dynamics, FGF19 dynamics and faecal bile acid excretion were compared between the two groups (patients with CC and disease controls) using Mann-Whitney U tests. For analysis of gene expression, fold-change relative to a group of untreated disease controls was compared between CDCA-treated CC patients and disease controls using a Mann-Whitney U test. Correlations between FGF19 levels and GB volumes or fecal bile acid excretion were explored by Spearman correlation. A two-sided P-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 15.0.

RESULTS

Clinical patient characteristics

Between September, 2009 and May, 2011 a total of nine patients with quiescent CC and 14 disease controls were enrolled in this study. (Figure 1)

Figure 1. Flowchart of patient inclusion in the study



Two disease controls were included but did not receive CDCA, since baseline gallbladder volumes could not be reliably assessed with ultrasound. These patients were excluded from further analysis. Mean age was 51 years (±SD 15) in CC patients and 50 years (±SD 12) in disease controls. Six CC patients (67%) and six disease controls (50%) were of male gender. In the CC group, mean duration of disease was 20 years (SD ± 10). Mean weight and BMI were respectively 75 kilograms (±SD 11) and 23.6 (±SD 2.2) in CC patients and 79 kilograms (±SD 15) and 24.8 (±SD 2.7) in disease controls. None of these characteristics were significantly different between the two groups. Disease characteristics of CC patients during previous and current colonoscopies are given in Table 1.

Table 1. Disease characteristics of patients with Crohn's colitis at prior investigations and current investigation

	Patients with Crohn's colitis (N=9)	
	Prior investigations (%)	**Current investigation (%)**
Endoscopic disease extent		
No inflammation	0 (0)	7 (78)
<50% of colon	6 (67)	1 (11)
>50% of colon	3 (33)	0 (0)
Unknown	0 (0)	1 (11)
Histological disease extent		
No inflammation	0 (0)	6 (67)
<50% of colon	2 (22)	2 (22)
>50% of colon	6 (67)	0 (0)
Unknown	1 (11)	1 (11)
Endoscopic disease severity		
No inflammation		7 (78)
Mild	3 (33)	2 (22)
Moderate	2 (22)	0 (0)
Severe	4 (44)	0 (0)
Maximum histological disease severity		
No inflammation	0 (0)	7 (78)
Mild	3 (33)	0 (0)
Moderate	3 (33)	2 (22)
Severe	3 (33)	0 (0)
Ileal involvement	3 (33)	1 (11)
Unknown	0 (0)	1 (11)
*Disease behaviour**		
Non-stricturing, non-penetrating (B1)	9 (100)	8 (89)
Stricturing (B2)	0 (0)	1 (11)
+ peri-anal disease	3 (33)	0 (0)

Data presented as numbers (% of group). Disease extent was divided in more or less than 50% colonic involvement; disease extent of more than 50% was defined as involvement of three or more anatomical parts of the colon; *disease behavior according to the Montreal Classification.[25]

In one patient from each patient group, the cecum was not reached and biopsies could therefore not be taken. In the short study period compliance with CDCA ingestion (based on pill counts at day 8) was 100% in all patients (taking into account dose reductions performed by the physician because of perceived side effects), except one who had by accident been taking 1 pill less for 1 day. Mean cumulative CDCA dose was 111 mg/kg * 8days (±SD 13.4) in CC patients and 117 mg/kg * 8 days (±SD 9.90) in disease controls (p=0.26). Three CC patients and six disease controls experienced side effects of CDCA. These consisted of loose stools and bowel complaints in two CC patients and in five disease controls, and heartburn in one CC patient and one disease control. In one CC patient and five disease controls, dose reductions were needed because of these side effects. Mean duration of the reduced CDCA treatment period was 2.3 days (±SD 1.5). There were no serious adverse events.

Plasma FGF19 levels

At baseline, fasting plasma FGF19 levels were not different between CC and disease controls (0.23 ± 0.14 resp. 0.21 ± 0.11 ng/mL, mean ± SD; Table 2). One hour after the first CDCA dosage, mean FGF19 levels decreased to 0.18 ng/mL in CC patients (±SD 0.07, p=0.24 compared to baseline) and to 0.14 ng/mL in disease controls (±SD 0.06, p=0.006 compared to baseline). Thereafter, FGF19 levels progressively increased in all patients (p=0.00, Figure 2) to an average of 576% and 537% of baseline levels in patients with CC and disease controls, respectively. No differences in FGF19 dynamics were found between CC patients and disease controls during the first 6 hours after CDCA ingestion (Table 2, Figure 2). After 8 days of CDCA ingestion FGF19 levels further increased to 1.18 ng/mL in CC patients (613% of baseline, p=0.002) and 1.29 ng/mL in disease controls (626% of baseline, p=0.000), again without differences between both groups.

Table 2. FGF19 dynamics in patients with Crohn's colitis and disease controls during the first 6 hours after CDCA ingestion and after 8 days of CDCA ingestion

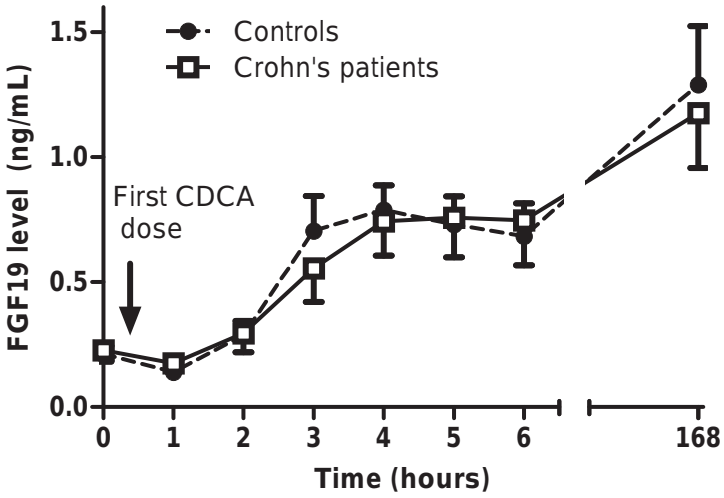
	Crohn's patients N=9	Disease controls N=12	p-value
During the first 6 hrs after CDCA ingestion			
FGF19_t0 (ng/mL)	0.23 (0.14)	0.21 (0.11)	1.00
$FGF19_{min}$ (ng/mL)	0.14 (0.05)	0.13 (0.05)	0.48
$FGF19_{min}$ (%)	70 (28)	66 (23)	1.00
$\Delta FGF19_{min}$ (ng/mL)	-0.09 (0.10)	-0.09 (0.08)	0.78
$\Delta FGF19_{min}$ (%)	-30 (28)	-31 (23)	0.89
Time $FGF19_{min}$ (hours)	1.33 (1.32)	1.33 (0.89)	0.82
$FGF19_{max}$ (ng/mL)	1.00 (0.41)	0.96 (0.41)	0.48
$FGF19_{max}$ (%)	576 (330)	537 (292)	0.72
$\Delta FGF19_{max}$ (ng/mL)	0.77 (0.46)	0.75 (0.37)	0.89

Table 2. FGF19 dynamics in patients with Crohn's colitis and disease controls during the first 6 hours after CDCA ingestion and after 8 days of CDCA ingestion *(Continued)*

	Crohn's patients N=9	Disease controls N=12	p-value
ΔFGF19$_{max}$ (%)	476 (330)	437 (292)	0.72
Time FGF19$_{max}$ (hours)	4.89 (1.05)	4.42 (1.24)	0.38
AUC (ng/mL*360min)	99.1 (107)	110 (81.0)	0.72
AUC (percent*360 min)	61352 (64680)	67291 (58258)	0.57
After 8 days of CDCA ingestion			
FGF19_t8 (ng/mL)	1.18 (0.66)	1.29 (0.88)	0.89
FGF19_t8 (%)	613 (301)	626 (193)	0.83
ΔFGF19_t8 (ng/mL)	0.95 (0.64)	1.08 (0.72)	0.72
ΔFGF19_t8 (%)	513 (301)	526 (193)	0.83

Values are in means ± SD; CDCA, chenodeoxycholic acid; *FGF19_t0,* baseline fasting FGF19 level; *FGF19$_{min}$* (ng/mL), minimal FGF19 level in ng/mL; *FGF19$_{min}$* (%), minimal FGF19 level as percentage of FGF_t0; *ΔFGF19$_{min}$* (ng/mL), difference between FGFmin and FGF_t0; *ΔFGF19$_{min}$* (%), percentage difference between FGFmin and FGF_t0; *Time FGF19$_{min}$* (hours), time to minimal FGF19 level from t0; *FGF19$_{max}$* (ng/mL),maximal FGF19 level; *FGF19$_{max}$* (%), maximal FGF19 level as percentage of FGF_t0; *ΔFGF19$_{max}$* (ng/mL), difference between FGFmax and FGF_t0; *ΔFGF19$_{max}$*(%), percentual difference between FGFmax and FGF_t0; *Time FGF19$_{max}$* (hours), time to maximal FGF19 level from t0; *AUC* (ng/mL*360 min), area under the curve of change of FGF19 level during 360 minutes; *AUC* (percent*360 min), area under the curve of percentage change of FGF19 level during 360 minutes; *FGF19_t8* (ng/mL), FGF19 level after 8 days of CDCA ingestion; *FGF19_t8* (%), FGF_t8 as percentage of FGF_t0; *ΔFGF19_t8* (ng/mL), difference between FGF19_t8 and FGF19_t0; *ΔFGF19_t8* (%), percentage difference between FGF19_t8 and FGF19_t0

Figure 2. Plasma FGF19 levels (SEM) as a function of time after ingestion of chenodeoxycholic acid in patients with Crohn's colitis and disease controls. FGF19 levels progressively rise in all patients (p=0.00) without differences between both groups



Gallbladder motility

GB dynamics after the first CDCA dosage are given in Table 3. Fasting, minimal and maximal GB volumes were slightly lower in patients with CC, although these differences did not reach statistical significance. In contrast to FGF19 dynamics, there was no initial decrease of GB volumes after the first CDCA dosage. Over time, a progressive increase in GB volume was seen in all CC patients and all but one disease controls (p=0.00, Figure 3) to an average of 190% and 178% of baseline GB volumes in CC patients and disease controls, respectively.

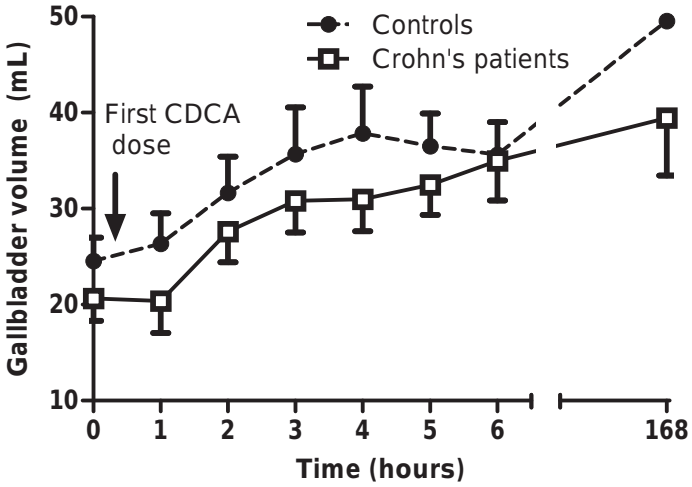
Table 3. Gallbladder dynamics in patients with Crohn's colitis and disease controls during the first 6 hours after CDCA ingestion and after 8 days of CDCA ingestion

	Crohn's patients N=9	Disease controls N=12	p-value
During the first 6 hrs after CDCA ingestion			
V0 (mL)	20.7 (7.02)	24.6 (8.48)	0.29
V_{min} (mL)	17.5 (7.17)	20.9 (7.11)	0.48
V_{min} (%)	86 (18)	89 (20)	0.59
ΔV_{min} (mL)	-3.1 (3.7)	-3.7 (6.4)	0.88
ΔV_{min} (%)	-15 (18)	-12 (20)	0.59
Time V_{min} (hours)	0.56 (0.53)	0.75 (1.42)	0.69
V_{max} (mL)	36.3 (11.1)	41.4 (15.1)	0.36
V_{max} (%)	190 (69)	178 (51)	0.83
V_{max} (mL)	36.3 (11.1)	41.4 (15.1)	0.36
V_{max} (%)	190 (69)	178 (51)	0.83
ΔV_{max} (mL)	15.7 (10.1)	16.9 (12.9)	1.00
ΔV_{max} (%)	90 (68)	78 (51)	0.83
Time V_{max} (hours)	5.11 (1.05)	3.67 (1.83)	0.04
AUC (mL*360 min)	2766 (2407)	3053 (3907)	0.89
AUC (percent*360 min)	16204 (15101)	15199 (15489)	0.94
After 8 days of CDCA ingestion			
V_t8 (mL)	39.5 (18.0)	49.6 (20.7)	0.23
V_t8 (%)	199 (79)	208 (94)	1.00
ΔV_t8 (mL)	18.8 (14.3)	25.0 (17.4)	0.32
ΔV_t8 (%)	99 (79)	108 (94)	1.00

Values are in means ± SD; CDCA, chenodeoxycholic acid; *V0*, baseline fasting gallbladder volume (mean of 3 measurements); V_{min} (mL), minimal gallbladder volume; V_{min} (%), minimal gallbladder volume as percentage of V0; ΔV_{min} (mL), difference between Vmin and V0; ΔV_{min} (%), percentual difference between Vmin and V0; *Time* V_{min} (hours), time to minimal gallbladder volume from t0; V_{max} (mL), maximal gallbladder volume; V_{max} (%), maximal gallbladder volume as percentage of V0; ΔV_{max} (mL), difference between Vmax and V0; ΔV_{max} (%), percentual difference between Vmax and V0; *Time* V_{max} (hours), time to maximal gallbladder volume from t0; *AUC* (mL*360 min), area under the curve of change of gallbladder volume during 360 minutes; *AUC* (percent*360 min), area under the curve of percentual change of gallbladder volume during 360 minutes; *V_t8* (mL), gallbladder volume after 8 days of CDCA ingestion; *V_t8* (%), *V_t8* as percentage of V0; *ΔV_t8* (mL), difference between V_t8 and V0; *ΔV_t8* (%), percentage difference between V_t8 and V0

No differences in GB dynamics were found between CC patients and disease controls during the first 6 hours after CDCA ingestion, with the exception of time until maximum GB volume (*Time V_{max}*), which was shorter in disease controls (Table 3, Figure 3). GB volumes further increased both in CC patients (199% of baseline, p=0.004) and disease controls (208% of baseline, p=0.000) at day 8.

Figure 3. Gallbladder volume as a function of time after ingestion of chenodeoxycholic acid in patients with Crohn's colitis and disease controls. Gallbladder volumes progressively rise in all patients (p=0.00) without differences between both groups



Correlations between plasma FGF19 levels, gallbladder volumes and CDCA doses

Although a significant increase in both GB volume and FGF19 level over the first 6 hrs after CDCA ingestion was found (Figures 2 and 3), there was no significant correlation between plasma FGF19 levels and corresponding GB volumes at the individual time points after the first CDCA ingestion, neither in the two subgroups nor in the total group (R varying from -0.38 to 0.52, p>0.05). In contrast, plasma FGF19 levels and corresponding GB volumes correlated significantly in disease controls after 8 days of CDCA ingestion (R=0.60, p=0.04), but significance was absent in the CC group (R=0.32, p=0.41). Changes in FGF19 levels on day 8 (*ΔFGF19_t8*) compared to baseline were significantly correlated with cumulative CDCA dose (mg/kg * 8 days) in CC patients (R=0.73, p=0.03), but not in disease controls. There was no significant correlation between cumulative CDCA dose (mg/kg * 8 days) and GB parameters on day 8 (results not shown).

Faecal bile acid excretion

In one patient with CC, faecal bile acid excretion could not be assessed due to the absence of bowel movement on the day before the colonoscopy. Mean 24 hrs-fecal bile acid excretion

after 7 days of CDCA ingestion was 2.23 mmol/24 hrs (± SD 2.54) in CC patients and 1.86 mmol/24 hrs (± SD 1.42) in disease controls (p=0.68). In 7 of 8 CC patients and in 10 of 12 disease controls fecal bile acid excretion exceeded normal reference values (0.0-0.4 mmol/24 hrs). Fecal bile acid excretion did not correlate with cumulative ingested CDCA dose (data not shown). No significant correlations between FGF19 levels or GB volumes after 8 days of CDCA ingestion and fecal bile acid excretion were found (data not shown).

FXR target gene expression

Transcript levels of FXR and FXR target genes for the two CDCA-treated groups are given in Table 4. Ileal expression of all investigated genes was not significantly different between both CDCA-treated groups. Compared to a separate untreated disease control group, in CDCA-treated CC and disease control groups mRNA expression of the FXR target genes IBABP (3.2 fold resp. 4.2 fold) and FGF19 (22 fold resp. 43 fold) were higher, and mRNA levels of ASBT (0.46 resp. 0.58 fold) were lower. SHP expression was only higher in CC patients (2.3 fold). Regarding FXR-dependent genes implicated in antibacterial defense: angiogenin 1 mRNA expression was lower in both CDCA treated groups (0.63 fold in both CC patients and disease controls). iNOS expression was only lower in CDCA treated CC patients. mRNA expression of FXR, FXR target and FXR dependent genes in cecum was much lower than in ileum, without differences between CDCA-treated CC and controls (data not shown).

Table 4. Ileal mRNA expression levels of FXR and FXR target genes in patients with Crohn's colitis and disease controls after CDCA stimulation

	Crohn's patients N=8	Disease controls N=11	p-value
FXR	0.48 [0.31-1.43]	0.61 [0.40-0.81]	0.39
SHP	2.32 [0.48-8.52]	1.04 [0.37-4.57]	0.56
IBABP	3.15 [1.85-5.54]	4.15 [2.31-7.66]	0.30
FGF19	21.7 [3.37-862)	42.8 [2.98-909]	0.87
iNOS	0.80 [0.09-18.8]	1.03 [0.36-4.10]	0.74
Angiogenin 1	0.63 [0.28-0.98]	0.63 [0.36-0.94]	0.56
ASBT	0.46 [0.15-1.14]	0.58 [0.17-1.06]	0.74

Data in median [range]; p-values according to Mann-Whitney-U test. Expression levels are given as fold change compared to a separate group of disease controls without CDCA stimulation (see text).

DISCUSSION

Since we previously observed dysregulation of FXR target gene expression in ileal biopsies of CC patients[13], in this study we aimed to explore whether pharmacological activation of ileal FXR is feasible in patients with CC. For this purpose we used CDCA, which is the most potent endogenous FXR ligand in man, in contrast to the hydrophobic bile salt ursodeoxycholic

acid, that has no effect on FXR activation. The main finding of our study is that activation of the bile acid-FXR-FGF 19 axis by the FXR ligand CDCA is feasible in patients with CC. The primary endpoint (increase of FGF19 levels after 8 days of CDCA ingestion) was not different between the two groups (0.95 ng/mL in CC patients resp. 1.08 ng/mL in disease controls). We included slightly less CC patients than originally intended, because we could not recruit more patients willing to undergo the demanding protocol. Nevertheless, it seems highly unlikely that different results regarding the primary endpoint would have been found, even with 12 CC patients. A post-sensitivity analysis revealed that inclusion of three more CC patients with the lowest increase in FGF19 of the CC patient group, would not have changed our results. Although we cannot exclude a difference in FXR activation between CD patients and controls in this study, our findings strongly direct to an adequate FXR activation in CC patients.

In order to get an impression of the extent of FXR activation in the enterocyte, we present mRNA levels as fold change from a separate control group not treated with CDCA. We found no difference in mRNA expression of FXR target genes in the ileal biopsies between the two CDCA-treated groups (Table 4). Although this lack of significant difference may have resulted from a small sample size, the increases in both FGF19 levels, which function as read-out of FXR-activation, and target gene expression support the appropriate activation of the bile acid-FXR-FGF19 axis in the CC group. CDCA treatment increased ileal IBABP and FGF19 expression while reducing expression of ASBT. These changes in bile acid transport proteins are all expected upon FXR activation and appropriate to avoid potentially toxic intracellular bile acid concentrations. It should be noted in this respect that expression of FXR target genes, rather than FXR itself serve as indicators for FXR activation.[19]

In the current study, no data are available on expression of FXR and target genes in the ileum of CC patients and disease controls before CDCA administration, since performing a second colonoscopy was considered not appropriate. Therefore, no conclusions can be made on FXR activity in the basal state. However, we previously reported that, under unstimulated conditions, there is lower expression of ileal FXR target genes in patients with CC.[13] This could be a secondary event due to a disturbed enterohepatic circulation of bile salts: absorption of bile acids in the ileum into the enterohepatic circulation may be impaired in inflammatory bowel disease, either through active ileal inflammation or through faster passage of intestinal contents through the intestinal tract.[20,21] In addition, lower FXR target gene expression in CC patients under unstimulated conditions could be secondary to minor intestinal inflammation, considering the existence of reciprocal inhibition of FXR and various genes encoding pro-inflammatory cytokines.[10,19] Although these mechanisms could have precluded FXR activation by CDCA or other FXR ligands in patients with Crohn's colitis, our current results clearly show that such activation is well feasible in this patient category. Finally, decreased FXR activation in CC patients could hypothetically be the result of overrepresentation of loss of function variants of the FXR gene in this patient category.[22]

Our current results argue against the latter possibility, which is in line with our recent study in which we genotyped seven common tagging SNPs and two functional SNPs (-1G>T and 518T>C) in FXR in 2355 Dutch IBD patients (1162 Crohn's disease and 1193 ulcerative colitis) and in 853 healthy controls. None of the SNPs was associated with inflammatory bowel disease, ulcerative colitis or Crohn's disease, nor with any clinical subgroup of Crohn's disease including CC.[13]

Our findings provide a rationale to further explore the potential beneficial effects of FXR ligands in CC patients. Dysregulation of the immune response to intestinal bacteria is supposed to be a key mechanism in the pathogenesis of Crohn's disease, as illustrated by mutations in autophagy genes, NOD2 mutations and IL23 pathway mutations, with a resulting shift from secretion of anti-inflammatory mediators towards pro-inflammatory molecules. Activation of the Nuclear Factor kappa B (NF-κB) was identified as one of the key factors in this shift, resulting in strongly enhanced expression of pro-inflammatory genes, and recruitment of excess inflammatory cells to the intestinal wall. It has been shown that FXR activation can inhibit NF-κB in the intestine as well as in other organs.[10,12,19] Phase 3 clinical trials are currently being performed to investigate the potential beneficial effects of a semisynthetic FXR ligand (6-ethyl-chenodeoxycholic acid) in chronic cholestatic liver diseases such as primary biliary cirrhosis. Of note, based on increase of plasma FGF19 levels, magnitude of FXR stimulation with CDCA 15 mg/kg in the current study appears similar to 10 mg 6-ethyl-chenodeoxycholic acid in primary biliary cirrhosis (the dose currently proposed in this cholestatic liver disease).[24]

Since FGF19 has been reported to induce gallbladder (GB) refilling in the mouse[3], we also determined concurrent plasma FGF19 levels and GB volumes during 6 hrs after the first CDCA ingestion and after 8 days CDCA. There were progressive increases with time for FGF19 levels and GB volumes, without any differences between CC and disease control groups. The initial decrease in plasma FGF 19 levels in a subset of CC patients and disease controls after first CDCA ingestion remains however unexplained. A similar phenomenon has been observed previously in normal subjects receiving an oral fat load.[24] After the first CDCA dosage, there were no significant correlations between FGF19 levels and GB volumes at the individual time points. A potential explanation could be that initially, there is a strong increase of hepatic bile salt secretion and bile flow (and thus gallbladder filling), due to relatively large amounts of exogenous CDCA entering the enterohepatic circulation. As a result, effects of FGF19 on GB volumes may not be evident initially. In contrast, in steady state conditions, after 8 days of CDCA ingestion, we found a significant positive correlation between FGF19 levels and GB volumes in the disease control group.

In summary, we found normal activation of the bile salt-ileal FXR axis in patients with CC using the endogenous FXR ligand chenodeoxycholic acid. These findings provide a rationale to further explore the potential therapeutic role of FXR agonists in this patient category.

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CHAPTER 3

Serological markers predict inflammatory bowel disease years before the diagnosis

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ABSTRACT

Background and aims

Anti-neutrophil cytoplasmic antibodies (ANCAs) and anti-Saccharomyces cerevisiae mannan antibodies (ASCA) have been detected in serum of ulcerative colitis (UC) and Crohn's disease (CD) patients as well as their unaffected family members. The aim of this study was to establish the value of serological markers as predictors of UC and CD.

Methods

Individuals who developed CD or UC were identified from the European Prospective Investigation into Cancer and Nutrition (EPIC) study. At recruitment, none of the participants had a diagnosis of CD or UC. For each incident case, two controls were randomly selected matched for center, date of birth, sex, date of recruitment and time of follow-up. Serum of cases and controls obtained at recruitment was analyzed for ASCA IgG, ASCA IgA, pANCA, antibodies against Escherichia coli outer membrane porin C (OmpC) and flagellin CBir1. Conditional logistic regression was used to determine risk of CD and UC. Receiver operating characteristics (ROC) curves were constructed to test accuracy.

Results

A total of 77 individuals were diagnosed with CD and 167 with UC after a mean follow-up of 4.5 (SD 3.2) and 4.4 (SD 3.1) years following blood collection, respectively. Combinations of pANCA, ASCA, anti-CBir1 and anti-OmpC were most accurate in predicting incident CD and UC (AUC 0.679 and 0.657, respectively). The predictive value of the combination of markers increased when time to diagnosis of CD or UC decreased.

Conclusion

A panel of serological markers is able to predict development of CD and UC in individuals from a low-risk population.

INTRODUCTION

Although the mechanisms underlying the development of inflammatory bowel disease (IBD) are still incompletely understood, it is biologically plausible that the aetiology of IBD involves an inappropriate response of the mucosal immune system to the commensal intestinal flora in genetically susceptible individuals. This abnormal response may be facilitated by failure of the intestinal epithelial barrier function and/or defects in the mucosal immune system.[1] These mechanisms probably facilitate the generation of antibodies against microorganisms including mannose epitopes from the yeast Saccharomyces cerevisiae (ASCA) and against proteins in the nuclear lamina of neutrophils (pANCA). The combination of these antibodies has been shown to differentiate Crohn's disease (CD) from ulcerative colitis (UC) with a sensitivity of 52-64% and specificity of 92-94%[2,3] and may be used in patients in whom the distinction between CD and UC cannot be made on clinical, histological and/or endoscopical grounds. More recently, other serological markers have been identified, including antibodies against the outer membrane porin C of Escherichia coli (ompC), antibodies against subtypes of flagellins (CBir1) and various anti-glycan antibodies. Both pANCA/ASCA and the more recent identified antibodies were found to be associated with specific clinical and phenotypical characteristics in CD and UC patients. For example, both ASCA and anti-OmpC have been linked to ileal disease, complicated disease behavior and early surgery in CD.[4-7] Patients with UC that have a high level of pANCA are at an increased risk of pouchitis after ileal pouch-anal anastomosis.[8,9]

The appearance of serum antibodies might precede the onset of clinical symptoms in autoimmune diseases by several years. For instance, anti-cyclic citrullinated peptide (CCP) antibodies and rheumatoid factor (RF) have been reported to predate clinical rheumatoid arthritis by up to 22 years[10,11] and the presence of these antibodies is associated with a significantly increased risk of development of rheumatoid arthritis in these individuals (OR 16.1, 95% CI 3.3-76.7 and OR 5.1, 95% CI 1.6-16.0 for anti-CCP and RF-IgA, respectively).[10] Whether serological markers predict the future development of IBD in asymptomatic individuals is presently largely unknown. Only one small study reported the presence of ASCA (IgG/IgA) and pANCA in 32 individuals with incident CD and 8 individuals with incident UC respectively, before the clinical diagnosis was made.[12] We aimed to investigate whether serological markers predict the development of either CD or UC in a large European cohort of individuals.

PATIENTS AND METHODS

Study population

Participants were enrolled in the European Prospective Investigation into Cancer and Nutrition (EPIC) study, which recruited ~520,000 volunteers from 23 centers in 10 European countries

between 1992 and 2000. The study design has been reported extensively elsewhere.[13] For the present study, data and serum were available from participants in 6 European countries, constituting a sub-cohort of 354,398 participants. Subjects were recruited from centers in Sweden, Denmark, The United Kingdom, France, Germany and the Netherlands.

Case and control identification

At recruitment, none of the participants had a diagnosis of CD or UC. Individuals who subsequently were diagnosed with CD or UC during follow-up until July 2010 (incident CD and UC cases) were identified by different measures in order to acquire the most complete coverage, including EPIC follow-up questionnaires, population-based disease registries, hospital-based registries, pathology records and health insurance schemes. For each case, local physicians confirmed the diagnosis of CD or UC on the basis of endoscopy, histopathology, surgery and/or radiology reports and reported the date of diagnosis and extent of disease.

For each incident case of IBD, two controls were randomly selected from the same EPIC center, matched for date of birth (± 6 months), sex and date of recruitment into EPIC (± 3 months). Controls were alive at the date that the matched case was diagnosed (incidence density matching), which ensured that the time of follow-up was similar for all subjects.

Antibody testing

Serum samples donated by participants at recruitment were stored in liquid nitrogen containers and shipped on dry ice to Prometheus Laboratories (San Diego, California, United States of America) where they were analyzed for ASCA IgG, ASCA IgA, pANCA, anti-OmpC and anti-CBir1.[14-17]

The laboratory technicians were blinded to the case/control status of participants.

Statistical analysis

For incident cases we defined time to event by calculating the difference between sampling date and date of diagnosis. To test the accuracy for each of the serological markers studied, receiver operating characteristics (ROC) curves were constructed by plotting sensitivity versus 1-specificity, using the titers of the individual markers. Furthermore, to study whether a combination of markers improved their predictive accuracy, a new ROC curve was compiled which included the relative contribution of each marker based on its concentration, as assessed by conditional logistic regression analysis with backward selection of all five markers. This resulted in 2 serologic scores; 1 for CD versus controls and 1 for UC versus controls. In order to assess risk of CD or UC in different time frames before the diagnosis using the serologic scores of the two cohorts, each cohort was divided into three equally-sized groups based on time to diagnosis. Risk of CD or UC was then calculated by conditional

logistic regression for each time frame and adjusted for cigarette smoking at recruitment. All statistical analyses were performed using SPSS 15.0 for Windows. A p-value <0.05 was considered statistically significant.

Ethical considerations

This study was carried out with the approval of the International Agency for Research and Cancer Institutional Review Board and that of local ethics committees in each center. At recruitment, all participants gave written informed consent for future use of their data and material.

RESULTS

Baseline characteristics

A total of 244 patients with incident IBD were identified, of whom 77 had CD (74% women) and 167 UC (53% women, Table 1). Mean age was 57.2 years (SD 10.0) at CD diagnosis and 58.0 years (SD 10.5) at UC diagnosis. At recruitment, 29 incident CD cases (38%) and 54 incident UC cases (33%) were cigarette smokers as compared to respectively 50 (33%) and 104 (31%) of matched controls. Of CD cases, 26 (34%) had colonic disease, 23 (30%) ileal disease and in 21 (27%) disease that was located in both the ileum and colon. Thirty-two UC cases (19%) had a proctitis, 74 (44%) a left-sided colitis, while in 46 cases (28%) disease extended beyond the splenic flexure. Individuals were diagnosed with CD and UC after a mean follow-up period of 4.5 (SD 3.2) and 4.4 (SD 3.1) years after serum sampling, with a maximum of 14.2 and 15.5 years, respectively.

Table 1. Demographics of cases and controls

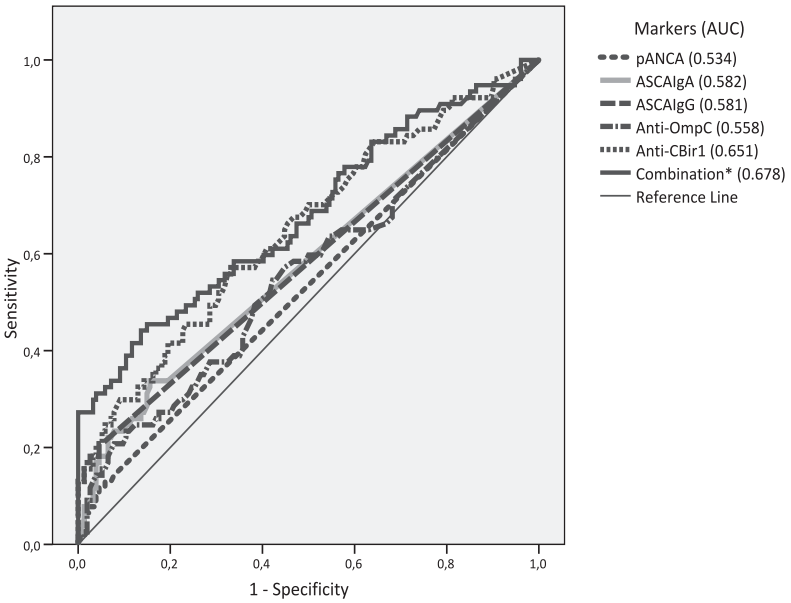
	CD	**CD controls**	**UC**	**UC controls**
	N= 77	N = 144	N= 167	N= 334
Male (%)	20 (26)	40 (26)	79 (47)	158 (47)
Age at recruitment, years (mean ± SD)	52.6 (10.0)	52.5 (9.9)	53.6 (10.1)	53.6 (10.1)
Age at IBD diagnosis, years (mean ± SD)	57.2 (10.0)	-	58.0 (10.5)	-
Time to IBD diagnosis, years (mean ± SD)	4.5 (3.2)	-	4.4 (3.1)	-
Extent of CD / UC (%)				
L1 ileal / L1 Proctitis	23 (30)	-	32 (19)	-
L2 colonic / L2 Left-sided colitis	26 (34)	-	74 (44)	-
L3 ileocolonic / L3 Pancolitis	21 (27)	-	46 (28)	-
L4 isolated upper disease	1 (1)	-	-	-
Unknown	6 (8)	-	15 (9)	-
Perianal disease (%)	6 (8)	-	-	-

IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis

Predictive accuracy of combination of markers

The ROC curves of both the individual markers and the combination of markers for differentiating between incident CD and controls are shown in Figure 1a. The combination of pANCA, ASCA IgG, anti-CBir1 and anti-OmpC (the relative contribution being reflected by the regression coefficient of each individual marker) resulted in a higher predictive accuracy (Area Under the Curve (AUC) 0.678) than that of each individual marker (AUC ranging from 0.534 to 0.651).

Figure 1a. Receiver Operating Charcteristic curves of individual markers and a combination of markers in incident Crohn's disease.



*0.024*pANCA+0.069*ASCAIgG+0.050*anti-OmpC+0.036*anti-CBir1

A serological score of 1.46 as cut-off value for a CD diagnosis was associated with a specificity and positive predictive value (PPV) of 90% and 64%, respectively, and a sensitivity of 39%. The numbers of seropositive individuals according to this cut-off value are shown in Figure 2. A higher serologic score yielded a lower sensitivity and higher specificity. A score of 2.41 was associated with a specificity and PPV of 100%.

The ROC curves of the individual and combination of markers for differentiating incident UC from controls are shown in Figure 1b. Although pANCA was the strongest predictor, the addition of anti-CBir1, anti-OmpC and ASCA IgA increased the predictive accuracy compared to pANCA alone (AUC 0.657 versus 0.621). The addition of ASCA IgG did not increase the accuracy of the model (AUC 0.657). With a serological score of 0.163 as cut-off value for

the UC diagnosis, sensitivity, specificity and PPV were 35%, 90% and 63%, respectively. The numbers of seropositive individuals according to this cut-off value are shown in Figure 2. A serologic score of 5.62 was associated with a specificity of 100% and a PPV of 100%.

Figure 1b. Receiver Operating Charcteristic curves of individual markers and a combination of markers in incident ulcerative colitis.

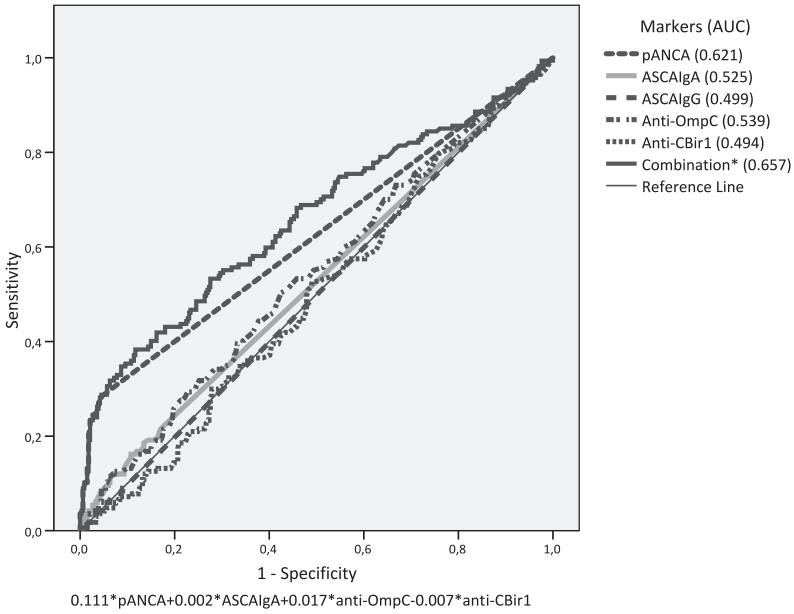
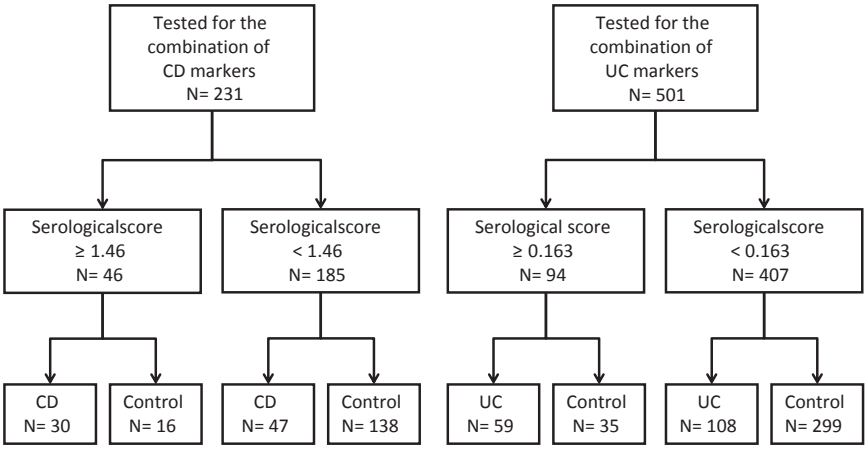


Figure 2. Flowchart of test results in individuals with incident Crohn's disease (CD) and ulcerative colitis (UC) and controls.

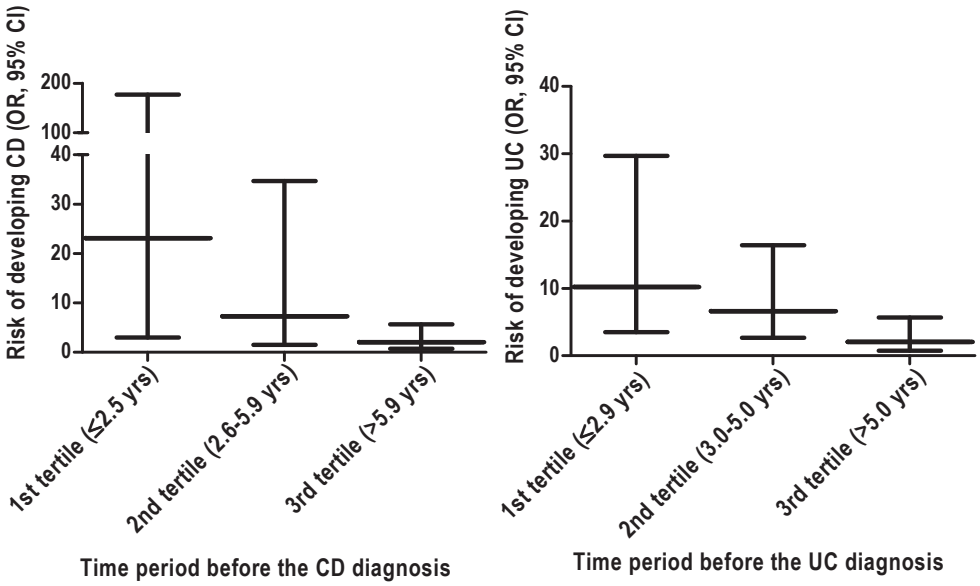


Association between serologic score and time to diagnosis

There was no specific pattern in the appearance of individual antibodies in relation to time to diagnosis in both incident CD and incident UC.

Figure 3 (left) shows the risk of CD in three time frames according to time to diagnosis, using 1.46 as cut-off value of the serological score for the CD diagnosis. The overall Odds Ratio (OR) for developing CD according to this cut-off value was 5.18 (95% CI 2.51-10.7). The risk of developing CD was twenty-three times greater (OR 23.1, 95% CI 3.00-177) amongst participants with a positive serological score who were diagnosed with CD in the first 2.5 years of follow-up. This association was smaller amongst individuals who were diagnosed within 2.6 to 5.9 years (OR 7.30, 95% CI 1.53-34.7) and amongst participants diagnosed more than 5.9 years after baseline (OR 2.05, 95% CI 0.774-5.67).

Figure 3. Risk of developing inflammatory bowel disease in three different time periods before the clinical and histopathological diagnosis using 1.46 as cut-off value for Crohn's disease (left) and 0.163 as cut-off value for ulcerative colitis (right).



1st tertile OR 23.1, 95% CI 3.00-177; 2nd tertile OR 7.30, 95% CI 1.53-34.7; 3rd tertile: OR 2.05, 95% CI 0.774-5.67.

1st tertile OR 10.2, 95% CI 3.51-29.7; 2nd tertile OR 6.61, 95% CI 2.67-16.4; 3rd tertile 2.05, 95% CI 0.774-5.67.

Figure 3 (right) shows the risk of UC in three time frames before the UC diagnosis, using 0.163 as cut-off value of the serological score for the UC diagnosis. The overall OR for developing UC according to this cut-off value was 5.57 (95% CI, 3.22-9.65). The risk of developing UC was ten times greater (OR 10.2, 95% CI 3.51-29.7) amongst participants with a positive serological score who were diagnosed with UC in the first 2.9 years of follow-up. As for CD,

this association was smaller amongst individuals who were diagnosed within 3.0 to 5.0 years (OR 6.61, 95% CI 2.67-16.4) and amongst participants diagnosed more than 5.0 years after baseline (OR 2.05, 95% CI 0.774-5.67).
Adjustment for smoking at recruitment into EPIC had no influence on ORs for each time period before the CD and UC diagnosis (data not shown).

DISCUSSION

The present study assessed the value of serological markers as predictors of inflammatory bowel disease (IBD). We were able to assemble a unique cohort of 77 individuals with incident CD and 167 individuals with incident UC in whom five serological markers were tested. We analyzed the predictive accuracy using the titers of the individual markers and combined these into an equation. The predictive accuracy of a combination of markers was found to be higher than the accuracy of each marker alone, with high specificities and PPVs. Interestingly, the risk of developing either CD or UC amongst individuals with a positive serological score for either CD or UC was found to be highly dependent on time to diagnosis of IBD. This finding, together with the high specificity and PPV suggests that these serologic scores can be used as screening tests for CD and UC in apparent healthy individuals.
The only other study that assessed the presence of serological markers before a clinical diagnosis of IBD examined ASCA and pANCA in recruits of the Israeli military corps of whom 32 developed CD and 8 UC.[12] The investigators reported ASCA positivity in 10 of 32 incident CD patients (31%) 3.2 years before the clinical diagnosis, and pANCA positivity in 2 of 8 subjects (25%) with incident UC. The panel of serological markers used in our study performed better: 30 of 77 (39%) CD patients and 58 of 167 (35%) UC patients tested positive years before the clinical diagnosis. In contrast to Israeli et al., we did not employ the cut-off values as proposed by the producers of the kit. These values were determined in patients with established IBD and could therefore not be used in the apparent healthy individuals in our cohort. The mean interval between serum sampling and time of diagnosis was longer in our study as compared to the Israeli study. The latter study reported an increase in ASCA levels and frequency of positivity in asymptomatic subjects when intervals between serum sampling and time of diagnosis decreased. A shorter mean interval between serum sampling and time of diagnosis might therefore have resulted in higher positivity rates for ASCA and the other antibodies in our study. This is highlighted by our observation that risk of CD or UC was higher among individuals with a positive serological score when the interval between recruitment in the study and diagnosis of IBD was shorter. However, based on individual antibody levels in three different time frames before the clinical diagnosis, we found no specific patterns in antibody levels.
Furthermore, in CD patients ASCAs have been associated with ileal and pANCAs with colonic disease.[6,18] Therefore, the predictive values of these serological markers might be higher in

these specific subgroups. The relative small number of patients with these phenotypes in our study, however, precludes a meaningful analysis.

Another striking difference to the Isreali study relates to the higher mean age at diagnosis of our IBD cohort, which resulted from the inclusion of predominantly middle-aged and elderly individuals in the EPIC study. In CD, ASCA positivity is more frequently encountered in patients diagnosed at a young age[6,19], while pANCA positivity seems to be observed more frequently in CD patients diagnosed at an age of 40 years or older.[18,20] In addition, our cohort included more women than men. Whether these patient characteristics had a major impact on our results is a matter of speculation.

In the present study, we did not have the date of onset of symptoms attributable to IBD at our disposal. Although cases were excluded if symptoms were noted before inclusion into the EPIC cohort, disease duration might have been longer than assessed in this study. However, since the mean interval between blood collection and time of diagnosis was 4.5 years in both cohorts, and the mean diagnostic delay of CD and UC is approximately 9 and 4 months[21], we do not think that this had a major impact on our results. Furthermore, no information was available on the presence of other diseases affecting antibody production, amongst others celiac disease and Behcet's disease.[22,23] These disorders can present with similar symptomatology and are associated with the presence of serological markers as well. We feel that we can safely assume, however, that the risk of confounding by other diseases is low. The IBD diagnosis was confirmed by local gastroenterologists, thereby minimizing the chance of misdiagnosis. The prevalence of Behçet's disease in Western Europa is too low to consider as a source of bias, but we cannot exclude co-existence of celiac disease in our cohort. Since this would have occurred in both the IBD group and controls, the impact on our results is probably small, even though celiac disease might be associated with a modest increased risk of developing IBD.

The reason for antibody generation in future IBD patients is still largely unclear. It has previously been postulated that ASCAs are detectable due to increased intestinal permeability in CD patients leading to an increased exposure to antigens. Several studies could not link intestinal permeability to ASCA levels in CD patients, however.[24-27] In more recent studies, mannan-binding lectin (MBL) has been associated with ASCA generation. MBL plays an important role in human innate immunity by binding to mannan epitopes on the surfaces of microorganisms.[28] Lack of MBL was found to promote a systemic adaptive immune response in mice treated with Candida albicans and was associated with higher ASCA levels in both mice and patients with CD.[29,30] Candida albicans has been shown to generate ASCAs due to the expression of similar cell wall mannan epitopes as the yeast Saccharomyces cerevisiae.[31,32] Standaert-Vitse et al. reported an increased colonization by Candida albicans in CD patients and their healthy relatives. However, when Candida albicans colonization was investigated in relation to ASCAs in CD patients and their healthy relatives,

increased Candida albicans colonization was found to correlate with ASCA levels in healthy relatives, but not in patients with established CD.[33] It thus seems that ASCAs are generated as a result of a deficiency in MBL in the presence of specific intestinal microorganisms. The exact contribution of both immunological and microbiological factors, however, still needs to be further unraveled.

ANCAs have been found in both patients with vasculitis as well as in patients with IBD. IBD patients show an atypical perinuclear staining by indirect immunofluorescence, that is DNAse sensitive. This pattern is different from the perinuclear, myeloperoxidase-reactive staining pattern found in patients with vasculitis.[17] Although various antigens have been suggested to be detected by atypical pANCAs in patients with IBD, including a nuclear histone (H1) and high-mobility group (HMG) non-histone chromosomal proteins (HMG1 and HMG2)[34,35], the main antigen for atypical perinuclear pANCA staining is still unknown. Newer serological markers include anti-CBir 1 and anti-OmpC. Flagellins are principal components of bacterial flagella that are recognized by Toll-like receptor (TLR) 5.[36] The flagellin CBir1 was identified as a dominant target antigen that elicits strong B-cell and CD4 T-cell reactions in mice with colitis. Moreover, CD4 T-cells specific for CBir1 were found to induce colitis when transferred to severe immune deficient mice. CBir 1 was found to induce IgG antibody responses in approximately 50% of CD patients.[15] OmpC, a transport protein in the membrane of Escherichia Coli, was first identified as bacterial antigen in pANCA-positive UC patients, and was then found to elicit antibody responses in up to 55% of CD patients.[3,14,37] Although knowledge on all these antigens is rapidly emerging, the specific role in the pathogenesis of IBD remains to be further investigated.

A major finding of our study is an increase of the predictive value of our panel of serological markers when time to diagnosis of CD or UC becomes shorter**.** We assume that in a significant number of individuals subclinical disease, and consequently, aberrant mucosal immune responses to microbiota antigens, is already present at an early stage. It can be envisioned that these abnormalities are more distinct if time to diagnosis decreases, thereby explaining a better performance of our serological scores. The clinical consequences of these findings are presently unclear.

In conclusion, this study demonstrates the value of a combination of serological markers in predicting CD and UC years before these diseases are being clinically overt. The predictive value of this combination of markers increases when time to diagnosis of CD or UC is shorter. The identification of individuals at risk of CD or UC using a combination of the present serological markers opens new avenues for pathogenetic studies in the 'prediagnostic IBD phase' and thereby introduces a window of opportunity for early intervention.

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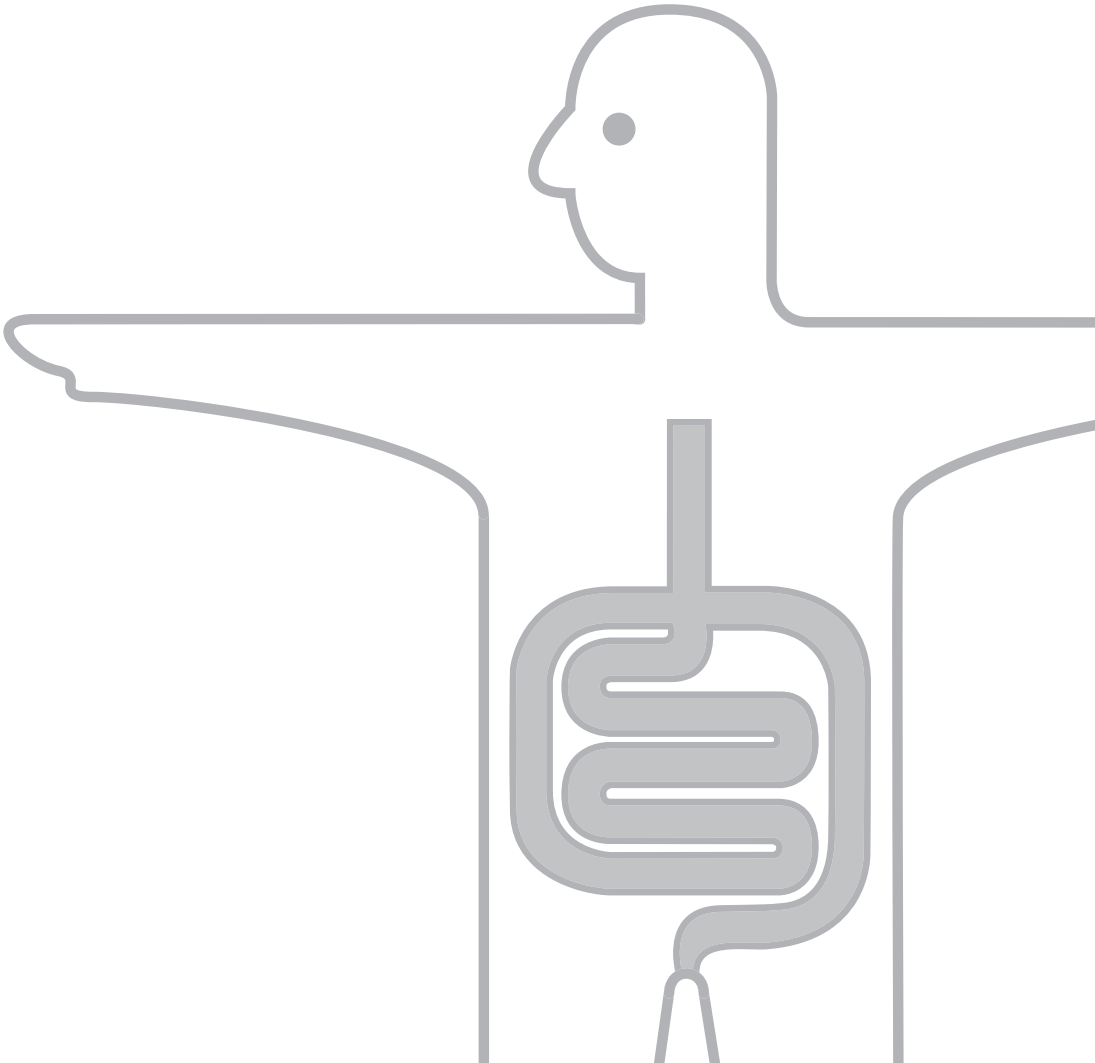
PART II

NEW INSIGHTS INTO COLITIS-ASSOCIATED NEOPLASIA



CHAPTER 4

Endoscopic and pathological aspects of colitis-associated dysplasia

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ABSTRACT

The risk of developing colorectal cancer in patients with colitis-associated dysplasia is considerable. Surveillance programs in patients with ulcerative colitis and Crohn's disease aim to detect dysplastic lesions early and rely heavily on taking random biopsy samples along the length of the colon. Diagnosing dysplasia can be difficult because of the heterogenous endoscopic appearance of dysplasia and the poor interobserver agreement among pathologists when grading dysplasia. Colitis-associated dysplasia may present as a dysplasia-associated lesion or mass (DALM), which may be indistinguishable from a sporadic adenoma in non-colitic tissue, or may arise in flat mucosa of endoscopically normal appearance. Information about the endoscopic appearance, the colonic distribution and the histopathological grade of colitis-associated dysplasia is required to define the optimal treatment. This review summarizes the endoscopic and histopathological features of colitis-associated dysplasia and the requirements for optimal interaction between endoscopists and pathologists, with the aim of reducing the uncertainties in the diagnosis of dysplastic lesions and improving the management of colitis-associated dysplasia.

INTRODUCTION

Patients with ulcerative colitis or Crohn's disease in the large intestine (Crohn's colitis) are at increased risk of developing inflammation-associated colorectal cancer (CRC). This type of CRC is hypothesized to develop in an inflammation–dysplasia–carcinoma sequence and therefore differs from the adenoma–carcinoma sequence seen in sporadic CRC.[1–4] The cumulative risk of developing CRC for patients with ulcerative colitis is 2%, 8% and 18% after 10, 20 and 30 years of having the disease, respectively.[5] This risk has been shown to be comparable for patients with Crohn's colitis.[6,7] In comparison, men and women in the general population have a risk of approximately 5% of developing a colorectal malignancy during their lifetime.[8] Surveillance has been introduced to detect dysplasia early. Since dysplasia can develop in endoscopically unremarkable mucosa, surveillance regimens rely heavily on random biopsy sampling along the length of the colon for examination by a pathologist. Depending on the severity of the dysplasia found, the time intervals between surveillance visits may be shortened or the patients may be referred for a colectomy.[9,10] Raised colitis-associated dysplastic lesions, coined dysplasia-associated lesions or masses (DALMs), are characterized by an endoscopically heterogeneous appearance and may be indistinguishable from sporadic adenomas arising in non-colitic tissue. Describing these lesions accurately and deciding whether they should be removed immediately or can be managed otherwise is a challenge for endoscopists. Furthermore, the surrounding mucosa that appears normal should be biopsied to determine the size of the dysplastic area and whether multifocal dysplastic sites are present. These variables also influence the management of these lesions.[11–13] Confirming dysplasia histopathologically is also difficult. A classification system was introduced to establish more uniformity in grading dysplasia (Riddell's criteria[14]), but the interobserver variation is still great, even among experienced gastrointestinal pathologists.[15,16] Good communication between endoscopists and pathologists to integrate endoscopic, histopathological and clinical factors might help physicians to reach an unequivocal diagnosis on the degree and extent of colitis- associated dysplasia. In this review we discuss the requirements that we consider to be vital for optimal interaction between endoscopists and pathologists when diagnosing colitis-associated dysplasia.

SURVEILLANCE PROGRAMS

Current guidelines

Establishing an effective surveillance program is the first step in enabling endoscopists and pathologists to detect and diagnose colitis-associated dysplasia accurately and early. Current surveillance programs in patients with ulcerative colitis or Crohn's colitis are based on UK and US guidelines.[9,10] These guidelines rely on expert opinions, as there are no prospective,

randomized controlled studies assessing the efficacy of surveillance in patients with IBD. Several retrospective and case–control studies indicate that adherence to surveillance results in a decreased risk of death from CRC and in the detection of cancers at earlier stages.[17–19] A colonoscopic surveillance program in the UK that spanned 30 years and included 600 patients with ulcerative colitis, reported remarkably lower cancer incidence rates (2.5% [95% CI 1.18–4.80] and 7.6% [95% CI 3.99–12.88] after 20 years and 30 years disease duration, respectively) than those reported previously by Eaden et al.[5] However, even in the setting of a dedicated referral center, development of CRC could not be completely prevented.[20] Both the UK and US guidelines advocate performing a screening colonoscopy 8–10 years after diagnosis to determine the extent of the disease in the patient. UK guidelines advise initiating periodic surveillance endoscopies immediately for pancolitis (ulcerative colitis that involves the entire large intestine), while for left-sided disease surveillance may be started after 15–20 years of disease. The presumed exponential increase in cancer risk over time is taken into account by shortening the surveillance intervals from every 3 years in the second decade of disease to annually by the fourth decade. The US guidelines are similar: surveillance should start 8 years after the colitis was diagnosed in patients with pancolitis and 15 years after the colitis was diagnosed in patients with left-sided colitis. However, unlike the UK guidelines, the US guidelines advise repeating surveillance every 1–2 years without any adjustment of the interval between each endoscopic procedure later on. Patients with concomitant primary sclerosing cholangitis have an even higher risk of developing CRC: the 10-year and 20-year risks have been reported to be 9–14% and 31%, respectively.[21,22]In these patients the UK and US guidelines concur that surveillance should be performed annually from diagnosis. We believe that surveillance is most effective when a patient has quiescent colitic disease, as inflammation interferes with the detection of dysplasia. Inflammation may hinder the identification of true dysplastic lesions during endoscopy, leading to underdiagnosis, while regenerative changes seen histopathologically may be misinterpreted as dysplasia, resulting in overdiagnosis. Guidelines generally advocate repeating the colonoscopy after remission of the inflammatory component of colitis.[9,14] During surveillance colonoscopy, the entire colonic mucosa has to be scrutinized and suspicious lesions, if present, should be extensively biopsied. In addition, random biopsy samples should be taken at 10 cm intervals to detect dysplasia in flat mucosa of endoscopically normal appearance. This recommendation is based on a study that estimated that at least 33–34 biopsy samples are needed to exclude dysplasia with a probability of 90%.[23] Although surveillance guidelines are based on data from patients with ulcerative colitis, patients with Crohn's colitis should benefit from surveillance as well.[19] The segmental distribution of Crohn's disease along the colon, however, complicates the assessment of the colonic extent of inflammation and thereby obscures the potential association of the detected dysplasia with previous inflammation at that site. This, in turn, hampers therapeutic decision-making in these patients.

New developments

Our group published a study in 2008 that casts doubt on the value of the current surveillance guidelines. We found that 22–28% of colitis-associated CRCs develop early in the course of disease and could, therefore, be missed if current guidelines to start surveillance ≥8 years after diagnosis of colitis are followed.[24] The need to take random biopsy samples is also debatable if new techniques that increase the sensitivity and specificity of endoscopic surveillance and enable targeted biopsy sampling are used (Box 1). An example of the use of one of these new techniques, chromoendoscopy, compared with conventional endoscopy is shown in Figure 1. Although these new techniques are major advances, they have not been introduced widely in clinical practice, mainly because the procedures require specific endoscopic tools and skills and take longer to perform.

Box 1. New developments in endoscopic surveillance

New techniques have been introduced that increase the sensitivity and specificity of endoscopic surveillance.

Chromoendoscopy (Fig. 1) is a technique that uses dyes to stain the colonic mucosa. This technique results in a higher yield of dysplasia per (targeted) biopsy sample and is altogether a more effective surveillance method.[63-65]

Narrow-band imaging, an endoscopic imaging technique using interference filters to illuminate the mucosa in narrowed red, green and blue bands of the spectrum, allows better visualization of the mucosal structure and vascular networks[66-68] but does not improve detection of dysplasia compared with conventional endoscopy.[69]

Autofluorescence imaging uses blue light for the excitation of tissue-specific autofluorescence. This technique is reportedly superior to conventional endoscopyfor detecting dysplasia.[70]

Endomicroscopy enables imaging of the mucosal layer during colonoscopy, providing *in vivo* data on the microarchitecture of the colonic mucosa and vasculature, with resolution at the cellular level.[71] Using a combination of chromoendoscopy and endomicroscopy, Kiesslich et al. reported a 4.75-fold increased detection rate of neoplastic lesions compared with conventional colonoscopy alone.[72]

Interestingly, in studies reporting results dating back to 1988, most dysplasia was found to be endoscopically visible in ulcerative colitis patients.[25] In our experience, new-generation colonoscopes provide markedly improved high-definition images and might therefore perform even better in surveillance protocols, but again, these may not always be available. We envisage that a surveillance strategy based on well-defined risk factors and using state-of-the-art endoscopy techniques will increase the accuracy of surveillance procedures, and

will render the need to take many random biopsy samples redundant. Nonetheless, until the development and validation of such revised guidelines, the effectiveness of surveillance relies on strict adherence to the current recommendations and optimal interactions between pathologists and endoscopists to reduce the uncertainties in the diagnostic process.

DYSPLASIA: THE ENDOSCOPIST'S PERSPECTIVE

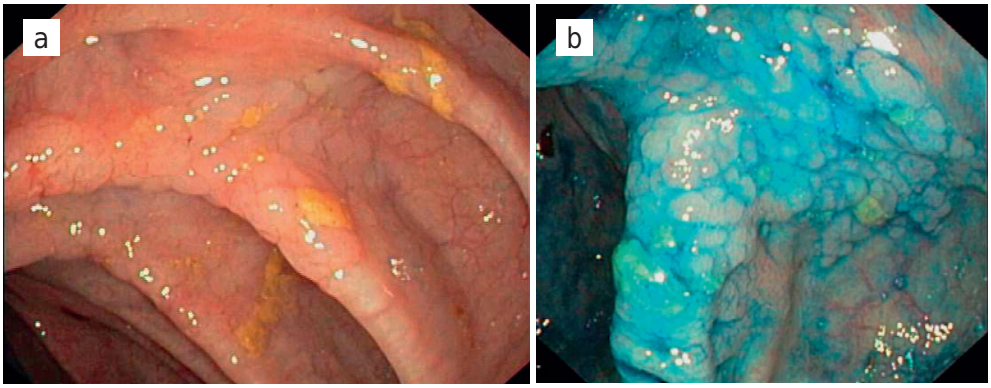
Colitis-associated dysplasia may appear as an endoscopically aberrant lesion or may be present in flat, normal appearing mucosa. During colonoscopy, endoscopists are therefore concerned with the detection, description and biopsy sampling of all endoscopically aberrant, suspicious lesions and the delivery of biopsy samples from the flat, endoscopically normal appearing mucosa to the pathologist.

Dysplasia in obviously aberrant mucosa

During colonoscopy, colitis-associated dysplasia may appear as a solitary adenoma, a discrete nodule, a mass or plaque-like lesion with a raised, irregular or nodular surface, or an area with multiple polypous lesions of different sizes.[11,25] In 1981, Blackstone et al. coined the term DALM for these endoscopically visible raised colitis-associated dysplastic lesions that are frequently associated with CRC. The term DALM, however, can be confusing and is used to describe a variety of lesions. We have noticed that some physicians apply this term to all endoscopically visible dysplastic lesions detected in an actively or previously inflamed segment of the colon, while others use it to describe the more suspicious-looking abnormalities with a perceived high risk of HGD or cancer. There are, in general, two endoscopic subtypes of DALM: adenoma-like and nonadenoma-like (Table 1). The nonadenoma-like subgroup refers to all irregular, diffuse masses or plaque lesions in actively or previously inflamed areas of the colon that generally cannot be removed by endoscopic resection and frequently harbor synchronous malignancy (Figure 1).[11,13] Blackstone et al. reported the presence of CRC in the colectomy resection material in seven out of 12 patients with a nonadenoma-like DALM. These lesions generally warrant a colectomy.[10] The adenoma-like subtype refers to all discrete polyps, either pedunculated or sessile, in actively or previously inflamed segments of the colon. The lesions of this subtype resemble sporadic adenomas, which may occur in individuals without IBD and in non-inflamed colonic segments in patients with colitis. The question arises whether all polyps in actively or previously inflamed segments of the colon in patients with colitis represent true inflammation- associated neoplasia. In a study by Odze et al., 30 polyps from previously inflamed colonic segments in patients without dysplasia in flat mucosa or CRC initially or during long-term follow-up, were compared with 16 sporadic adenomas located outside an area of colitis.[26] With the exception of an increased inflammatory infiltrate of mononuclear cells in the lamina propria of the mucosa of polyps within an area of colitis, no significant differences were found regarding clinical, endoscopic or histological features. It can, therefore, be argued that adenoma-like DALMs with no evidence of dysplasia in the flat mucosa represent age-related sporadic adenomas.

Other data, showing that the molecular features of sporadic adenomas and adenoma-like DALMs are similar, underscore the concept that these lesions may represent a similar pathogenetic entity.[27,28]

Figure 1. Endoscopy image showing a nonadenoma-like dysplasia-associated lesion or mass.



The slightly raised, poorly circumscribed dysplastic lesion was seen in a 53-year-old man with a 20-year history of extensive colitis and primary sclerosing cholangitis. Previously, multifocal indefinite dysplasia was found in random biopsy samples taken during surveillance colonoscopy. The histology of this lesion revealed the presence of high-grade dysplasia (Figure 2). **a** conventional endoscopy. **b** chromoendoscopy.

Dysplasia in flat mucosa

Although a meticulous endoscopic inspection using up-to-date (high-definition) endoscopes will identify most dysplastic areas,[25,29,30] dysplasia may also be detected in random biopsy samples taken from mucosa of normal appearance during surveillance colonoscopies. Random biopsy sampling of the entire colon is therefore considered important. Low-grade dysplasia (LGD) in flat mucosa has been shown to be associated with a 53–54% 5-year risk of progression to high-grade dysplasia (HGD) and CRC.[18,31] (The differentiation of LGD from HGD by histopathology is discussed in the following section.) The risk of progression to HGD and CRC seems to be similar for unifocal and multifocal LGD.[31] Moreover, two studies have reported the occurrence of synchronous HGD or CRC in 19% and 27% of patients with colitis who underwent a colectomy immediately or within 6 months of a diagnosis of flat LGD, respectively.[13,31] These data seem to justify a colectomy in patients with flat LGD, even if only seen in a single biopsy sample. However, considerably lower risks of progression have been reported in two other studies.[32,33] In one of these studies outdated pathology definitions were used, which may have affected the outcome.[32] In the other study no progression was observed during 17.8 years of follow-up of six cases of flat LGD in which the diagnosis was revised retrospectively using the current dysplasia classification criteria.[33] So, although flat LGD is reportedly associated with a high risk of progression to HGD or CRC, different risk strata for the progression of flat LGD in patients with ulcerative colitis or Crohn's colitis may exist. A consensus on the optimal diagnostic and therapeutic approach to LGD in patients

Table 1. Clinical characteristics of different types of dysplasia in patients with inflammatory bowel disease.

	Endoscopic appearance	Pathological features	Risk for CRC	Management
Sporadic adenoma	Circumscribed polypoid lesion, pedunculated or sessile, typically outside the (previously) inflamed colonic mucosa	Circumscribed lesion with tubular, tubulovillous or villous architecture and crypts uniformly lined with adenomatous epithelium	0-6%[20,59]	Polypectomy
DALM, adenoma-like	More or less circumscribed polypoid lesion, mostly sessile, in (previously) inflamed areas of the colonic mucosa Often undistinguishable from sporadic adenomas	Tubular, tubulovillous or villous architecture with dysplastic mucosa and generally lamina propria inflammation Dysplastic crypts may be admixed with normal crypts.	0-10%[12,59]	Local excision or colectomy, depending on degree of dysplasia and presence of multifocal flat dysplasia
DALM, nonadenoma-like	Irregular, diffuse masses or plaque-like lesions in (previously) inflamed areas of the colonic mucosa	Dysplastic mucosa with crypts lined with dysplastic epithelium, occasionally admixed with non-dysplastic crypts and inflamed lamina propria	58%[11]	Colectomy
LGD in flat mucosa	No gross abnormality	Crypts lined with dysplastic epithelium; high nucleus/cytoplasm ratio but nuclei remain confined to the basal half of the cell	0-28%[18,31,33]	Intensification of surveillance or colectomy
HGD in flat mucosa	No gross abnormality	Nuclei extend into the luminal parts of the dysplastic epithelium and lay in a disorganised pattern comparable with cancer, but confined within the basement membrane	33-37%* [13,20]	Colectomy

DALM, dysplasia-associated lesions or mass; LGD, low-grade dysplasia; HGD, high-grade dysplasia. *Risk may apply to HGD in both flat and raised mucosa.

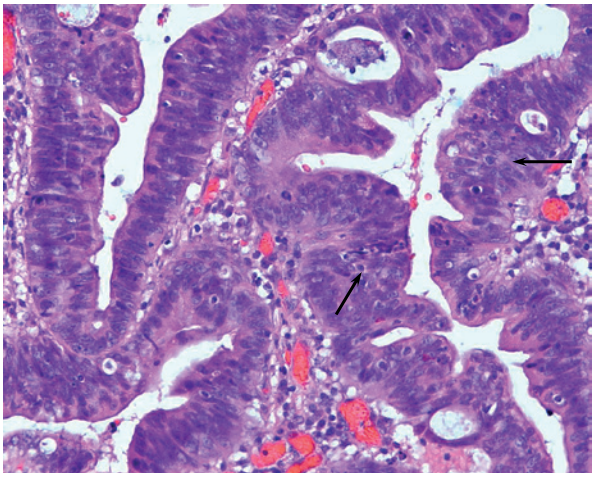
with ulcerative colitis or Crohn's colitis has not been reached. The management of flat LGD should, therefore, be based on careful decision making that integrates clinical, histopathological and patient-related factors.

DYSPLASIA: THE PATHOLOGIST'S PERSPECTIVE

After delivery of paraffin-embedded biopsy samples to the pathologist, the pathologist is concerned with the microscopic examination of the biopsy samples, the determination and classification of dysplasia and the confirmation of the suspicions of the endoscopist. In 1983, Riddell and colleagues defined dysplasia as an unequivocal neoplastic alteration of the colonic epithelium with the potential to become invasive.[14] A classification system was created consisting of three categories: negative, indefinite and positive for dysplasia. The 'negative' category applies to normal mucosa, regenerative mucosa and mucosa with active or chronic inflammation. The category 'indefinite for dysplasia' includes all epithelial changes that are too aberrant to be classified as negative for dysplasia, but do not fulfill all the criteria of unequivocal dysplasia. This category was introduced to group indeterminate inflammation-induced mucosal changes. The 'positive' category is characterized by specific nuclear, cellular and architectural changes to the epithelium. Nuclear changes consist of enlargement of the nuclei, pleomorphism, hyperchromatism, fragmentation of chromatin, excessive mitotic activity, pathological mitoses and a marked stratification of nuclei. The cellular changes are characterized by high ratios of nucleus to cytoplasm, resulting from the enlargement of the nuclei, and decreasing, altered or complete lack of mucus production. The architectural changes often resemble the glandular arrangement of adenomas. The positive category is subdivided into LGD and HGD. Differentiating HGD from LGD is based on the nuclear localization in the cells of the epithelial layer: in LGD nuclei remain confined to the basal half of the cell, whereas in HGD nuclei extend into the luminal parts of the cell in a strikingly disorganized pattern (Figure 2, Table 1). Grading dysplasia is difficult and has shown to be subject to poor inter-observer agreement, even among pathologists who are experts in gastrointestinal pathology. Two studies dating back to the 1980s reported good agreement for the negative category, while poor agreement was observed with regard to all grades in the positive category.[16,34] By contrast, two more recent studies reported poor levels of agreement for the indefinite and low-grade categories compared with the high-grade and negative categories.[15,35] Pathologists frequently use immunohistochemistry to support the diagnosis of dysplasia, including staining for p53, Ki-67 and β-catenin. Mutations in the p53 tumor suppressor gene can be found in 50% of all human cancers.[36] Although p53 mutations are a late event in sporadic carcinogenesis, patients with IBD have been found to harbor mutations in p53 even before the development of dysplasia.[37–40] The monoclonal antibody to Ki-67 identifies the proliferation fraction of tissues and tumors. In normal colonic epithelium proliferating cells are restricted to the basal third of the crypts. However,

in neoplastic epithelium, proliferating cells extend into the superficial part of the crypt.[41–44] β-catenin is a proto-oncogene with a membranous expression in normal epithelium. A decrease in membranous β-catenin expression and an increase in cytoplasmic and nuclear expression is observed in colitis-associated dysplasia and cancer.[45,46] Although these markers may be helpful in diagnostically difficult cases, none are, to date, of proven predictive or diagnostic value. In patients with IBD, the expression of a novel marker, alpha-methylacyl-CoA racemase (AMACR), was shown to be significantly increased in 96% of cases with LGD, 80% of cases with HGD and 71% of cases with CRC, compared with no expression in any cases with mucosa negative for dysplasia.[47] These data were confirmed by two later studies, which showed moderate to strong AMACR expression in neoplastic lesions in patients with IBD.[48,49] However, in the latter studies, weak to moderate AMACR expression was also found in a wide range of IBD patients with no neoplasia. The exact diagnostic value of this biomarker, alone or as part of a panel of markers, awaits further evaluation.

Figure 2. Histopathology of high-grade dysplasia.



Histopathology of the high-grade dysplastic changes in the mucosa of the lesion shown in Figure 1. The crypts are covered by highly atypical epithelial cells (arrows) with a high mitotic activity, concomitant with large hyperchromatic, partly vesicular, and polymorphic nuclei. The orientation of the nuclei is completely lost and the mucus production is almost completely lacking; however, no invasive growth is seen.

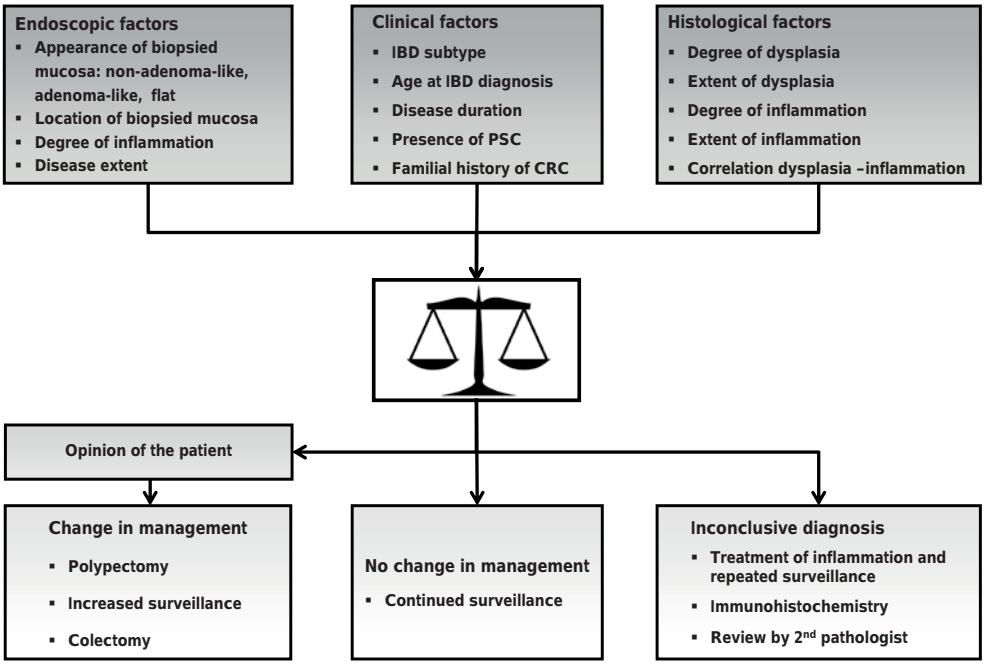
CLINICAL DECISION MAKING

Diagnosis

Identifying and diagnosing dysplasia is a difficult process that depends on the accuracy of both endoscopists and pathologists (Figure 3). Grading dysplasia is purely based on the pathologist's assessment of the sample on the microscopic slide according to Riddell's criteria. However, since this grading has been shown to be difficult, adding relevant clinical

and endoscopic information can be expected to increase the reliability of the pathologist's diagnosis and the validity of the management approach proposed by the endoscopist.

Figure 3. Algorithm for the management of colitis-associated dysplasia.



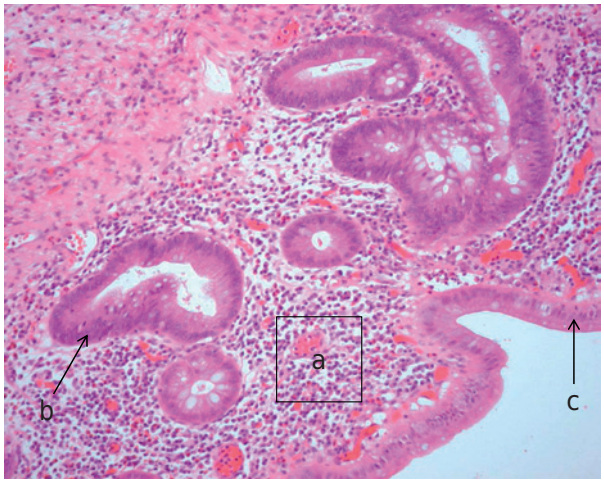
Endoscopic, histopathological and clinical factors should be integrated to reach an unequivocal diagnosis and improve the management of colitis-associated dysplasia. When there are factors of uncertain prognostic significance present (for example, low-grade dysplasia) the patient's opinion may be decisive. CRC, colorectal cancer; PSC, primary sclerosing cholangitis.

The resectability of a lesion, suspicion of HGD or cancer, extent of colitis and diagnosis of Crohn's disease or ulcerative colitis should all be discussed in this setting. Detailed endoscopic information—including a description of the appearance and location of the lesion that was biopsied or endoscopically removed, with photographs of all suspicious areas—may be of additional value to the pathologist interpreting the origin and extent of dysplasia throughout the colon. An adenoma-like DALM often poses a diagnostic problem because differentiation between this and a sporadic adenoma is extremely difficult. The endoscopist should, therefore, inform the pathologist whether the lesion was detected in inflamed or non-inflamed mucosa and should supply the pathologist with several biopsy samples of the surrounding mucosa in order to exclude dysplasia in flat mucosa. To make matters even more complicated, there is the potential for discrepancies between the endoscopic and histopathological assessment of the extent of the inflammatory process, which hinders the estimation of the origin of dysplasia: in patients with ulcerative colitis,

microscopic inflammatory activity is frequently seen proximal to the maximum endoscopic extent of the colitis.[50,51] As dysplasia and CRC can develop in areas of histopathologically proven areas of active inflammation without endoscopic evidence of disease,[52] and since more extensive disease is associated with a greater risk of CRC, the histopathologically documented extent of disease may predict the risk of neoplasia in patients with IBD better than the endoscopic assessment.

We feel that all these clinical, endoscopic and histopathological data should be taken into account when making management decisions about patients with colitis-associated dysplasia. As a consequence of the difficulties in differentiating between regeneration abnormalities in areas of active inflammation and the changes associated with dysplasia, a pathologist might not be able to make a definitive diagnosis based on the biopsy samples obtained from an area of active colitis. In these cases, the pathologist will categorize the histopathological abnormalities as 'indefinite for dysplasia' (Figure 4). The clinical importance of this category remains uncertain because longitudinal data on the risk and optimal treatment associated with this category are sparse. Treatment of the active inflammation followed by repeated colonoscopic surveillance is advised in these cases.

Figure 4. Histopathology of indefinite or low-grade dysplasia.



Histopathology of a biopsy sample from the large intestine of a 56-year-old male with longstanding IBD. The mucus membrane shows a mixed inflammatory infiltrate (a). Low-grade dysplastic changes are seen mainly in deeper parts of the crypts (b). The superficial epithelial cells have more regenerative abnormalities (c). Because of the slightly active inflammatory component, the dysplastic changes were classified as indefinite dysplasia and the biopsy sampling was repeated after treatment of the IBD.

Management

If dysplasia is detected, treatment should be tailored according to the grade of dysplasia, the endoscopic appearance of dysplasia and the extent of disease (Table 1). Moreover, clinical

factors including young age at diagnosis,[53,54] a positive family history of CRC[55,56] and concurrent PSC[21,57] should also influence management decisions, since these are all associated with an increased risk of CRC. A proctocolectomy is recommended if HGD is found, since this diagnosis has been associated with a high prevalence of synchronous CRC and high incidence of subsequent CRC. In a review of 10 prospective studies, Bernstein et al. reported a 42% prevalence of CRC in resection material from patients operated on for HGD.[13] However, no consistent policy exists for the treatment of LGD. Either intensification of the surveillance program or a proctocolectomy, especially for multifocal LGD, is recommended.[13,31,58] Both adenoma-like DALMs and sporadic adenomas, when not accompanied by dysplasia in the surrounding flat mucosa, can be effectively treated by polypectomy and continued endoscopic surveillance.[26,59,60] We recommend that endoscopic resectability, not the location in inflamed or non-inflamed mucosa, should guide the treating physician in clinical decision making for polypous lesions in patients with Crohn's colitis. Whenever a polyp is removed by endoscopic excision, biopsy samples from the flat mucosa surrounding the removed lesion should be taken to assess the completeness of the resection. Tattooing the resection site of the lesion may be helpful to identify the remnants of the lesion during colonoscopic follow-up.[61,62] In all other situations, including dysplasia in flat mucosa surrounding polyps or elsewhere in the colon, technically unresectable polyps, polyps harboring carcinoma, and non-adenomatous DALMs, a colectomy is advocated. The segmental distribution of Crohn's disease along the colon further complicates therapeutic decision making, such as whether to perform a proctocolectomy for a dysplastic lesion, especially if only a segment of the colon has been previously inflamed. So far no consensus has been reached on this topic. In our view, a partial colectomy might be a good alternative to complete colectomy in patients with Crohn's colitis and a confirmed, unresectable DALM in an isolated area of previous inflammation.

CONCLUSIONS

An extensive surveillance program including periodic colonoscopy and a comprehensive biopsy sampling regimen has been advocated to detect colitis-associated neoplasia early. When the new techniques to increase the sensitivity and specificity of endoscopic surveillance become more widely available, there will be a need to revise the current guidelines. The identification, grading and interpretation of dysplastic lesions can be troublesome for both pathologists and endoscopists. The combination of endoscopic, histopathological and clinical factors should guide treating physicians deciding if dysplasia should be managed by continued surveillance with or without increased frequency, endoscopic removal or colectomy (Figure 3). The importance of careful cooperation and communication between pathologists and endoscopists to reduce uncertainties in the diagnosis of dysplastic lesions, and to improve the management of colitis-associated dysplasia cannot be overestimated.

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CHAPTER 5

Misclassification of dysplasia in patients with inflammatory bowel disease: consequences for progression rates to advanced neoplasia

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ABSTRACT

Background and aim

The natural behavior of flat low-grade (LGD) and indefinite dysplasia (IND) in patients with inflammatory bowel disease (IBD) remains uncertain and seems to be dependent on the interpretation of the pathologist. We studied the progression rate of flat LGD and IND to advanced neoplasia (high-grade dysplasia (HGD) or colorectal cancer (CRC)) before and after histopathological review by a panel of gastrointestinal expert pathologists.

Methods

A nationwide pathology database was used to identify IBD patients with dysplasia in six Dutch university medical centers between 1990 and 2006. Medical charts of patients with recorded flat LGD or IND were reviewed. Histological slides from three university medical centers were reviewed by a panel of three expert GI pathologists.

Results

We identified 113 flat LGD patients and 26 flat IND patients. Advanced neoplasia was found in 18 flat LGD patients (16%) after a median follow-up of 48 months resulting in a 5-year progression rate of 12%. Five IND patients (19%) developed advanced neoplasia after a median follow-up of 24 months resulting in a 5-year progression rate of 21%. Review of 1547 histological slides from 87 patients resulted in an increase of the 5-year progression rate of flat LGD to advanced neoplasia to 37%, whereas the progression rate of IND decreased to 5%.

Conclusions

A diagnosis of flat LGD that is confirmed by a panel of expert GI pathologists is associated with a substantial risk of progression to advanced neoplasia while confirmed IND is associated with a low risk of progression.

INTRODUCTION

Patients with longstanding ulcerative colitis (UC) or Crohn's disease of the colon (CD) are at increased risk of developing colorectal cancer (CRC).[1-3] Colitis-associated CRC is supposedly developing along an inflammation-dysplasia-carcinoma sequence.[4] Dysplasia is classified microscopically as low-grade (LGD), high-grade (HGD) or indefinite dysplasia (IND).[5] HGD is associated with a high risk of synchronous or metachronous CRC[6] and is therefore generally considered an unambiguous indication for colectomy. Decision making in case of flat LGD and IND is, however, not straightforward. Progression rates of flat LGD to HGD or CRC varied greatly in previous reports, ranging from no progression to 5-year progression rates of more than 50%.[7-10] These diverging results may have their origin in the selections of study populations and design, and endoscopy-related matters. Another pivotal contribution to this phenomenon, however, may be the (mis)interpretation of the pathologists when grading dysplasia. Dysplasia in the indefinite and low-grade categories in particular is associated with a poor interobserver agreement.[11,12] The impact of the different interpretations of dysplasia on the progression rate of flat LGD is unknown. Moreover, data on the natural course of IND are scarce. We studied the frequency of progression of flat LGD and IND to advanced neoplasia (HGD, CRC) before and after histopathological review of the diagnosis by a panel of expert gastrointestinal pathologists. Furthermore, we determined which clinical factors were associated with the risk of progression.

MATERIAL AND METHODS

Study population

The nationwide pathology archive (PALGA), containing all pathology reports from the Netherlands dating back to 1971, was used to identify patients with IBD-related dysplasia. The PALGA database has a complete nationwide coverage since 1990. A PALGA search for diagnoses of IBD and dysplasia or atypia was performed in six Dutch university medical centers for the time period of January 1990 until April 2006, using diagnostic terms in line with SNOMED® terminology. The following combinations of search terms were used to search for dysplasia: colon AND all epithelial dysplasias, colon AND atypia, rectum AND all epithelial dysplasias and rectum AND atypia. These terms were combined with the following terms to search for dysplasia in patients with IBD: colitis, ulcerative colitis, indeterminate colitis, idiopathic colitis and Crohn's disease. Only patients with LGD or IND in flat mucosa were included in this study. A biopsy was defined as 'flat' when the histopathological diagnosis of LGD or IND was made in a biopsy that was taken from endoscopically normal mucosa, as documented in the colonoscopy report. Patients were recruited in the study at the first time flat LGD or IND was detected. Patients with LGD or IND in raised or otherwise suspect mucosa, those who were diagnosed with HGD or CRC previously or synchronously

at the time of flat LGD/IND diagnosis, patients with a previous or concomitant subtotal or total colectomy, patients without any endoscopic or surgical follow-up and patients with incomplete or missing medical charts were excluded.

Data collection

A single reviewer collected the following characteristics from medical charts, endoscopy, pathology and surgery reports: gender, date of birth, date of IBD diagnosis, presence of primary sclerosing cholangitis (PSC), date of diagnosis of flat LGD or IND, location of flat LGD or IND, maximum endoscopic and histological extent of disease, the finding of LGD or advanced neoplasia during follow-up and duration of follow-up. Disease extent was defined as the maximum extent according to histology and endoscopy reports. In UC patients or patients with indeterminate colitis (IC), proctitis was defined as disease limited to the rectum, left-sided colitis as disease proximal to the rectum but distal to the splenic flexure and extensive colitis as disease proximal to the splenic flexure. In CD patients, disease extent was defined as ileal if disease was located in the ileum, colonic if disease was located in the colon and ileocolonic if disease was located in both the ileum and colon. Dysplasia was considered 'unifocal' if only one specimen jar contained dysplasia and 'multifocal' if two or more specimen jars contained dysplasia. Advanced neoplasia was defined as the finding of HGD or CRC in a biopsy or colectomy specimen. Progression was defined as the development of advanced neoplasia during follow-up. Duration of follow-up was measured in months and defined as the time from first flat LGD/IND diagnosis to one of the following endpoints: 1. end of follow-up (1st of December 2007); 2. death; 3. subtotal or total colectomy, or 4. lost-to-follow-up. When dysplasia was found during follow-up, location, grade and mucosal appearance were documented. When CRC had evolved, location, stage and therapy were documented.

Pathology review

Histology slides from patients with flat LGD or IND were retrieved from three academic centers. The selection of centers for histopathological review was based on the availability of histological slides from these centers. All slides belonging to the original pathology reports that reported dysplasia or CRC were distributed among three expert gastrointestinal pathologists (JAO, FJWtK, MEIS) and reviewed by one of these three pathologists in a blinded fashion. Slides were reviewed according to the criteria and definitions as articulated by Riddell et al.[5] Each slide was scored as negative, positive or indefinite for dysplasia, and in case of positive for dysplasia as LGD or HGD. The indefinite category was subdivided in 'probably positive' and 'probably negative'. The most advanced degree of neoplasia observed in a histological slide during review and the relation to the underlying IBD was documented. When a pathologist doubted the degree of dysplasia or when disagreement existed between

the original and the revised diagnosis, slides were discussed and reassessed by all three pathologists during a regular scheduled meeting in order to obtain a consensus diagnosis. We compared original diagnoses with revised diagnoses and assessed progression rates before and after histopathological review. Patients with missing slides containing dysplasia according to the original diagnosis were excluded from further follow-up analysis.

Statistical analysis

Patients were entered in this study at the first finding of flat LGD/IND and were followed for the progression to advanced neoplasia. Time to progression was measured in months. Patients who did not develop HGD or CRC during follow-up were censored at the moment of the last colonoscopy or colectomy. Five-year progression rates were calculated using Kaplan-Meier survival analysis and comparisons between progression rates were made using log-rank testing. Univariate and multivariate testing was performed using Cox regression analysis. Proportions were compared using the Chi-Square test or Fisher's Exact test, where appropriate. Continuous variables were compared using the Student's t-test or Mann-Whitney U test, where appropriate. A p-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 15.0 for Windows.

Ethical considerations

This study was carried out with approval of and in accordance with the ethical guidelines of the research review committee of our institution.[13]

RESULTS

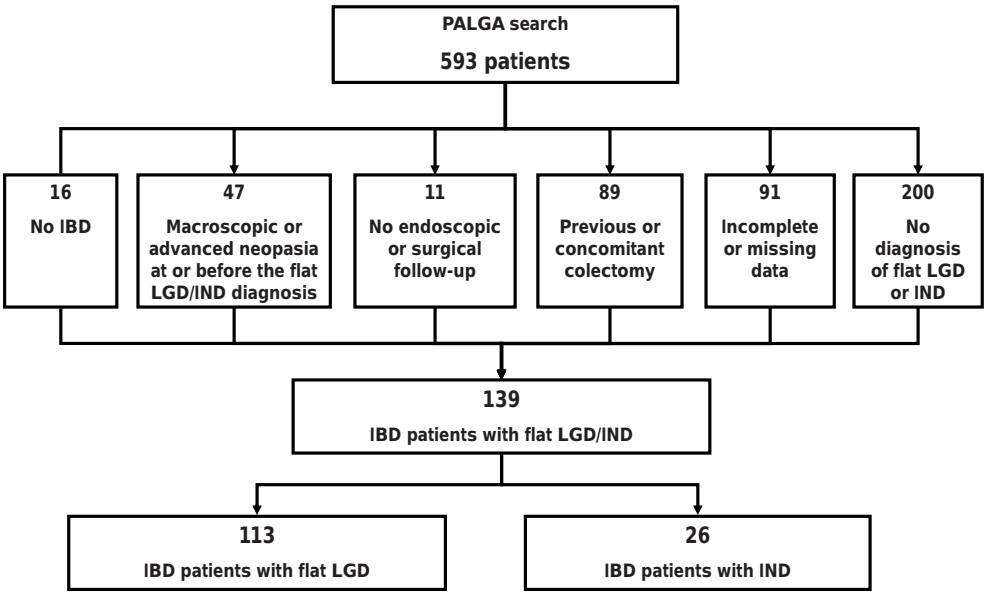
Patients

Our search yielded 593 IBD patients with dysplasia or atypia in the initial report. Review of medical charts, endoscopy, pathology and surgery reports resulted in exclusion of 454 patients, leaving 113 IBD patients with flat LGD and 26 IBD patients with IND for further analysis. The reasons of exclusion are shown in the flowchart (Figure 1).

Progression of flat LGD to advanced neoplasia before review

An initial diagnosis of flat LGD was made in 92 UC patients (81%), in 18 CD patients (16%) and in 3 IC patients (3%). Clinical characteristics of these patients are shown in Table 1. In more than half of the patients, flat LGD was found to be unifocal, predominantly located in the rectum. Forty patients had multifocal flat LGD, which was located distally to the splenic flexure in 22 patients (55%). Patients were followed for a median duration of 71 months (range, 0-209). In the whole group of 113 patients with flat LGD, 18 patients (16%) developed advanced neoplasia.

Figure 1. Flowchart of PALGA search



Eleven patients progressed to HGD, 5 of whom developed CRC subsequently. Of these 5 patients, 3 were operated on after the detection of HGD and CRC was found in the surgical specimen. In 1 patient, CRC was found during colonoscopy performed 4 months after the detection of HGD while in another patient CRC was diagnosed after 11 years during a follow-up endoscopy. In another 2 patients HGD and CRC were detected synchronously. Five patients progressed to CRC without prior HGD. In 16 patients (89%), colectomy was performed whereas in 1 patient only the rectum harbouring CRC was resected. Another patient underwent a transanal endoscopic microsurgery procedure to resect a non-adenoma-like DALM with HGD. In 6 patients with no endoscopic or histological evidence of progression, advanced neoplasia was found in the colectomy specimen. Median time to progression was 48 months (range, 1-169 months). The 5-year progression rate to HGD or CRC for all 113 patients with flat LGD was 12% (95% Confidence Interval (CI), 0.05-0.19) (Figure 2).

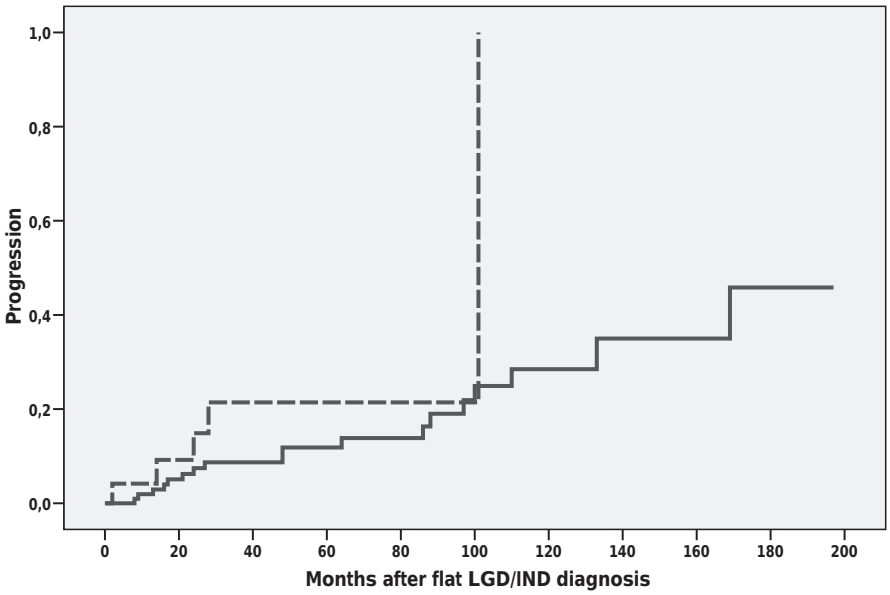
In 95 patients no progression to HGD or CRC was found during a median duration of follow-up of 65 months (range, 0-209) and a median of 3 colonoscopies per patient (range, 0-24). In 27 patients (28%) one or more foci of LGD were detected during follow-up, but none progressed to HGD or CRC.

Table 1. Patient characteristics of flat LGD and IND patients before histopathological review of dysplasia

Variable	Flat LGD N = 113	IND N = 26
Sex (%)		
Male	60 (53)	19 (73)
Female	53 (47)	7 (27)
IBD diagnosis (%)		
Ulcerative colitis	92 (81)	21 (81)
Crohn's disease	18 (16)	4 (15)
Indeterminate colitis	3 (3)	1 (4)
Age at diagnosis, years (median, range)	28 [7-78]	25 [13-66]
Age at initial dysplasia diagnosis, years (median, range)	41 [12-78]	37 [18-73]
Duration of IBD at initial dysplasia diagnosis, years (median, range)	11 [0-43]	14 [0-30]
Extent of IBD (%)		
Ulcerative colitis – Proctitis	1 (1)	1 (5)
Ulcerative colitis – Left-sided	16 (17)	6 (29)
Ulcerative colitis – Extensive	75 (82)	14 (76)
Crohn's disease – Colonic	11 (61)	3 (75)
< 50% of colon	1 (9)	0 (0)
> 50 % of colon	10 (91)	3 (100)
Crohn's disease – Ileocolonic	7 (39)	1 (25)
< 50% of colon	1 (14)	0 (0)
> 50% of colon	6 (86)	1 (100)
Indeterminate colitis – Proctitis	0 (0)	0 (0)
Indeterminate colitis – Left-sided	0 (0)	0 (0)
Indeterminate colitis – Extensive	3 (100)	1 (100)
Location of dysplasia (%)		
Unifocal	61 (54)	17 (65)
Rectum	30 (49)	8 (47)
Sigmoid	15 (25)	7 (41)
Descending colon	8 (13)	2 (12)
Transverse colon	2 (3)	0 (0)
Ascending colon	3 (5)	0 (0)
Cecum	3 (5)	0 (0)
Multifocal	40 (35)	7 (27)
Unknown	12 (11)	2 (8)
Mean no. of biopsies sampled for detection of dysplasia (±SD)	12 (11)	14 (12)
Primary sclerosing cholangitis (%)	14 (12)	2 (8)
Duration of follow-up, months (median, range)	71 [0-209]	54 [0-111]

IBD, inflammatory bowel disease; LGD, low-grade dysplasia; IND, indefinite dysplasia

Figure 2. Kaplan-Meier curve comparing the progression rate to advanced neoplasia in patients with flat LGD (—) and IND (---) (log-rank test p = 0.079) before pathology review. Patients with no progression are censored at the moment of their last colonoscopy or colectomy. Vertical lines represent events of advanced neoplasia. LGD, low-grade dysplasia; IND, indefinite dysplasia.



Progression of IND to advanced neoplasia before review

IND was initially found in 21 UC patients (81%), 4 CD patients (15%) and 1 IC patient (4%). Clinical characteristics of these patients are shown in Table 1. Unifocal flat IND was identified in 17 patients (65%), all located in the left colon. Multifocal IND was found in 7 patients (27%) and was distributed over all segments of the colon. Patients were followed for a median duration of 54 months (range, 0-111). Five UC patients (19%) progressed to advanced neoplasia over a median time of 24 months (range, 2-101). All 5 patients developed HGD. In 1 of these patients concurrent CRC was found whereas in another patient CRC was diagnosed later on. All 5 patients underwent colectomy. In 1 patient HGD was detected in the colectomy specimen, following resection for therapy refractory disease. In both CRC cases, the colectomy was performed because of HGD with CRC identified in the colectomy specimen. The 5-year progression rate to HGD for all 26 patients with flat IND was 21% (95% CI, 0.02-0.40) (Figure 2). Twenty-one patients (81%) showed no progression to advanced neoplasia during a median follow-up of 62 months (range, 0-111) and a median of 2 colonoscopies (range, 0-6) per patient. In 8 patients (38%) 1 or more foci of LGD were found during follow-up, but none progressed to HGD or CRC.

Pathology review

A total of 1547 histology slides from 87 patients were reviewed. The original diagnoses were flat LGD in 70 patients (80%) and IND in 17 patients (20%). Percentages of progression to advanced neoplasia in these 87 patients before histopathological review are shown in Table 2A. In 56 patients (64%), slides were discussed and reassessed by all three pathologists.

Table 2. Development of advanced neoplasia in patients with flat LGD and IND before (A) and after (B) histopathological review of the diagnosis

Variable	A. BEFORE REVIEW		B. AFTER REVIEW	
	FLAT LGD N = 70	IND N = 17	FLAT LGD N = 25	IND N = 39
Advanced neoplasia during follow-up (%)	13 (19)	4 (24)	11 (44)	3 (8)
HGD	3 (23)	2 (50)	4 (36)	0 (0)
HGD with concurrent CRC	2 (15)	1 (25)	3 (27)	1 (33)
HGD with subsequent CRC	4 (31)	1 (25)	3 (27)	1 (33)
CRC	4 (31)	0 (0)	1 (9)	1 (33)
Median time to progression (range)	27 [1-169]	21 [2-101]	24 [1-102]	133 [37-169]

LGD, low-grade dysplasia; IND, indefinite dysplasia; HGD, high-grade dysplasia; CRC, colorectal cancer

Figure 3 shows the differences between the diagnoses after the first review by 1 expert pathologist and the diagnoses after assessment by the panel of pathologists. After review of the flat LGD cases the number of confirmed flat LGD patients fell from 70 to 21. In 29 of these patients (41%), the diagnosis was downgraded to IND, in 17 (24%) no dysplasia could be detected at all and in 3 flat LGD was found to be not related to the underlying IBD. Review of the flat IND cases resulted in a decrease of confirmed flat IND to 10 (59%). In 3 flat IND patients (18%) no dysplasia could be detected after review. However, 4 patients were found to have flat LGD instead of flat IND. Overall, histopathological review reduced the number of flat LGD patients from 70 to 25, but increased the number of flat IND from 17 to 39. (Table 3) Characteristics of colitis patients in whom the diagnosis was changed were not found to differ from patients in whom the diagnosis was confirmed. However, in the biopsies from LGD patients in whom the diagnosis was downgraded to 'no dysplasia' or 'IND', severe inflammation was more frequently encountered than in patients in whom the LGD diagnosis remained the same after review by the expert panel (50% versus 10%, $p<0.01$).

Figure 3. Flowchart of diagnoses of 44 patients with flat low-grade dysplasia (LGD) and 12 patients with indefinite dysplasia (IND) after review by one expert pathologist and after assessment by the panel of pathologists.

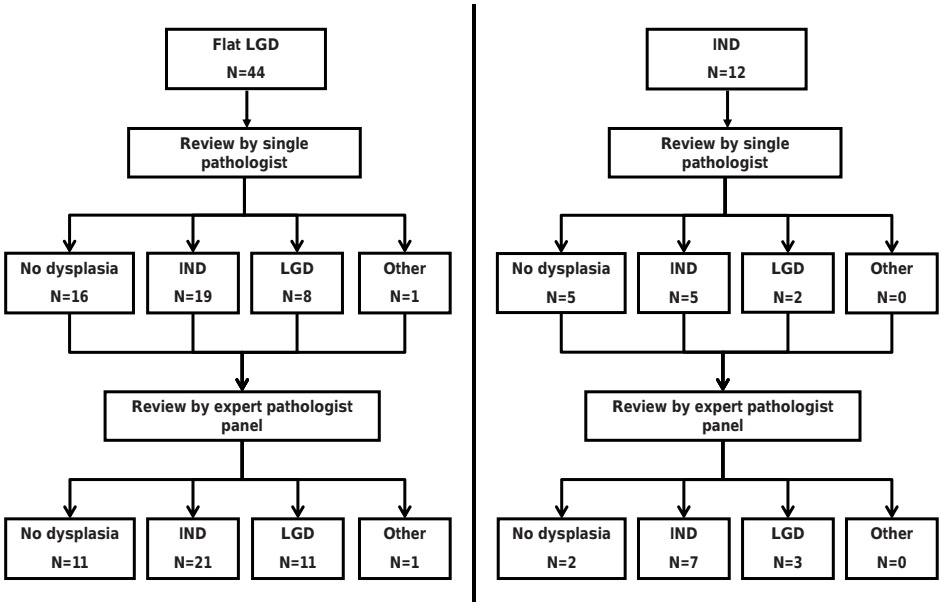


Table 3. Results of histopathological review of histological slides from 70 flat LGD patients and 17 flat IND patients

Original diagnosis	Diagnosis after histopathological review				
	No dysplasia	IND-PN	IND-PP	LGD	Other
70 flat LGD (%)	17 (24)	19 (27)	10 (14)	21 (30)	3 (4)
17 flat IND (%)	3 (18)	5 (29)	5 (29)	4 (24)	0 (0)

IND-PN, indefinite dysplasia, probably negative; IND-PP, indefinite dysplasia, probably positive; LGD, low-grade dysplasia

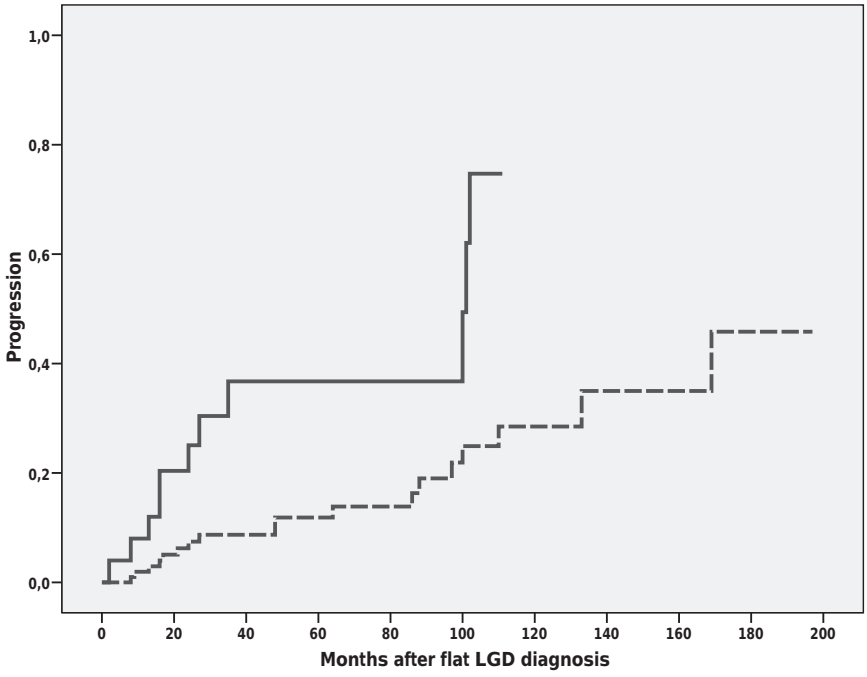
Progression of flat LGD to advanced neoplasia after review

After reviewing all histological slides showing dysplasia or CRC, 11 of 25 flat LGD patients developed advanced neoplasia (44%) during a median follow-up time of 24 months (range, 2-102) (Table 2B). Eight patients were diagnosed with UC (73%), 1 with CD (9%) and 2 with IC (18%). The advanced neoplasia was classified as HGD in 4 patients (36%), as HGD with concurrent CRC in 3 patients (27%), as HGD with subsequent CRC in 3 patients (27%) and as a single CRC in 1 patient (9%). From the 11 flat LGD cases that developed advanced neoplasia, 7 had advanced neoplasia detected during colonoscopy and 4 had advanced neoplasia detected during colectomy. In 1 of the latter, HGD was found in the specimen of

the colectomy that was performed 1 month after the detection of multifocal flat LGD. In the other 3 patients the indications for colectomy (performed 100, 101 and 35 months after flat LGD detection respectively) were a villous lesion containing LGD, repeated multifocal flat LGD during colonoscopy and a stenosis in the sigmoid.

Overall, the rate of progression to advanced neoplasia in patients with a confirmed flat LGD diagnosis was found to be 37% at 5 years (95% CI, 0.16-0.58), compared to 16% at 5 years (95% CI, 0.05-0.26) in these patients before histopathological review (Figure 4). This rate was not statistically significantly different between UC and CD patients (p=0.11). The incidence rate for advanced neoplasia for all patients with a confirmed flat LGD diagnosis was 13.1 per 100 person years at risk.

Figure 4. Kaplan-Meier curve comparing the progression rate to advanced neoplasia in patients with flat LGD before (---) and after (—) histopathological review (log-rank test, p<0.05). Patients with no progression are censored at the moment of their last colonoscopy or colectomy. Vertical lines represent events of advanced neoplasia. LGD, low-grade dysplasia

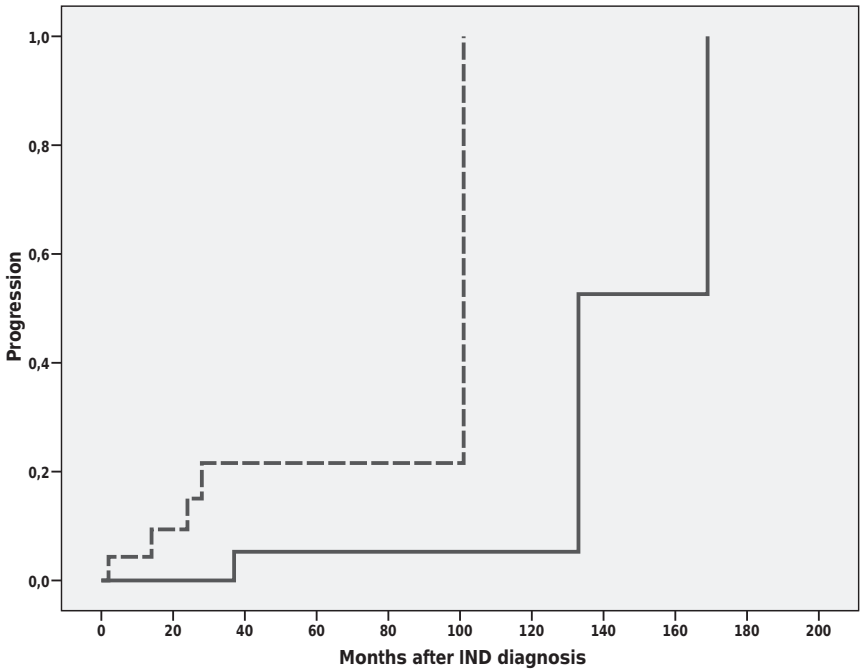


Progression of flat IND to advanced neoplasia after review

After review of the histological slides, 4 patients with a confirmed diagnosis of flat IND developed LGD during follow-up. Of these, 2 progressed to HGD and CRC. In the whole group of 39 patients with a confirmed diagnosis of flat IND, 3 UC patients (8%) developed advanced neoplasia during a median follow-up time of 133 months (range, 37-169) (Table

2B). IND was classified as 'probably positive' in 2 of these patients and as 'probably negative' in the other. All 3 patients progressed to CRC. From the 3 IND patients that developed advanced neoplasia, 2 had advanced neoplasia detected during colonoscopy. In 1 patient, CRC was detected in the specimen of a colectomy that was performed 133 months after the flat LGD diagnosis, because of an LGD-bearing adenoma. In the group of 36 IND patients that showed no progression to advanced neoplasia, IND was classified as 'probably positive' in 15 patients (42%) and as 'probably negative' in 21 patients (58%). Overall, the rate of progression to advanced neoplasia in patients with a confirmed flat IND diagnosis was 5% at 5 years (95% CI, 0 to 0.15), compared to 21% at 5 years (95% CI, 0 to 0.43) in these patients before histopathological review (Figure 5). The incidence rate for advanced neoplasia for patients with a confirmed IND diagnosis was 2.4 per 100 person-years at risk.

Figure 5. Kaplan-Meier curve comparing the progression rate to advanced neoplasia in patients with IND before (---) and after (—) histopathological review (log-rank test, $p<0.05$). Patients with no progression are censored at the moment of their last colonoscopy or colectomy. Vertical lines represent events of advanced neoplasia. IND, indefinite dysplasia.



Patients with no dysplasia after review

Of the 87 patients with flat LGD or flat IND, 20 patients (23%) were downgraded to 'no dysplasia' after review of the histological slides (Table 3). None of these patients developed

LGD during follow-up. One patient (5%) developed an adenomatous lesion with HGD that appeared to harbour CRC in the colectomy specimen. Originally, this patient was reported to have a focus of flat LGD 2 years previously. Histopathological review could not confirm the presence of LGD in this focus. None of the other patients that were downgraded to 'no dysplasia' developed HGD or CRC during follow-up.

Risk factors of neoplastic progression

After univariate and multivariate analysis of potential factors associated with neoplastic progression in patients with flat LGD or IND, no independent predictors for progression to advanced neoplasia were identified. Multifocal dysplasia was found to be a predictor of neoplastic progression in the univariate analysis, but after multivariate testing this was not confirmed (data not shown).

DISCUSSION

This study demonstrates that a diagnosis of LGD in patients with IBD largely depends on the interpretation of the consulting pathologist. Furthermore, the study demonstrates that thorough histological classification of dysplasia can significantly improve the predictive value of LGD with regard to the development of advanced neoplasia. We found remarkable differences in both the numbers of flat LGD and IND patients and the progression rates associated with these diagnoses before and after histopathological review by expert pathologists. Based on the original pathology reports, the 5-year progression rate to advanced neoplasia in 113 patients with flat LGD was found to be 12%. Remarkably, this was lower than the 5-year progression rate in 26 IND patients (21%). After histopathological review we found that the 5-year progression rate to advanced neoplasia in the remaining 25 patients with flat LGD had increased to 37%, whereas the 5-year progression rate in 39 patients with IND had decreased to only 5%. Moreover, 20 patients with an initial diagnosis of flat LGD or IND were downstaged to 'no dysplasia'.

We did not identify clinical or pathological factors that were associated with the risk of progression. Although multifocal dysplasia seemed to be a predictor of progression in the univariate analysis, this did not reach significance in multivariate analysis. This may be due to the relatively low numbers of patients with confirmed LGD available for Cox-analysis after histopathological review. Mesalazine treatment has been suggested to exert chemoprotective effects in longstanding colitis patients, although this is solely based on data from retrospective studies.[14-16] We were, however, not able to assess the contribution of drug-related parameters in the current analysis, as we were confronted with a high percentage of missing data regarding medication use.

In previous studies, conflicting data with respect to the clinical course of flat LGD have been reported ranging from no increased progression to 5-year progression rates of more

than 50%. Compared to prior data reported by Ullman[10], flat LGD patients in this study had more extensive disease, were more frequently suffering from PSC and had a longer duration of follow-up. In spite of an apparently more severe phenotype in our study and the absence of histopathological confirmation by a second expert pathologist in the Ullman study, the 5-year progression rate of flat LGD was lower in our series. Whether differences in mesalazine prescription or other risk factors might have played a role in this respect is a matter of speculation. Another high progression rate was reported by Connell and colleagues.[8] In this study, the histopathological diagnosis was reviewed, but the progression rate was based on only nine patients. Both Connell and Ullman reported progression rates for UC patients only. Our study included both UC and CD patients. As the inflammation-dysplasia-carcinoma sequence is thought to be the pathogenetic denominator of CRC in both subgroups, we feel that inclusion of UC and CD patients is justified and that this does not explain the lower progression rates in our study. In contrast, in 60 patients with flat LGD included in the study of Befrits et al.[7], only two progressed to advanced neoplasia (HGD). In this paper the diagnoses were made by two expert pathologists, but they refrained from use of the IND category which may have led to a higher percentage of patients upgraded to LGD. Pekow et al. included this category and found a higher incidence rate for advanced neoplasia in patients with IND confirmed by two expert pathologists compared to patients with flat LGD.[17] However, this study suffered from low patient numbers in both groups, which may have influenced the results. Histopathological review of the IND diagnoses in our study revealed a 5-year progression rate of only 5% for patients with IND. This low risk of progression is in line with several other, previously published studies.[18,19]

The mean number of biopsies per colonoscopy was rather low in our study (12 and14 for LGD and IND respectively). This was due to the fact that all cases of LGD or IND identified were included in the study, starting from the late eighties until a few years ago. Thus, biopsies from both surveillance endoscopies and colonoscopies for other indications were used, resulting in a lower mean number of biopies than guidelines generally advise.[20,21] This might have resulted in sampling errors and thereby in an underestimation of the presence of multifocal LGD or synchronous flat HGD. However, since most cases of advanced neoplasia can easily be identified endoscopically, the effect of this phenomenon on our results is probably small. All original pathology reports used in this study were produced by pathologists working in one of six university medical centers in the Netherlands. Histological slides from 87 patients in three university medical centers were reviewed for this study. The selection of centers for histopathological review was based on the availability of histological slides from these centers. To our knowledge no differences exist between the patient populations of the six tertiary referral centers included in this study. Baseline progression rates (i.e. before histopathological review) of the 87 patients of whom histological slides were reviewed for this study were not significantly different from the progression rates of the total population

of 139 (data not shown). Our objective was to assess a potential discrepancy in progression rates of flat LGD and IND before and after review by a panel of expert gastrointestinal pathologists. This panel of expert pathologists was able to significantly better distinguish patients at an increased risk and patients at no or low risk for progression to advanced neoplasia. The total number of patients with a diagnosis of flat LGD decreased, while confirmed LGD lesions displayed an increased progression rate to advanced neoplasia. As could be expected, the reverse phenomenon was encountered in patients with confirmed IND, a diagnosis characterized by epithelial changes not significant enough to be classified as LGD. Twenty patients were downgraded to 'no dysplasia', of which all but one showed no development of any neoplasia during follow-up.

The large discrepancy in this study between the total number of patients with LGD before and after histological review reflects the poor inter-observer agreement for dysplasia, which is a well-known phenomenon in literature.[11,12] In a study by Lim et al., LGD slides from 40 patients were reviewed by five specialist gastrointestinal pathologists.[22] A consensus diagnosis, defined as agreement between at least 3 of 5 pathologists, was reached in 15 of 40 cases (38%), which is in line with our results. Although the present study focussed on academic hospitals where slides are often assessed by dedicated GI pathologists, we do not expect other outcomes if the study would have been performed in general hospitals, since agreement has been shown to be not significantly better between expert pathologists.

The management of patients with flat LGD remains a challenge. No consistent policy exists for the treatment of flat LGD; either intensification of the surveillance program or a proctocolectomy is recommended.[6,10,23] The differences in progression rates of flat LGD before and after histopathological review in this study leave room for doubt regarding the progression rates reported in previous studies that did not perform a methodical histopathological review of the histopathological slides. In our opinion, every histological slide with (a suspicion of) dysplasia should be reviewed by a panel of expert pathologists. In clinical practice this may cause a logistic problem, and therefore we would opt for a review by at least one expert GI pathologist who, in case of doubt, forwards the slide to an expert panel. Obviously, one cannot rely on the histopathological diagnosis of dysplasia alone. The large discrepancy between the number of flat LGD patients before and after histopathological review and the fact that not all flat LGD patients develop advanced neoplasia stresses the importance of identifying other characteristics that reliably identify patients at an increased risk of CRC.

In conclusion, this study indicates that patients with a *confirmed* diagnosis of flat LGD are at a significant increased risk of developing HGD or CRC, whereas patients with IND carry a low risk. This has great implications for clinical decision making in colitis patients with LGD. Although histopathological review by a team of dedicated pathologists increases the value of LGD as a predictive parameter as shown in the present study, its use in daily practice

might be hampered by logistics and costs and time constraints. Unfortunately, we were not able to identify other clinical parameters reliably predicting the development of advanced neoplasia in these patients. Evaluation of the use of potentially interesting biomarkers, such as p53, might provide valuable additional tools in this respect.

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CHAPTER 6

Role of immunohistochemical markers in predicting progression of dysplasia to advanced neoplasia in patients with ulcerative colitis

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ABSTRACT

Background

Although dysplasia is thought to be the precursor lesion in the development of colitis-associated colorectal cancer (CRC), a significant proportion of patients with ulcerative colitis (UC) and low-grade (LGD) or indefinite (IND) dysplasia remains cancer-free during endoscopic follow-up. There is a need for biomarkers that predict neoplastic progression. We studied the value of a series of immunohistochemical markers in UC patients with flat LGD or IND with regard to neoplastic progression.

Methods

Tissue samples were collected from 12 UC patients (6 flat LGD, 6 IND) without progression and from 10 UC patients (8 flat LGD, 2 IND) with documented progression to HGD and/or CRC during a median of 25 and 23 months of colonoscopic follow-up, respectively. Immunohistochemistry using monoclonal antibodies was performed for p53, CD44, Ki67, AMACR, β-catenin, Cyclin D1, p21 and ALDH. Positive and negative staining patterns were compared for progression to advanced neoplasia.

Results

When patients showed co-expression of p53 and AMACR, 6/7 patients (86%) developed advanced neoplasia, compared to 4/15 patients (27%) without p53/AMACR co-expression (p=0.02). Patients with p53/AMACR co-expression developed advanced neoplasia in a time period of 19 months (median, range 1-101) compared to 80 months (median, range 8-169) in patients without p53/AMACR co-expression (p=0.14). Interestingly, in 3 patients with progression and previous dysplasia-negative biopsies, 2 out of 3 biopsies were p53 positive a median of 12 months (range 10-14) before the LGD/IND diagnosis.

Conclusion

This study suggests a role for p53/AMACR co-expression as potential marker of neoplastic progression in patients with UC.

INTRODUCTION

Patients with ulcerative colitis (UC) are at an increased risk of developing colorectal cancer (CRC), which is supposed to develop along an inflammation-dysplasia-carcinoma sequence.[1] Although dysplasia is thought to be the precursor lesion in this sequence, a significant proportion of patients with low-grade (LGD) or indefinite (IND) dysplasia remain cancer-free during endoscopic follow-up.[2-8] Based on clinical features, it is still not possible to predict which patients with flat LGD or IND will develop advanced neoplasia (high-grade dysplasia (HGD) or CRC) and therefore should be treated by colectomy or should receive intensified surveillance. Thus, there is a need for parameters that predict neoplastic progression in patients with flat LGD and IND. Many previous studies have focused on the expression of tumor markers in different degrees of dysplasia or CRC. For example, immunohistochemical p53 overexpression has frequently been observed in areas of dysplasia and CRC but is rarely encountered in epithelium without histological evidence of neoplasia.[9-12] Other studies focused on Ki67, p21, Cyclin D1, beta-catenin and Alpha-MethylAcyl-CoenzymeA Racemase (AMACR).[12-24] Although these immunohistochemical markers have been shown to correlate to some degree with dysplasia or CRC at a certain time point, the predictive value of these markers for progression from LGD or IND to advanced neoplasia later on still needs to be evaluated. Recently, an American Gastroenterological Association (AGA) technical review on the diagnosis and management of colorectal neoplasia in IBD underlined the importance of longitudinal studies that investigate the role of biomarkers for the future development of advanced neoplasia.[25] Stimulated by this, we assessed the value of a series of immunohistochemical markers in UC patients with flat LGD or IND with regard to progression to advanced neoplasia during follow-up.

PATIENTS AND METHODS

Patients and tissue samples

Patients with UC and flat LGD or IND with and without progression to advanced neoplasia were recruited from 2 Dutch university medical centers. A biopsy was defined as 'flat' when a histopathological diagnosis of LGD or IND was made in a biopsy that was taken from endoscopically normal mucosa, as documented in the colonoscopy report. Patients were recruited in the study at the moment that flat LGD or IND was detected for the first time. Histological diagnoses were confirmed by a panel of three expert gastrointestinal pathologists as described previously[26], using the histological criteria of Riddell et al.[27] Paraffin embedded tissue specimens from biopsies with LGD or IND were obtained from the Departments of Pathology of the University Medical Center Utrecht and the Academic Medical Center Amsterdam, the Netherlands. Tissue samples were collected from 2 groups of patients: 1. UC patients with flat LGD (n=6) or IND (n=6) and no progression to HGD or

CRC; 2. UC patients with flat LGD (n=8) or IND (n=2) and documented progression to HGD and/or CRC. For the latter group, the tissue samples and surgical resection material with advanced neoplasia were collected as well. Clinical and pathological characteristics of both patient groups are listed in Table 1.

Table 1. Clinical and histopathological characteristics of patients with and without progression

	LGD/IND without progression n=12	LGD/IND with progression n=14
LGD:IND	6:6	8:2
Sex – male (%)	5 (42)	6 (60)
Mean age at IBD diagnosis (years ± SD)	31 (12)	27 (9)
Mean duration IBD (years ± SD)	20 (15)	11 (8)
Mean age at dysplasia diagnosis (years ± SD)	52 (13)	38 (5)*
Dysplasia detected during surveillance colonoscopy	5 (42)	2 (20)
Mean no. of biopsies during surveillance (SD)	27 (9)	38 (1)
Dysplasia detected during colonoscopy for other reasons	7 (58)	8 (80)
Pancolitis (%)	10 (83)	9 (90)
Degree of active inflammation (%)		
None	4 (33)	0 (0)
Mild	7 (58)	7 (70)
Severe	1 (8)	3 (30)
Median duration of colonoscopic follow-up / time to progression (months, range)	25 [1-199]	23 [1-169]
HGD during follow-up (%)	0 (0)	9 (90)
CRC during follow-up (%)	0 (0)	7 (70)
Primary sclerosing cholangitis (%)	1 (8)	1 (10)

*p=0.005 compared to UC patients without progression. UC, ulcerative colitis; LGD, low-grade dysplasia; HGD, high-grade dysplasia; CRC, colorectal cancer; IND, indefinite dysplasia; SD, standard deviation.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using monoclonal antibodies for p53, CD44, Ki67, AMACR, β-catenin, Cyclin D1, p21/waf1 and aldehyde dehydrogenase (ALDH). The antibodies and staining conditions are summarized in Table 2. For the CD44 V6 and ß-catenine staining, antibody binding was visualized using the Powervision + poly-HRP detection system (ImmunoVision Technologies, Co, Daly City, CA, USA) with 3,3-DiAmino Benzidine tetrachloride (DAB; Sigma D5637). For the Cyclin D1 staining we used PowerDAB (Immunologic Cat. No. BS03-25) as chromogen. Sections were counterstained with hematoxylin. Samples of colorectal carcinoma were used as positive controls for p53,

β-catenin and ALDH1 IHC. Samples of mantle cell lymphoma, prostate carcinoma and colon adenoma were used as positive controls for Cyclin D1, AMACR and CD44 IHC, respectively. Normal colorectal mucosa was used as a positive control for Ki67 and p21/waf1 IHC. In the latter samples, Ki67 was located in the basal zone of the crypts whereas p21/waf1 was confined to the upper parts and superficial epithelium of the crypts.

Scoring

All slides were coded and scored by an expert gastrointestinal pathologist (FtK) and PhD student (FvS) separately and, subsequently, jointly. Scoring methods were confirmed by a second expert gastrointestinal pathologist (GO). Inflammation was classified as none, mild to moderate or severe. No inflammation was characterized by the absence of inflammatory cells in the lamina propria and the absence of basal plasmocytosis; mild to moderate inflammation by an increased inflammatory infiltrate with crypt distortion and only occasionally active cryptitis, and severe inflammation by a regularly active cryptitis, crypt abscesses and crypt destruction, often accompanied by erosion or ulceration. Staining patterns were only evaluated in dysplastic areas with well-conserved epithelial morphology. Immunostaining for CD44, Cyclin D1, AMACR and ALDH was assessed according to the intensity of staining and divided into four categories: negative (-), weak (+), moderate (++) or strong (+++), with moderate or strong IHC staining being regarded as positive. Beta-catenin staining was evaluated in the cell membrane and nucleus. A membranous staining pattern was considered to be negative, whereas a reduced membranous expression accompanied by an increased cytoplasmic and nuclear staining was scored as positive. For staining frequency of CD44, Cyclin D1, AMACR, ALDH1 and β-catenin, the number of positive (moderate or strong) cells were expressed as the percentage of the total number of cells per high-power field and categorized as <5%, 5-25%, 25-50%, 50-75% and >75%.[19] In order to avoid a biased assessment of immunostaining intensity and frequency, areas displaying the strongest immunostaining were selected for analysis. Immunostaining for p53 was only scored as positive when the epithelial cells showed an intense brown nuclear staining in at least 5% of the total number of epithelial cells in a high-power field.[9] In all other cases, slides were scored as negative. Ki67 staining was assessed by the presence of nuclear staining in the surface epithelium. A diffuse or uninterrupted nuclear staining in neighboring cells in the surface epithelium was scored positive. If there was only scattered staining in the surface epithelium or if there was no surface epithelial staining, slides were scored negative. P21/waf1 staining was scored as positive when nuclear staining was found in the lower half of the crypt; when staining was confined to the upper part of the crypt, slides were scored negative.

Statistical analysis

Since patient numbers were low, categories of staining frequency were re-categorized into 0%, <25% and >25%. For p53, categories were re-categorized into <5%, 5-25% and >25%. Expression patterns of the individual immunohistochemical markers were compared between patients with progression to advanced neoplasia and those without progression, using Fisher's exact test or Chi Square test, where appropriate. Continuous variables were compared using Student's t-test or Mann-Whitney U test, where appropriate. A p-value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 15.0 for Windows.

Table 2. Antibodies and staining conditions used for immunohistochemistry.

Antigen	Source	Clone	Dilution	Method
p53	Neomarkers / MS-738-P	DO7+BP53-12	1:2000	ARS
Ki67	DAKO / M7240	MIB-1	1:100	ARS
p21/waf1	Zymed / 18-0401	AE10	1:150	ARS
ß-Catenin	BD Biosciences / 610153	14	1:5000	ARS / overnight incubation at 4°C
Cyclin D1	Neomarkers / RM-9104-S	100	1:100	ARS / overnight incubation at 4°C / DAB++
CD44	Mender Med Syst / BMS125	VFF18/V6	1:300	ARS
AMACR	DAKO / M3613	13H4	1:75	ARS
ALDH1	BD Biosciences / 611195	44	1:50	No ARS

ARS, Antigen Retrieval System

Ethical considerations

This study was carried out in accordance with the ethical guidelines of our institution.[28]

RESULTS

Expression of individual markers

Staining patterns of the individual immunohistochemical markers in each patient group are shown in Table 3.

Expression of p53

Positive p53 staining was found in 6 patients (60%) with progression and in 2 patients (17%) without neoplastic progression. In patients with progression, p53 overexpression was exclusively found in patients with LGD, whereas in patients without progression p53 overexpression was detected in 1 LGD patient and in 1 IND patient. The p53 staining pattern was not related to the presence of active inflammation (p=1.00). Positive and negative staining patterns for p53 are shown in Figure 1A and 1B.

From 4 patients with LGD (3 with progression, 1 without progression) and a positive p53 staining non-dysplastic tissue samples obtained before the development of LGD were available and stained for p53. Samples were obtained a median of 17 months (range 10-28) before the LGD diagnosis. In 3 patients a positive p53 staining pattern was found. Two of these patients developed advanced neoplasia during follow-up. To compare these results with p53 staining patterns in patients without any neoplasia during follow-up, tissue samples of 6 UC patients who did not develop neoplasia during a mean of 22 years (SD 8) were collected and stained for p53. In these 6 patients no p53 overexpression was found.

Expression of CD44

A moderate to strong CD44 staining pattern was detected in all patients (100%) with progression and in 10 patients (83%) without progression. In the group of patients without progression, a positive CD44 staining was found in 6 LGD patients and 4 IND patients. The CD44 staining pattern was not related to the presence of active inflammation (p= 0.34). A positive staining pattern for CD44 is shown in Figure 1C.

Expression of β-catenin

In the group of patients without progression to advanced neoplasia, nuclear β-catenin expression was detected in 7 patients (58%), of which 4 were diagnosed with LGD and 3 with IND. Six LGD patients and 1 IND patient with progression to advanced neoplasia (70 %) were β-catenin positive. Beta-catenin staining was found in a higher percentage of cells in patients with progression to advanced neoplasia. No significant relationship was found between β-catenin staining and the presence of active inflammation (p= 0.12). A positive staining pattern for β-catenin is shown in Figure 1D.

Expression of AMACR

In the group of patients without progression to advanced neoplasia, a moderate to strong AMACR staining intensity was found in 5 patients (42%; 2 LGD, 3 IND patients). Six UC patients with LGD and progression to advanced neoplasia (60%) were AMACR positive. In patients without progression AMACR staining was often focally found in less than 25% of epithelial cells (Table 3). No significant relation was found between AMACR staining and the presence of active inflammation (p=0.59). A positive staining pattern for AMACR is shown in Figure 1F.

Expression of Cyclin D1

A moderate or strong staining intensity for Cyclin D1 was detected in 9 patients (90%) with and in all patients without progression to advanced neoplasia. One patient with LGD and progression showed a completely negative Cyclin D1 staining pattern. This patient had a

negative staining pattern for all the other markers as well. Cyclin D1 staining was not related to the presence of active inflammation (p=1.00). A positive staining pattern for Cyclin D1 is shown in Figure 1E.

Table 3. Staining patterns of each immunohistochemical marker in patients with low-grade dysplasia (LGD) or indefinite dysplasia (IND) with and without progression to advanced neoplasia, represented as the number and percentage of patients with a positive immunostaining according to staining intensity and the percentage of stained cells in these patients.

Marker	LGD/IND no progression n=12 (%)	LGD/IND progression n=10 (%)	p-value
p53 +	2 (17)	6 (60)	0.07
5-25%	1 (50)	1 (17)	
>25%	1 (50)	5 (83)	
CD44 +	10 (83)	10 (100)	0.48
<25%	1 (10)	1 (10)	
>25%	9 (90)	9 (90)	
β-catenin nucleus +	7 (58)	7 (70)	0.68
<25%	7 (100)	3 (43)	
>25%	0 (0)	4 (57)	
Cyclin D1 +	12 (100)	9 (90)	0.46
<25%	7 (58)	4 (44)	
>25%	5 (42)	5 (56)	
AMACR +	5 (42)	6 (60)	0.39
<25%	5 (100)	3 (50)	
>25%	0 (0)	3 (50)	
ALDH1 +	3 (25)	2 (20)	1.00
<25%	2 (67)	1 (50)	
>25%	1 (33)	1 (50)	
p21/waf1 +	6 (50)	5 (50)	1.00
Ki67 +	2 (17)	2 (20)	1.00

Expression of AMACR

In the group of patients without progression to advanced neoplasia, a moderate to strong AMACR staining intensity was found in 5 patients (42%; 2 LGD, 3 IND patients). Six UC patients with LGD and progression to advanced neoplasia (60%) were AMACR positive. In patients without progression AMACR staining was often focally found in less than 25% of epithelial cells (Table 3). No significant relation was found between AMACR staining and the presence of active inflammation (p=0.59). A positive staining pattern for AMACR is shown in Figure 1F.

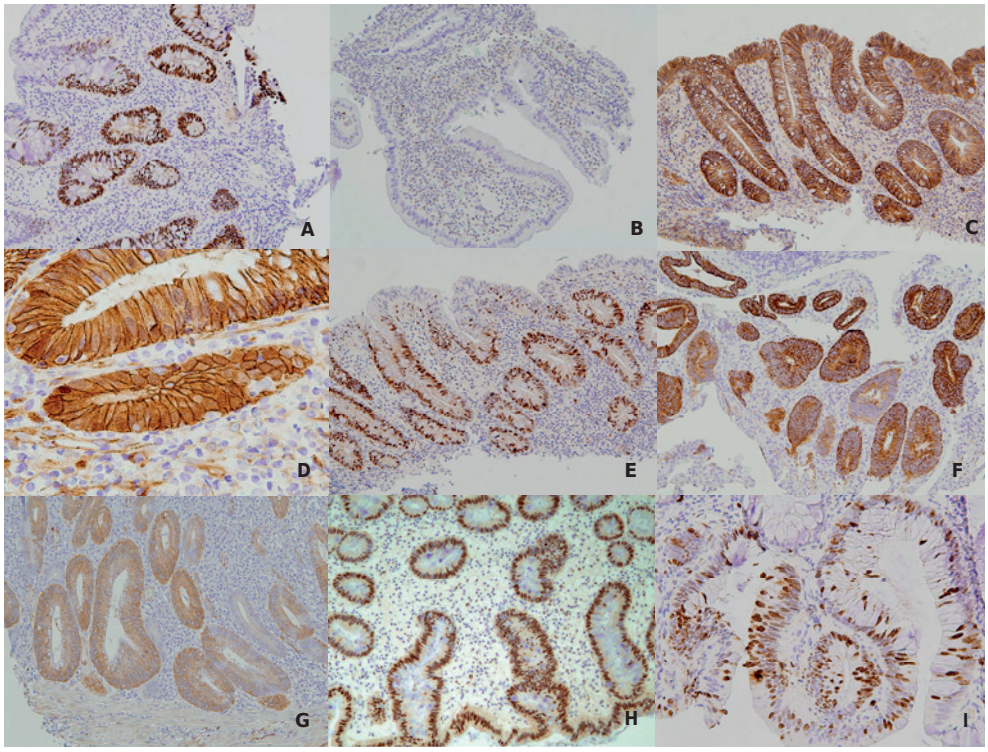

Figure 1. Images illustrating immunohistochemical expression patterns of p53 (A and B), CD44 (C), β-catenin (D), Cyclin D1 (E), AMACR (F), ALDH1 (G), p21/waf1 (H) and Ki67 (I). Sample (A) shows p53 overexpression in a patient with low-grade dysplasia (LGD). Sample B shows a completely negative nuclear p53 staining. This biopsy is suspect of harboring a stop codon mutation. Sample C shows a positive CD44 staining in a patient with LGD. Sample D shows nuclear β-catenin expression in a patient with indefinite dysplasia (IND). Sample E shows strong Cyclin D1 expression in a patient with LGD. Sample F shows strong AMACR expression in a patient with high-grade dysplasia (HGD). Sample G shows moderate ALDH expression in a colectomy specimen of a patient with HGD. Sample H shows p21/waf1 expression in the lower half of the crypt in a patient with LGD. Sample I shows nuclear Ki67 expression extending into the surface epithelium in a patient with LGD.

Expression of ALDH

ALDH expression was characterized by a clear brown color of the nucleus and cytoplasm and was localized near the base of the crypt or extended to the superficial parts of the crypt. In 3 patients without neoplastic progression (25%; 2 LGD and 1 IND), a positive ALDH staining pattern was detected. Two LGD patients with neoplastic progression (20%) were ALDH positive. Immunostaining for ALDH was not related to active inflammation (p=0.21). A positive staining pattern for ALDH is shown in Figure 1G.

Expression of p21/waf1

In 6 patients without neoplastic progression (50%, 3 LGD, 3 IND) p21/waf1 staining was detected in the lower part of the crypt. In the group of patients with progression to advanced neoplasia, staining in the lower part of the crypt was found in 5 patients (50%; 3 LGD, 2 IND). Positive p21/waf1 immunostaining was not related to the presence of active inflammation (p=0.59). A positive staining pattern for p21/waf1 is shown in Figure 1H.

Expression of Ki67

A diffuse or uninterrupted nuclear staining of Ki67 in neighboring cells in the surface epithelium was found in 2 patients (17%) without progression; both were diagnosed with LGD. In the group of patients with progression, 2 patients (20%) with LGD showed a positive Ki67 staining pattern. Overall, in patients with a positive Ki67 staining, the presence of surface epithelial Ki67 staining was accompanied by a decrease of staining in the germinative zone of the crypt. A positive Ki67 staining pattern was not related to the presence of active inflammation (p=0.55). A positive staining pattern for Ki67 is shown in Figure 1I.

Predictive value for progression to advanced neoplasia

Of all 8 patients with a positive p53 staining pattern, 6 (75%) showed progression to advanced neoplasia compared to 4/14 patients (29%) with a negative p53 staining (p=0.07). When patients showed co-expression of p53 and AMACR, 6/7 patients (86%) developed advanced neoplasia, compared to 4/15 patients (27%) without p53/AMACR co-expression (p=0.02). Patients with p53/AMACR co-expression at the moment of LGD/IND diagnosis developed advanced neoplasia in a time period of 19 months (median, range 1-101) compared to 80 months (median, range 8-169) in patients without p53/AMACR co-expression (p=0.14). Other combinations of markers had no enhancing effect on the predictive value of these markers for progression to advanced neoplasia in LGD/IND patients.

Expression of individual markers per degree of neoplasia

Of the 10 patients with progression to advanced neoplasia, 3 developed HGD, 1 developed CRC and 6 developed both HGD and CRC. A completely negative p53 immunostaining was found in 1 sample with LGD, 3 with HGD and in 2 with CRC. Sequence analysis of the p53 gene was started to search for a stop codon (null mutation) in these samples. However, due to a lack of sufficient tissue material the sequence analysis could not be performed. Expression of CD44 was increased in LGD compared to CRC (p=0.03). ALDH1 expression was increased in HGD compared to IND (p=0.04). The expression pattern of Ki67 was significantly increased in both HGD and CRC (p=0.03 and 0.003, respectively). Table 4 shows the individual immunohistochemical marker frequencies in subsequent degrees of neoplasia from IND to CRC.

Table 4. Expression of immunohistochemical markers in indefinite dysplasia (IND), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and colorectal cancer (CRC), represented as the number and percentage of patients with a positive immunostaining according to staining intensity and the percentage of stained cells in these patients

Immunohistochemical marker	IND n=8 (%)	LGD n=14 (%)	HGD n=8 (%)	CRC n=7 (%)
p53 +	1 (13)	7 (50)	3 (38)	4 (57)
5-25%	1 (100)	1 (14)	0 (0)	0 (0)
>25 %	0 (0)	6 (86)	3 (100)	4 (100)
CD44 +	6 (75)	14 (100)	7 (88)	4 (57)*
<25%	0 (0)	2 (14)	1 (14)	1 (25)
>25%	6 (100)	12 (86)	6 (86)	3 (75)
β-catenin nucleus +	4 (50)	10 (71)	7 (88)	6 (86)
<25%	4 (100)	6 (60)	2 (29)	3 (50)
>25%	0 (0)	4 (40)	5 (71)	3 (50)
AMACR +	3 (38)	8 (57)	5 (63)	6 (86)
<25%	3 (100)	5 (63)	1 (20)	2 (33)
>25%	0 (0)	3 (37)	4 (80)	4 (67)
Cyclin D1 +	8 (100)	13 (93)	6 (75)	5 (71)
<25%	6 (80)	4 (31)	3 (50)	2 (40)
>25%	2 (20)	9 (69)	3 (50)	3 (60)
ALDH1 +	1 (13)	4 (29)	6 (75)**	3 (43)
<25%	1 (100)	2 (50)	4 (67)	2 (67)
>25%	0 (0)	2 (50)	2 (33)	1 (33)
p21/waf1 +	5 (63)	5 (36)	3 (38)	5 (71)
Ki67 +	0 (0)	4 (29)	5 (63)***	5 (71)****

*p=0.03 compared with patients with LGD; **p=0.04 compared with patients with IND; ***p=0.03 compared with patients with IND; ****p=0.003 compared with patients with IND

DISCUSSION

This study investigated the role of immunohistochemistry for predicting risk of progression to advanced neoplasia in UC patients with flat LGD or IND. We found that LGD/IND patients with p53/AMACR co-expression at the moment of LGD/IND diagnosis developed significantly more often advanced neoplasia during follow-up compared with patients without this co-expression. Moreover, in 3 out of 4 patients with previous dysplasia-negative tissue samples available we detected immunohistochemical p53 expression in non-dysplastic mucosa already 2 years before the LGD/IND diagnosis and found that 2 of these patients developed advanced neoplasia during follow-up. This study is the first to show that a combination of

immunohistochemical markers is of potential value for predicting progression of flat LGD or IND to advanced neoplasia in patients with UC.

The selection of immunohistochemical markers in this study was based on the specific characteristics of these markers in neoplastic tissues, as reported in previous studies. These studies focussed on the expression of markers in normal and neoplastic tissue at the same time point. The present longitudinal study investigated both the predictive value of different markers for progression to advanced neoplasia and the frequency of expression in different neoplastic stages at different time points.

AMACR is an enzyme located in mitochondria and peroxisomes and involved in the β-oxidation of branched chain fatty acids. Expression of AMACR has been reported in prostate carcinoma[29,30] and was recently reported in patients with Barret's esophagus and patients with IBD-associated neoplasia.[31,32] In both studies the reported sensitivity of AMACR for the detection of dysplasia and cancer in IBD was high and therefore AMACR was suggested to be a reliable diagnostic tool to detect or confirm IBD-associated neoplasia. In the present study a positive AMACR immunostaining was detected in 57% of patients with LGD, 63% of patients with HGD and 86% of patients with CRC. Although the percentage of AMACR expression in CRC is comparable to both previous studies, the low frequency of AMACR expression in both LGD and HGD does not support the value of this marker for detecting or confirming dysplasia in clinical practice. In the study by Marx et al.[32], clinical follow-up data were obtained from 10 IND patients with p53/AMACR co-expression. After a follow-up of less then 4 months, 2 patients (20%) were found to have developed HGD or CRC. The early progression of these AMACR/p53-positive patients might indicate the predictive potential of these markers in patients with IBD-associated neoplasia. We indeed found co-expression of AMACR and p53 to be of potential value for predicting neoplastic progression in UC patients with flat LGD or IND.

Of all p53 mutations in human cancer, eighty percent are missense mutations that result in a prolonged half-life of the mutant p53 protein, allowing their detection by immunohistochemistry. Stop codon or nonsense mutations constitute less than 20% of p53 mutations in human tumours. These mutations cause a shortened, unstable or absent p53 protein product, resulting in a completely negative p53 expression.[33,34] In the present study, one patient with LGD and progression to advanced neoplasia showed a completely negative immunohistochemical staining for p53. Unfortunately, sequence analysis could not be performed in this patient due to lack of tissue. However, since sequence analysis in our previous study showed a p53 mutation in a significant proportion of tissue samples with a completely negative p53 expression[24], this patient may well have had a p53 mutation. None of the other studied immunohistochemical markers predicted progression to advanced neoplasia in our LGD/IND patients better than co-expression of p53/AMACR. P21/waf1 was studied as a downstream effector of p53; it regulates cell cycle control by inhibition of cyclin-

dependent kinases, thereby preventing progression of the cell through the G1/S phase.[13] However, p21/waf1 was not able to accurately predict neoplastic progression in patients with already existing IND or flat LGD.

To determine whether Wnt-signaling proteins can predict neoplastic progression at the moment of LGD or IND, Cyclin D1 and β-catenin were investigated. In the Wnt-pathway, loss of APC function results in the intracellular accumulation of β-catenin, which leads to translocation of β-catenin to the nucleus, where it activates the transcription of several target genes, including Cyclin D1.[17] In a previous study, our group found nuclear β-catenin and Cyclin D1 expression in non-dysplastic mucosa in a significant proportion of IBD patients with synchronous neoplasia elsewhere in the colon. The present study, however, found no important role for these proteins for predicting future progression to advanced neoplasia in UC patients with flat LGD or IND.

Recently, ALDH, a detoxifying metabolic enzyme, was identified as a marker for tumor-initiating cells in colitis patients. Immunohistochemically, an increased ALDH expression at the base of the crypt was found in colitis and widespread expression was found in colitis-associated CRC tissue.[35] For predicting neoplastic progression in flat LGD or IND, ALDH was not found to be useful, however.

In the present study we focussed on the predictive value of immunohistochemistry, since this technique is a straightforward and practical tool in clinical practice. Only a few studies have previously investigated the role of immunohistochemistry for the detection of progression of colitis-associated dysplasia. One study suggested a role for p53 as a marker of neoplasia in 14 patients with Crohn's disease.[36] However, in that study development of HGD or CRC could be investigated in only 8 patients, since 6 patients were lost to follow-up. Progression to HGD was found in 2 of 4 p53 positive patients that were found to have LGD at the moment of positive p53 immunostaining; none of the p53 negative patients developed advanced neoplasia during follow-up. In a cohort study of 95 patients with longstanding UC, Lashner et al. found that patients with a positive p53 immunostaining had a 4.5 times higher risk of developing dysplasia or cancer as compared to p53 negative patients.[9] In this study both patients with and without neoplasia, including LGD, HGD and CRC, were enrolled. The predictive value of p53 immunohistochemistry for the development of any dysplasia or cancer was 73%, while for the development of HGD or CRC this value was found to be 41%. These authors also showed that p53 overexpression might be present before the appearance of LGD at histopathological evaluation, which is in line with our results.

Our study has limitations that need to be addressed. First, the number of patients evaluated was low. Moreover, the duration of follow-up was limited, respectively 23 and 25 months in patients with and without progression. Results should therefore be interpreted with caution and a larger study including more patients with a longer follow-up is needed to confirm our results. Second, the degree of immunoreactivity was frequently heterogeneously distributed

throughout the tissue sample. To avoid selection bias, we therefore selected in each tissue sample the area with the strongest immunoreactivity. Since one tissue sample may contain multiple foci of dysplasia, a less intense immunoreactivity can thus be found in other dysplastic foci. Third, in this study the initial LGD or IND was more often detected during colonoscopies performed for other reasons than surveillance. This might be explained by the fact that we included tissue samples obtained in the time period between the eighties and a few years ago. In the eighties and nineties surveillance was less often performed, and therefore dysplasia might have been detected more often during colonoscopies performed for other reasons. This might have resulted in sampling errors and thereby in an underestimation of the presence of advanced neoplasia. However, since most cases of advanced neoplasia can be identified endoscopically, the effect of this phenomenon on our results is probably small.

Furthermore, we were unable to assess whether the advanced lesion originated from the incident flat LGD or IND, since this study investigated progression of LGD or IND in flat, endoscopically normal mucosa. However, since IBD-associated neoplasia is characterized by the development of metachronous neoplastic lesions, detection of LGD or IND at a single location probably reflects the malignant potential of the whole diseased colon. Assessment of the predictive value of tumour markers in patients with LGD or IND for progression to advanced neoplasia anywhere in the colon is therefore of even more importance.

In conclusion, based on a spectrum of immunohistochemical markers, this study suggests a role for a combination of p53 and AMACR as a potential marker of neoplastic progression in patients with UC and flat LGD or IND. In clinical practice, assessment of p53 and AMACR expression might help the physician to identify patients with dysplasia at an increased risk of HGD or CRC, enabling a more personalized approach with regard to surveillance strategies and therapy. However, larger studies are needed to confirm our observations and to identify other markers of potential value in predicting progression risk in patients with UC.

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CHAPTER 7

Adenomas in patients with inflammatory bowel disease are associated with an increased risk of advanced neoplasia

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ABSTRACT

Background

It is still unclear whether IBD patients with adenomas have a higher risk of developing high-grade dysplasia (HGD) or colorectal cancer (CRC) than non-IBD patients with sporadic adenomas. We compared the risk of advanced neoplasia (AN, defined as HGD or CRC) in IBD patients with adenomas to IBD patients without adenomas and patients without IBD with adenomas.

Methods

IBD patients with a histological adenoma diagnosis (IBD + adenoma), age-matched IBD patients without adenoma (IBD-nonadenoma) and adenoma patients without IBD (nonIBD + adenoma) were enrolled in this study. Medical charts were reviewed for adenoma characteristics and development of AN. The endoscopic appearance of the adenomas was characterized as *typical* (solitary sessile or pedunculated) or *atypical* (all other descriptions).

Results

A total of 110 IBD + adenoma patients, 123 IBD-nonadenoma patients and 179 nonIBD + adenoma patients were included. Mean duration of follow-up was 88 months (SD ±41). The 5-year cumulative risks of AN were 11%, 3% and 5% in IBD + adenoma, IBD-nonadenoma and nonIBD + adenoma patients, respectively ($p<0.01$). In IBD patients atypical adenomas were associated with a higher 5-year cumulative risk of AN compared to IBD patients with typical adenomas (18% versus 7%, $p=0.03$).

Conclusions

IBD patients with a histological diagnosis of adenoma have a higher risk of developing AN than adenoma patients without IBD and IBD patients without adenomas. The presence of atypical adenomas in particular was associated with this increased risk, although patients with typical adenomas were found to carry an additional risk as well.

INTRODUCTION

It is commonly accepted that both Crohn's colitis and ulcerative colitis (UC) are associated with an increased risk of colorectal cancer (CRC).[1] The cumulative risk was found to be 18% after 30 years of disease duration in patients with UC and similar risks have been reported in patients with Crohn's colitis.[2,3] Colitis-associated CRC is thought to be preceded by dysplasia, which can be found in flat mucosa or in endoscopically visible lesions. For the latter the term dysplasia-associated lesion or mass (DALM) was coined in 1981.[4] DALMs are heterogenous in their endoscopic appearance and are therefore subdivided in adenoma-like and non-adenoma-like lesions. The non-adenoma-like subtype refers to all irregular, diffuse masses or plaque lesions that generally cannot be removed by endoscopic resection. These lesions were reported to be frequently accompanied by synchronous malignancy and guidelines therefore generally recommend colectomy, although data supporting these guidelines are limited.[5,6] The adenoma-like DALM refers to all discrete, either pedunculated or sessile polyps which resemble sporadic adenomas in patients without IBD. Previous studies reported that CRC risk is low in these patients and that polypectomy with regular surveillance is an adequate treatment strategy.[7,8] Although these initial studies were based on small patient numbers with limited follow-up, two larger studies published more recently seemed to confirm these data.[9,10] However, little is known about the additional risk of adenomas in IBD patients as compared to IBD patients without an adenoma and patients with an adenoma but without IBD.

The aim of this study was therefore to describe the risk of subsequent colorectal neoplasia in a large cohort of IBD patients with an adenoma, and to compare this risk to that in IBD subjects without adenomas as well as non-IBD patients with adenomas.

METHODS

Patients

The nationwide pathology archive (PALGA) that contains all pathology reports from the Netherlands dating back to 1971, was used to identify three cohorts of patients: 1. IBD patients with an adenoma (IBD + adenoma); 2. IBD patients without an adenoma (IBD-nonadenoma) and 3. subjects with an adenoma but without IBD (nonIBD + adenoma). A PALGA search was performed in seven university medical centres for the period between 1995 and 2005.

IBD-nonadenoma patients were matched to IBD + adenoma patients for age at inclusion and centre. Year of inclusion was matched to the year of adenoma diagnosis in the IBD + adenoma patients. The latter was performed in order to obtain an equal follow-up time between these groups. Exclusion criteria were a history of any dysplasia (low-grade dysplasia (LGD) or high-grade dysplasia (HGD)) or CRC, a diagnosis of HGD or CRC at the moment

of adenoma diagnosis, a history of (sub)total colectomy, no endoscopic or surgical follow-up and incomplete or missing data. Furthermore, patients with Crohn's disease without involvement of the colon were excluded.

Data collection

Demographic and clinical data were collected from medical charts for all three cohorts. For the IBD + adenoma and IBD-nonadenoma cohorts, clinical data included date of IBD diagnosis, type of IBD, extent of IBD, medication use and the presence of primary sclerosing cholangitis. Disease extent was defined as the maximum extent according to either histology or endoscopy reports. In UC and indeterminate colitis (IC) or IBD-unclassified (IBD-U) patients disease extent was defined as either left-sided or extensive (inflammation distal or proximal to the splenic flexure, respectively). In patients with Crohn's colitis, involvement of 3 or more anatomical parts of the colon was considered extensive disease, whereas involvement of 1 or 2 sections was considered limited disease. In all three cohorts a family history of CRC was documented. For the IBD + adenoma and nonIBD + adenoma cohorts, histopathology and endoscopy reports were reviewed to identify the date of first adenoma diagnosis and to collect information about size, location within or outside an area of previous inflammation and endoscopic appearance. We selected patients based on their histological adenoma diagnosis. However, in the endoscopy reports we encountered a wide variety of endoscopic descriptions of adenomas. Therefore, for practical purposes, adenomas were classified as either 'typical' or 'atypical'. Typical adenomas included all lesions described as discrete solitary, sessile or pedunculated polyps resembling sporadic adenomas. All other endoscopic lesions, including adenomatous fields (i.e. areas of multiple, clustered polyps), lesions with an irregular surface and lesions endoscopically described as post-inflammatory polyps (but histologically classified as adenoma) were characterized as atypical adenomas. Dysplasia was classified as either low-grade or high-grade, according to the criteria and definitions articulated by Riddell et al.[11]

Follow-up

Histopathology and endoscopy reports were reviewed to detect whether patients developed LGD, HGD or CRC during follow-up. Advanced neoplasia (AN) was defined as a finding of HGD or CRC. Duration of follow-up was measured in months and defined as time from the first adenoma diagnosis (IBD + adenoma and nonIBD + adenoma cohorts) or from the moment of inclusion in the study (IBD-nonadenoma cohort) to one of the following endpoints: 1. end of follow-up 2. end of study period (1st of December, 2010), 3. death, or 4. subtotal or total colectomy.

Statistical analysis

All analyses were restricted to the period beyond the first 6 months of follow-up to exclude patients with prevalent AN. Baseline characteristics were analyzed with standard descriptive statistics and compared between the three groups. Continuous variables were analyzed using ANOVA or Kruskall-Wallis analysis, where appropriate. Categorical variables were analyzed using Pearson's chi-squared or Fisher's exact test, where appropriate. Five-year cumulative incidences of AN were calculated using Kaplan-Meier survival analysis and comparisons between groups were made using log-rank testing. Patients who did not develop AN during follow-up were censored at the moment of last colonoscopy or colectomy. Factors associated with the development of AN during follow-up in IBD patients (IBD + adenoma and IBD-nonadenoma patients) were assessed in a Cox proportional hazard model. A p-value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 15.0 for Windows.

Ethical considerations

This study was carried out with the approval of and in accordance with the ethical guidelines of the research review committee of our institution.[12]

RESULTS

Patients

Our search yielded 617 IBD + adenoma patients, 472 IBD-nonadenoma patients and 902 nonIBD + adenoma patients. Review of medical charts, endoscopy, pathology and surgery reports yielded 110 IBD + adenoma, 179 nonIBD + adenoma and 123 IBD-nonadenoma patients eligible for enrolment. The reasons for exclusion are shown in the flowchart (Figure 1). Clinical characteristics of the three patient groups are given in Table 1.

Adenoma characteristics

A total of 216 adenomas were identified in 179 nonIBD + adenoma patients and 133 adenomas in 110 IBD + adenoma patients. Adenoma characteristics are given in Table 2. Adenomas were characterized as typical in 82 (75%) and atypical in 28 IBD + adenoma patients (25%). The latter consisted of 15 lesions endoscopically classified as post-inflammatory polyps (54%) and 13 non-adenoma-like lesions (46%). In nonIBD + adenoma patients 8 adenomas (4%) were characterized as atypical and 171 (96%) as typical. Polypectomy was performed in 146 nonIBD + adenoma patients (82%) and in 68 IBD + adenoma patients (62%) (p<0.01). Median adenoma size was 5 mm (range 2 – 20) in the IBD + adenoma cohort and 6 mm (range 1 – 65) in the nonIBD + adenoma cohort (p=0.11).

Table 1. Baseline characteristics

	IBD Adenoma N = 110	IBD No adenoma N = 123	No IBD Adenoma N = 179
Diagnosis (%)			
Ulcerative colitis	73 (66)	53 (43)	NA
Proximal to splenic flexure	41 (56)	31 (58)	
Distal to splenic flexure	31 (43)	22 (42)	
Unknown	1 (1)	0 (0)	
Crohn's disease	32 (29)	52 (42)	NA
Extensive disease	14 (44)	32 (62)	
Limited disease	17 (53)	20 (38)	
Unknown	1 (3)	0 (0)	
Indeterminate colitis / IBDU	5 (5)	18 (15)	NA
Proximal to splenic flexure	3 (60)	8 (44)	
Distal to splenic flexure	1 (20)	10 (56)	
Unknown	1 (20)	0 (0)	
Age at IBD diagnosis, yr (mean ± SD)	42 (17)	39 (15)	NA
Age at initial adenoma diagnosis / start follow-up, yr (mean ± SD)	56 (12)	53 (12)	59 (12)
Duration of IBD, yr (mean ± SD)	14 (13)	14 (12)	NA
Gender – male (%)	64 (58)	67 (55)	84 (47)
History of PSC (%)	3 (3)	6 (5)	0 (0)
Family history of CRC[a] (%)	4 (4)	10 (8)	43 (24)
Medication use (%)			
5-ASA > 6 months	97 (88)	105 (88)	NA
Thiopurines > 6 months	32 (29)	40 (33)	NA
Infliximab	7 (6)	16 (13)	NA
Duration of follow-up, months (mean ± SD)	84 (43)	96 (40)	85 (40)
Number of follow-up colonoscopies (median, range)	3 [1-15]	3 [0-11][b]	2 [1-12]
Partial or total colon resection during follow-up (%)	18 (16)	14 (11)	7 (4)
Indication - neoplasia	14 (77)	5 (36)	7 (100)
Indication - IBD	4 (23)	9 (64)	0 (0)

IBD, inflammatory bowel disease; IBDU, IBD-unspecified; PSC, primary sclerosing cholangitis; SD, standard deviation. [a] Unknown in 188 patients. [b] One patient in the IBD-nonadenoma cohort underwent colectomy during follow-up but no colonoscopy

Table 2. Characteristics of adenomas in patients with and without IBD

	IBD Adenoma N = 110 (%)	No IBD Adenoma N = 179 (%)	p-value
Treatment of adenoma			
Biopsy	41 (37)	33 (18)	<0.01
Endoscopic removal	68 (62)	146 (82)	
Partial or total colon resection	1 (1)	0 (0)	
Endoscopic appearance			
Typical polyp	82 (75)	171 (96)	
Atypical polyp	28 (25)	8 (4)	NA
Post-inflammatory polyp	15 (54)	NA	
Non adenoma-like	13 (46)	8 (100)	
Adenoma located in area of IBD	89 (81)	NA	
Number of adenomas	133 (%)	216 (%)	
Location of adenoma			
Rectum	19 (14)	48 (22)	
Sigmoid	55 (41)	91 (42)	
Descending colon	10 (8)	15 (7)	0.21
Transverse colon	24 (18)	19 (9)	
Ascending colon	13 (10)	17 (8)	
Cecum	11 (8)	21 (10)	
Unknown	1 (1)	5 (2)	
Architecture			
Tubular	76 (57)	131 (61)	
Tubulovillous	39 (29)	70 (32)	0.22
Villous	8 (6)	4 (2)	
Serrated	1 (1)	7 (3)	
Other	9 (7)	4 (2)	
Size of adenoma, mm (median, range)	5 [2-20]	6 [1-65]	0.11

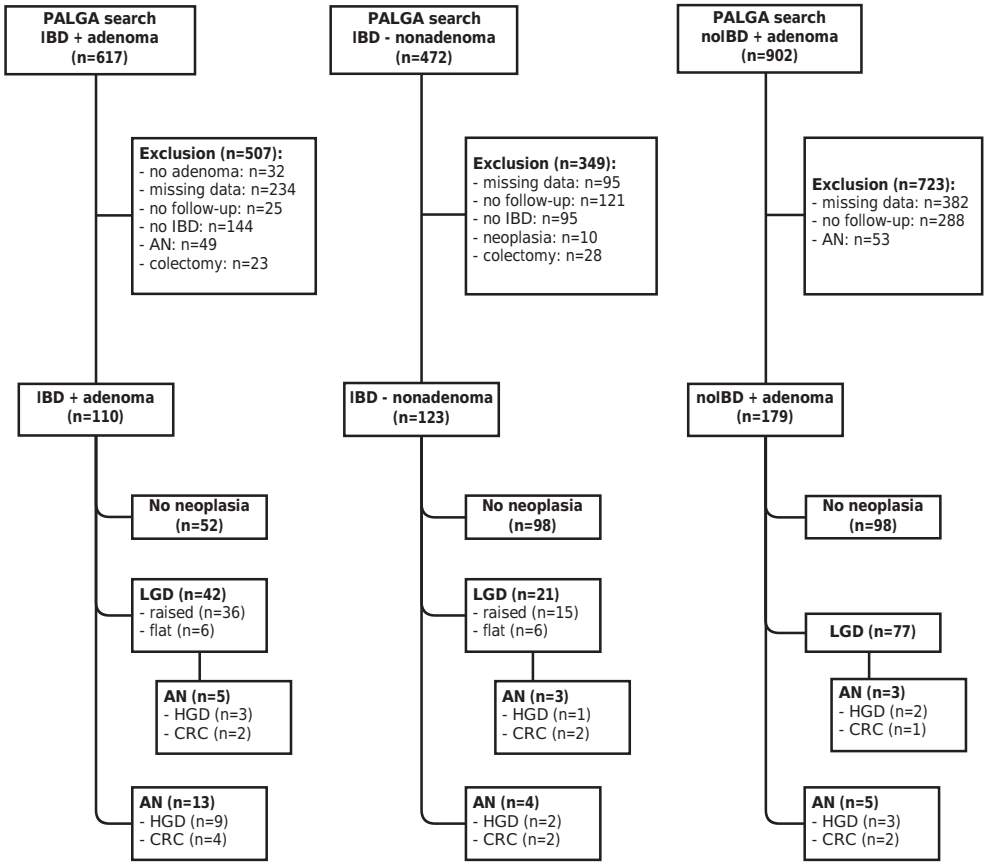
IBD, inflammatory bowel disease

There were no significant differences between IBD + adenoma and nonIBD + adenoma patients regarding adenoma location or architecture. In 21 IBD + adenoma patients (19%) adenomas were located outside the maximum endoscopic or histological extent of inflammation.

Development of neoplasia in IBD + adenoma patients during follow-up

In 36 IBD + adenoma patients (33%) adenomas were detected during follow-up, which were endoscopically classified as typical in 29 (81%) and atypical in seven patients (19%). Four other patients (4%) developed flat LGD and in two patients (2%) both flat and raised dysplasia was detected.

Figure 1. Flow chart of patient selection and development of neoplasia during follow-up.



IBD, inflammatory bowel disease; LGD, low-grade dysplasia; AN, advanced neoplasia.

AN was diagnosed in 18 IBD + adenoma patients (16%) after a median follow-up of 53 months (range 7 - 86) (Figure 1). Twelve patients developed HGD and six developed CRC, of which three were preceded by HGD. In 10 patients HGD was detected in typical adenomas, which were treated by polypectomy in seven and by subtotal colectomy in two patients. In one of these patients, CRC was detected in the resection specimen. One patient with HGD in a large typical adenoma was not treated with endoscopic or surgical resection due to advanced age. HGD was diagnosed in atypical lesions in three patients and in flat mucosa in two. Of these five patients, three were treated by colectomy. HGD was confirmed in the colectomy specimen of one patient while in the other two patients CRC was detected. In the remaining two patients no polypectomy or colectomy was performed due to advanced age and because the diagnosis of HGD could not be confirmed during follow-up colonoscopies. In 89% of patients AN was located in the colonic segment where the baseline adenoma was situated. Two patients, of whom one was originally treated by polypectomy and one was not, developed AN in another colonic segment.

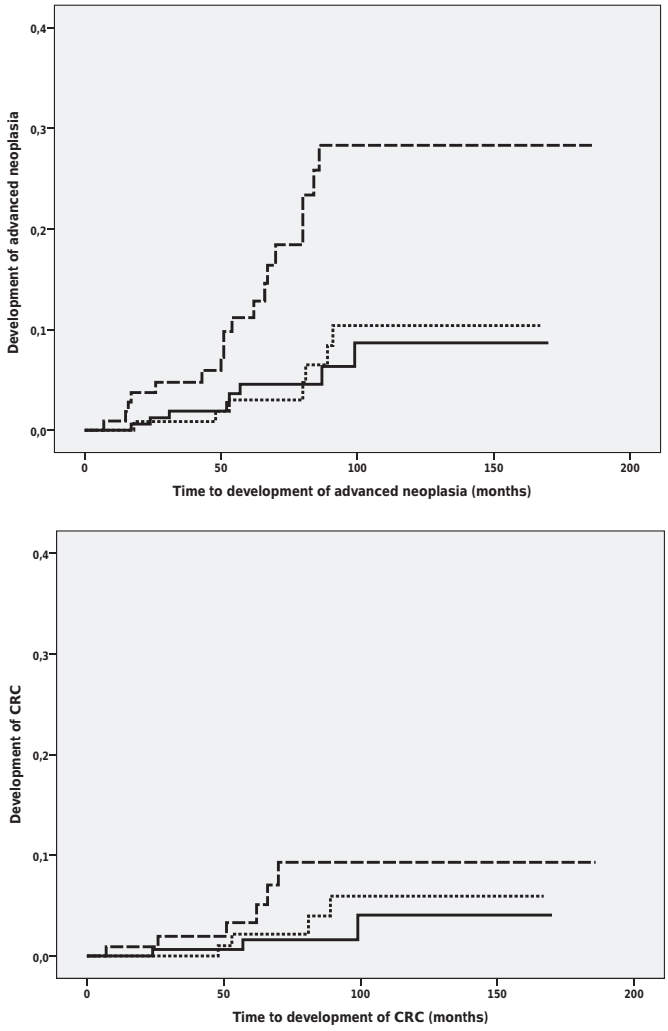
The 5-year cumulative incidence of AN in the IBD + adenoma cohort was 11% (Figure 2). This was similar in patients treated with polypectomy and patients in whom the adenoma was only biopsied (11% versus 10% respectively, p=0.99). Patients with an atypical adenoma at baseline had a higher risk of developing AN compared to patients with a typical adenoma (Figure 3). The 5-year cumulative incidence of AN was not different between patients with an adenoma located inside and patients with an adenoma outside an area of inflammation (10% and 19% respectively, p=0.91). No differences were found between CD and UC patients regarding development of AN (p=0.11).

Development of neoplasia in IBD-nonadenoma patients during follow-up

Within the IBD-nonadenoma cohort 15 patients (12%) developed adenomas during follow-up, which were endoscopically classified as typical adenomas in ten patients and atypical adenomas in five. Two patients developed flat LGD and four developed both flat and raised dysplasia.

A total of seven patients (7%) developed AN after a median duration of follow-up of 80 months (range 18 – 91 months, Figure 1). Three patients developed HGD in a typical adenoma. Of these, two were treated by colectomy and one by polypectomy. None of these patients developed CRC during follow-up. Four patients developed CRC. One of these developed CRC 4 years after an endoscopically resected flat adenoma whereas three developed CRC without evidence of prior LGD or HGD. The 5-year cumulative incidence of AN was 3% in the IBD-nonadenoma cohort (Figure 2). This was significantly lower compared to the IBD + adenoma cohort (p<0.01). No differences were found between CD and UC patients regarding development of AN (p=0.12). The 5-year cumulative incidence of CRC alone was not significantly different between the IBD-nonadenoma and IBD + adenoma groups (2% vs 4% respectively, p=0.23, Figure 2).

Figure 2. Kaplan-Meier curve comparing the development of the composite endpoint advanced neoplasia (HGD and CRC, upper figure) and CRC alone (lower figure) between IBD + adenoma patients (---), nonIBD + adenoma patients (—) and IBD-nonadenoma patients (····). Vertical lines represent events of advanced neoplasia (p <0.01, IBD + adenoma versus nonIBD + adenoma and IBD + adenoma versus IBD-nonadenoma, log-rank test)



Factors associated with development of AN

The presence of an adenoma in patients with IBD was associated with an increased risk of developing AN during follow-up (HR 3.6, 95% CI 1.5 – 8.7) (Table 3). In the multivariate analysis, this effect remained borderline significant after adjustment for duration and type

of IBD, extent of inflammation, age, gender, concomitant diagnosis of PSC and medication use (HR 2.8, 95% CI 1.0 – 8.2). A diagnosis of UC (compared to CD) was associated with an increased risk of developing AN as well, (unadjusted HR 6.1, 95% CI 1.4 – 26.4) which remained borderline significant in the multivariate analysis (adjusted HR 4.5, 95% CI 1.0 – 25.4). Medication use, including 5-ASA and thiopurines, was not associated with the development of AN.

Figure 3. Kaplan Meier curve comparing the development of advanced neoplasia between IBD patients with typical (—) and atypical (---) adenomas (p=0.02, log-rank test).

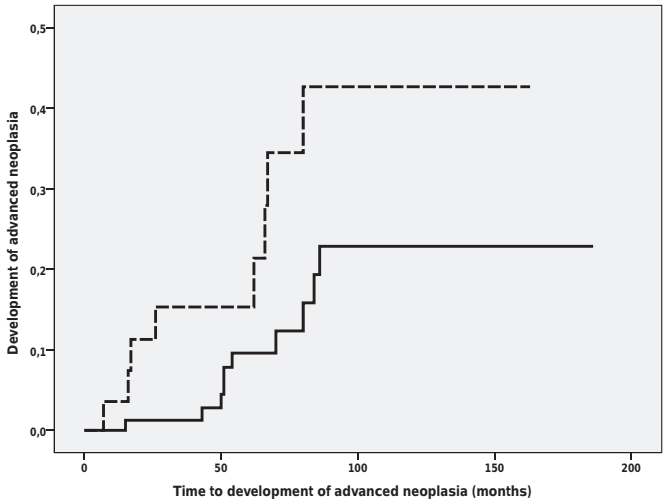


Table 3. Cox proportional hazard analysis of the association between the presence of an adenoma at baseline and the incidence of advanced neoplasia in the IBD patient groups (IBD + adenoma and IBD - nonadenoma)

	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
Presence of adenoma	3.6 (1.5-8.7)	2.8 (1.0-8.2)
Male gender	1.1 (0.5-2.4)	0.8 (0.3-2.1)
Ulcerative colitis (vs. Crohn's disease)	6.1 (1.4-26.4)	5.1 (1.0-25.4)
Extensive colitis	1.5 (0.6-3.5)	1.7 (0.6-4.7)
5-ASA use	0.8 (0.3-2.3)	0.8 (0.2-5.5)
Thiopurine use	0.8 (0.3-1.9)	1.1 (0.4-3.5)
History of PSC	2.0 (0.5-8.4)	3.9 (0.7-21.3)
Duration of IBD, years	1.0 (0.9-1.0)	1.0 (0.9-1.0)

PSC, primary sclerosing cholangitis; IBD, inflammatory bowel disease

Development of neoplasia in nonIBD + adenoma patients during follow-up

In 77 nonIBD + adenoma patients (42%) adenomas were detected during follow-up. A total of eight patients (4%) developed AN after a median follow-up of 53 months (range 17-99 months, Figure 1). In five patients HGD was detected in an adenoma that was treated endoscopically in all five patients. None of these patients developed CRC during follow-up. Three nonIBD + adenoma patients developed CRC, which were all treated by surgical resection.

The 5-year cumulative incidence of AN in the nonIBD + adenoma cohort was 5% (Figure 2). This was significantly lower compared to the IBD + adenoma cohort ($p<0.01$), as well as to the subset of IBD + adenoma patients with a typical adenoma resembling sporadic adenomas ($p=0.03$). The 5-year cumulative incidence of CRC alone in the nonIBD + adenoma cohort did not differ from the IBD + adenoma cohort (1% vs 3% respectively, $p=0.06$, Figure 2).

DISCUSSION

This study demonstrates an increased risk of AN in IBD patients with an adenoma, when compared to non-IBD patients with adenomas and IBD patients without adenomas. Notably, atypical as well as typical adenomas in patients with IBD were found to be associated with an increased risk of AN. This finding is in contrast with most previously published studies, which consistently reported a low risk of AN in IBD patients with polypoid dysplasia.[8,10,13,14]

The high incidence of AN among IBD + adenoma patients during follow-up in our study was mainly caused by the development of HGD rather than the development of CRC. HGD developed in 12% of IBD + adenoma patients as compared to 2% and 3% of IBD-nonadenoma and nonIBD + adenoma patients, whereas CRC developed in 6% of IBD + adenoma patients compared to 3% and 2% of IBD-nonadenoma and nonIBD + adenoma patients, respectively. Although previous studies primarily focused on CRC incidence, the reported incidences of HGD after a diagnosis of adenoma were low in these studies and varied between 0% and 5%.[8-10,15] Since HGD will undoubtedly progress to CRC when left untreated, we opted to use a composite endpoint including both HGD and CRC.[16-18] Furthermore, we report a substantially higher CRC incidence in IBD + adenoma patients than reported in several previous studies.[8,10,13]

One might assume that the inclusion of both patients treated by polypectomy and patients in whom adenomas were not endoscopically removed accounts for the higher risk of AN in our cohort. Eighty-one percent of all nonIBD + adenoma patients were treated by polypectomy, compared to only 62% of IBD + adenoma patients. An explanation for the relative low polypectomy rate in the IBD + adenoma cohort might be that post-inflammatory-like and small flat lesions are more frequently encountered and not deemed serious enough for polypectomy. Our rates are in line with recent data from Vieth et al.,[9] who found a

polypectomy rate of 59% in IBD patients with adenomas, although we could not confirm the higher incidence of neoplasia and CRC among patients in whom no polypectomy was performed as reported in that study. It is therefore conceivable that AN developed either as a result of field cancerization or due to residual dysplastic tissue.

Another possible explanation for the high incidence of AN might be the design of our study. We selected cases using a pathology database, whereas in previous studies selection was based on the endoscopic identification of solitary polyps.[8,10,13] Therefore, our study comprised a heterogeneous group of endoscopic lesions including a large subgroup of "non-adenoma-like" lesions, which have previously been associated with a high risk of synchronous CRC.[4,19-21] Current guidelines therefore generally recommend colectomy for these types of lesions.[5,6] In our IBD + adenoma cohort, 25% of patients had an atypical adenoma, including both non-adenoma-like lesions and lesions considered to be post-inflammatory polyps during endoscopy. The cumulative incidence of AN was substantially higher in these patients: eight patients with an atypical adenoma (29%) developed AN compared to ten patients (12%) with a typical adenoma. Interestingly, in 15 patients histologically diagnosed adenomas were endoscopically characterized as post-inflammatory polyps. Of these, 30 % developed AN during follow-up, which highlights the difficulty of differentiation between raised dysplasia and post-inflammatory polyps. Inexperience of the endoscopists who assessed the types of polyps may have contributed to this misclassification. Another explanation may be the fact that post-inflammatory polyps are proxies for longstanding and severe inflammation and may therefore be associated with an increased risk of CRC.[22,23]

Of note, IBD + adenoma patients with typical adenomas displayed a significantly increased 5-year cumulative incidence of advanced neoplasia as well: 6% compared to 5% in nonIBD + adenoma patients (p=0.03 log rank test). This conflicts with data from previous studies that reported no difference in development of subsequent neoplasia between these patient groups.[7,13] Our results stress the importance of complete removal and close follow-up of either type of adenoma.

The IBD + adenoma cohort contained patients with adenomas detected both inside and outside colonic areas (previously) involved in inflammation. Since by definition IBD-associated dysplasia only develops in areas of chronic inflammation, one should classify adenomas outside an area of previous inflammation as sporadic. Remarkably, the incidence of AN was not different between IBD patients with adenomas detected within or outside an area of previous inflammation.

The comparison of the IBD + adenoma and IBD-nonadenoma cohorts enabled us to assess the additional risk of adenomas in patients with colitis. IBD + adenoma patients had a higher risk of AN compared to IBD-nonadenoma patients. The presence of an adenoma seemed an independent predictor of AN when corrected for several known risk factors of IBD-associated neoplasia, although with borderline significance (HR 3.0, 95% CI 1.0 – 8.5).

Our study has some limitations. First, due to the retrospective design of the study we relied on the descriptions provided in endoscopy reports with regard to endoscopic characteristics and treatment of the adenomas. Second, IBD + adenoma patients and IBD-nonadenoma patients were solely matched on age at inclusion, centre and date of inclusion according to the date of adenoma diagnosis in the IBD + adenoma cohort. We do not think that this introduced important bias, though, since these cohorts were quite well-matched with regard to duration and extent of IBD. Third, a signficant proportion of patients were excluded due to the lack of follow-up or missing endoscopy or pathology reports. Since patients in the nonIBD + adenoma and IBD-nonadenoma cohort were more frequently excluded than patients in the IBD + adenoma cohort, this might have resulted in a higher risk of AN in these two cohorts, thereby introducing a selection bias. Obviously, this would only strengthen our conclusion that the risk of AN is increased in the IBD + adenoma patients. At last, although patients underwent a complete colonoscopy at inclusion in this study and at least one colonoscopy or a colectomy during follow-up, surveillance colonoscopies with random biopsy sampling were performed in only 52% of IBD + adenoma patients and 55% of IBD-nonadenoma patients. This might have resulted in an underestimation of the presence of AN. However, since most cases of AN can be identified endoscopically, we do not feel that this had a major influence on our results.

In conclusion, this study shows that IBD patients with a histological diagnosis of an adenoma have an increased risk of developing AN compared to nonIBD + adenoma patients and IBD-nonadenoma patients. The presence of atypical adenomas in particular was associated with this increased risk, although patients with typical adenomas were found to carry an additional risk as well. Thus, complete removal of adenomas and subsequent strict surveillance is warranted in IBD patients with atypical as well as typical colonic adenomas.

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CHAPTER 8

Thiopurines prevent advanced colorectal neoplasia in patients with inflammatory bowel disease

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ABSTRACT

Background and aims

Previous studies have suggested a chemopreventive effect of 5-aminosalicylic acid (5-ASA) therapy in patients with inflammatory bowel disease (IBD). This effect has not been reported in IBD patients using thiopurines. We investigated the association between thiopurine or 5-ASA use and the risk of advanced neoplasia, including high-grade dysplasia and colorectal cancer, in a large cohort of IBD patients in the Netherlands.

Methods

PALGA, the nationwide network and registry of histo- and cytopathology in the Netherlands was linked to an anonymized computerized database of a Dutch health insurance company to identify IBD patients with or without advanced neoplasia. Pharmaceutical data, including type and duration of medication use, were collected between January, 2001 and December, 2009. Cox proportional hazard regression analysis was used to calculate risk of advanced neoplasia in patients with and without thiopurine or 5-ASA use.

Results

A total of 2578 IBD patients were included. Of these, 973 patients (38%) used 5-ASA, 314 (12%) thiopurines, 456 (18%) both 5-ASA and thiopurines and 835 (32%) none of these drugs. Twenty-eight patients (1%) developed advanced neoplasia during 16,289 person years of follow-up. Of these, 11 patients (39%) had used 5-ASA, 2 (7%) thiopurines and 1 (4%) both drugs. Thiopurine use was associated with a significantly decreased risk of developing advanced neoplasia (adjusted Hazard Ratio (HR) 0.10, 95% CI 0.01-0.75). 5-ASA therapy also had a protective effect on developing advanced neoplasia, but this was not statistically significant (adjusted HR 0.56, 95% CI 0.22-1.40).

Conclusion

Thiopurine use protects IBD patients against the development of advanced neoplasia. The effect of 5-ASA appeared to be less pronounced.

INTRODUCTION

Patients with inflammatory bowel disease (IBD) have an increased risk of developing colorectal cancer (CRC). The cumulative probability of developing CRC in patients with ulcerative colitis (UC) is 2% after 10 years, 8% after 20 years and 18% after 30 years of disease duration.[1] A comparable CRC risk has been reported for patients with Crohn's colitis.[2,3] Since the neoplastic potential of the diseased colon is supposed to be the consequence of inflammation-induced genetic mutations[4], treatment of inflammation should reduce CRC risk in IBD patients.

Previous studies have indeed reported a chemopreventive effect of 5-aminosalicylic acid (5-ASA) drugs on colitis-associated CRC. 5-ASA drugs are amongst the most commonly prescribed anti-inflammatory drugs for patients with Crohn's or ulcerative colitis. Pooled analysis of 3 cohort and 6 case-control studies reported a 49% risk reduction of CRC and the composite endpoint dysplasia/CRC in UC patients using 5-ASA drugs.[5] However, two more recent studies reported no effect of 5-ASA use on CRC risk.[6,7] These conflicting results may be caused by heterogeneity with regard to patient populations, study designs and data sources used in each study. Moreover, only a few studies corrected for concurrent use of systemic immunomodulators[7-9], which might have influenced the magnitude of the effect of 5-ASA agents.

Systemic immunomodulators, such as the thiopurines azathioprine (AZA) and 6-mercaptopurine (6MP), are commonly used for maintenance of remission in both UC and Crohn's disease (CD). As a consequence of the reported malignancy risk associated with long-term thiopurine use in renal transplant patients[10,11], studies in IBD have mainly focused on the pro-carcinogenic potential of these compounds. Patients with IBD using thiopurines have an approximately fourfold increased risk of lymphoma[12,13] and possibly also an increased risk of non-melanoma skin cancer.[14] However, thiopurines might exert chemopreventive effects through reduction of colonic inflammation as well. In the few studies primarily investigating the putative association between thiopurine use and colorectal neoplasia risk in IBD patients, no beneficial effect of thiopurine use was found.[8,15-19] These studies had, however, some methodological shortcomings, including 1) a retrospective design; 2) they were based on prescription rates in medical records; and 3) pathology reports for validated endpoints were lacking.

In order to overcome these limitations we chose to combine a health claims database with a nationwide pathology database. This enabled us to study patients' 5-ASA and thiopurine use over time and to censor on endpoints based on pathology reports. The aim of this study was therefore to investigate the association between 5-ASA or thiopurine use and the risk of advanced neoplasia in patients with IBD, using a health claims database linked to a nationwide pathology network.

PATIENTS AND METHODS

Study population

For this cohort study, IBD patients were identified in the Agis Health database, an anonymized computerized database of a large Dutch health insurance company covering 1.2 million Dutch inhabitants, which is approximately 8% of the Dutch population.[20] This database represents the urbanized area of the Netherlands with regard to gender, age, ethnicity and socio-economic status, and contains data on diagnoses and medication use. Registration of drugs in this database is based on reimbursement of costs that is strictly controlled. Each drug delivered by a pharmacist to an insured individual is recorded, including information about the type of drug according to the Anatomical Therapeutic Chemical (ATC) code, the number of dosages provided and the date of delivery.

Patients with prevalent or incident IBD between January 2006 and December 2007 were identified, using the Dutch Diagnosis Treatment Combinations (DTC) for IBD which are based on the International Classification of Disease, 9th Revision. In the Netherlands, inpatient services provided by hospitals and physicians are paid for mostly on the basis of DTCs, for which prospectively fixed amounts are charged per episode of care. For a part of DTCs (e.g. Crohn's disease), health insurers and care providers are allowed to negotiate prices freely. The DTCs were introduced in 2005 and can be considered the Dutch version of the Diagnostic Related Groups (DRGs) as used in other countries, e.g. United States.[21,22]

Personal data of each IBD patient were linked to PALGA, the nationwide network and registry of histo- and cytopathology in the Netherlands, which contains pathology reports from all Dutch pathology institutes and has nationwide coverage since 1990. This allowed us to both verify the IBD diagnosis and to assess whether a patient had developed advanced neoplasia. Linkage was performed by a third trusted party in order to protect privacy during the linkage procedure. To ensure confidentiality, personal data were removed from the merged database before delivery to the researchers. Patients were included in this study when the pathology reports confirmed the presence of IBD. Patients with a history of advanced neoplasia, patients with Crohn's disease, but no colonic involvement and patients with a previous subtotal or total colectomy were excluded from this study.

Data collection

From the Agis database the number of daily defined doses (DDD) of 5-ASA compounds, thiopurines, folic acid and calcium and demographic data of each patient including gender, date of birth and mortality were collected. From the PALGA database the following data were extracted: date of IBD diagnosis, type and extent of IBD, development of advanced neoplasia, date of advanced neoplasia diagnosis, type of advanced neoplasia and colonic resections. Date of IBD diagnosis was defined as the date of the first pathology report confirming the presence of IBD. Disease extent was divided in more or less than 50% colonic

involvement. A disease extent of more than 50% was defined as disease that extended beyond the splenic flexure in UC patients and as involvement of 3 or more anatomical parts of the colon in CD patients.

Exposure

Patients were included at the date of insurance in the Agis database, starting from January 1st, 2001. Whenever patients developed IBD after the date of insurance, patients were included at the moment of IBD diagnosis.

In this study 5-ASA drugs included mesalazine, sulfasalazine and olsalazine. For sulfasalazine and olsalazine users equivalent dosages of mesalazine were calculated. Patients were defined as being exposed to 5-ASA if they had used at least 1.2 grams mesalazine per day during 6 months, according to previously published results.[23] For exposure calculation of the studied thiopurines (i.e. AZA and 6MP), we defined exposure as use of at least 50 mg AZA and/or 6MP per day during 6 months.

Endpoints

Patients were followed over time until one of the following endpoints: 1. end of follow-up (December 31st, 2009); 2. death; 3. advanced neoplasia; 4. subtotal or total colectomy or 5. loss to follow-up.

Date of death was collected from the national death registry, which is linked to the Agis Health database. Advanced neoplasia was defined as the finding of high-grade dysplasia (HGD) or CRC in a biopsy or colectomy specimen.

Statistical analysis

All analyses were restricted to the period beyond the first 6 months of follow-up. Patients with a follow-up duration of less then 6 months were excluded from further analyses.

Baseline characteristics of included patients were analyzed with standard descriptive statistics, and compared between patients using 5-ASA, thiopurines, a combination, and patients not using any of these drugs. Continuous variables were compared between these 4 groups using ANOVA or Kruskall-Wallis analysis, where appropriate. Categorical variables were analyzed using Pearson's chi-squared or Fisher's exact test, where appropriate.

Cox proportional hazard regression analysis was used to calculate hazard ratios (HR) and 95% Confidence Intervals (CI) for the association between 5-ASA or thiopurine use and the risk of advanced neoplasia in patients with IBD. In the multivariable model we adjusted for type of IBD, gender, age, duration of IBD, disease extent, history of dysplasia, history of partial colonic resection and concurrent folic acid or calcium use, since these may influence neoplasia risk. These procedures were repeated for different durations of use to analyze whether the length of exposure influenced the HRs. A p-value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 15.0 for Windows.

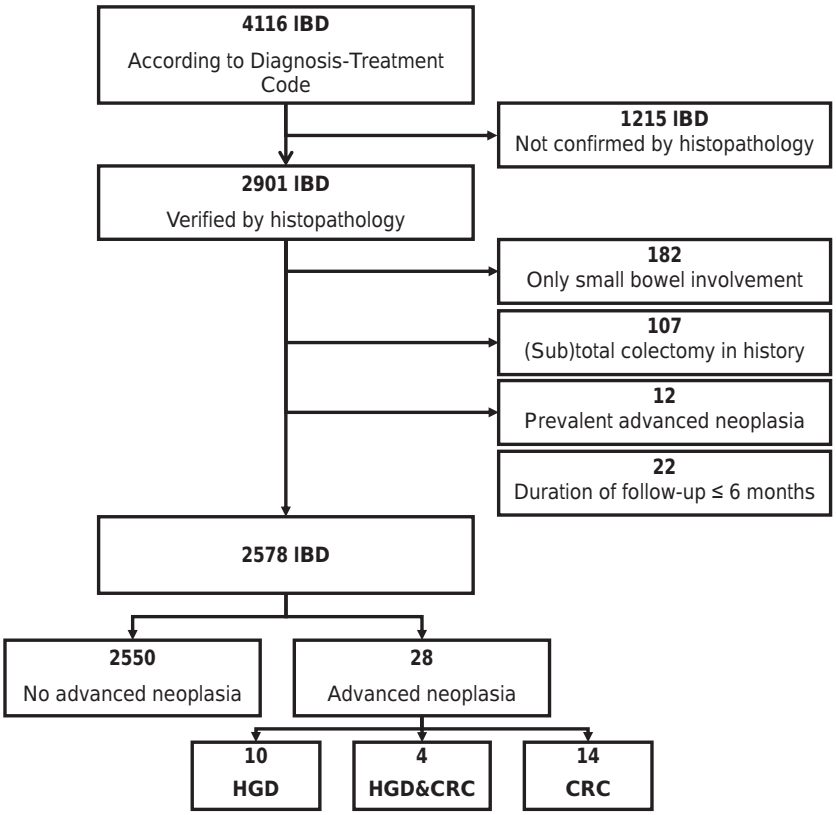
Ethical considerations

This study was carried out with the approval of and in accordance with the privacy and ethical guidelines of the privacy committee of PALGA and the research committee of the Agis Health Database.

RESULTS

We identified 4116 patients with a DTC for IBD. After linkage to the PALGA database and analysis of the pathology reports, 2901 patients with a verified IBD diagnosis were included. Application of the exclusion criteria excluded 323 patients, leaving 2578 IBD patients for further analysis (Figure 1).

Figure 1. Flowchart.



IBD, inflammatory bowel disease; HGD, high-grade dysplasia; CRC, colorectal cancer

According to the predefined definitions for 5-ASA and thiopurine use, 973 patients (38%) were defined as 5-ASA users, 314 (12%) as thiopurine user, and 456 (18%) as both 5-ASA and thiopurine user, while 835 patients (32%) were defined as non-user. Baseline characteristics of each treatment group are presented in Table 1. No differences between treatment groups were found for gender, age, type of IBD, duration and extent of IBD, and history of dysplasia. Total duration of follow-up was 16,289 person years, wherein 28 patients (1%) developed advanced neoplasia. Of these, 10 (36%) developed HGD, 14 (50%) CRC and 4 (14%) both HGD and CRC. Among the 28 patients who developed advanced neoplasia, 11 (39%) had used 5-ASA drugs, 2 (7%) thiopurines and 1 (4%) both drugs. The distribution of the types of advanced neoplasia among the different exposure groups is depicted in Table 2. The incidence rates are presented in Table 3. Figure 2 shows the Kaplan-Meier curves of each treatment group.

Table 1. Baseline characteristics of 5-ASA, thiopurines, 5-ASA and thiopurines and no medication users

	None N=835	**5-ASA** N=973	**Thiopurines** N=314	**5-ASA & Thiopurines** N=456	**p-value**
Type of IBD (%)					
Ulcerative colitis	388 (47)	440 (45)	150 (48)	201 (44)	0.18
Crohn's colitis	363 (43)	447 (46)	123 (39)	215 (47)	
IBD – unspecified	84 (10)	86 (9)	41 (13)	40 (9)	
Male (%)	360 (43)	416 (43)	132 (42)	192 (42)	0.98
Age, years (mean ± SD)	43 (17)	43 (17)	42 (17)	44 (17)	0.21
Duration IBD, months (mean ± SD)	35 (48)	36 (47)	34 (47)	40 (45)	0.19
Disease extension (%)					
> 50% of colon	242 (29)	263 (27)	98 (31)	125 (27)	0.40
< 50% of colon	344 (41)	430 (44)	120 (38)	208 (46)	
Unknown	249 (30)	280 (29)	96 (31)	123 (27)	
History of dysplasia (%)	19 (2)	20 (2)	9 (3)	13 (3)	0.74
History of partial colon resection (%)	9 (1)	10 (1)	6 (2)	8 (2)	0.46

IBD, inflammatory bowel disease; 5-ASA, 5-aminosalicylic acid

Table 2. Distribution of types of advanced neoplasia among different exposure groups

	None N=14	**5-ASA** N=11	**Thiopurines** N=2	**5-ASA & Thiopurines** N=1
HGD	5	5	0	0
HGD&CRC	2	1	1	0
CRC	7	5	1	1

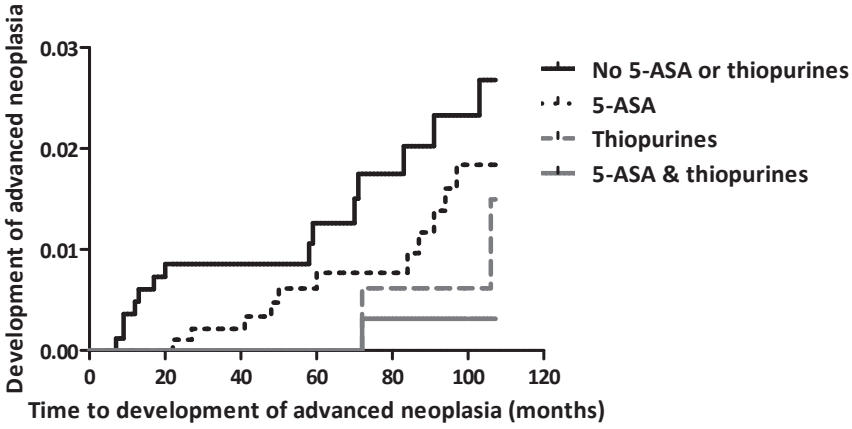
HGD, high-grade dysplasia; CRC, colorectal carcinoma; 5-ASA, 5-aminosalicylic acid

Table 3. Incidence rates per 1000 person-years for advanced neoplasia

	No. of patients	Person years exposed	No. of events (% of patients)	Incidence rate (per 1000 person years)
Total population	2578	16,289	28 (1)	1.72
Non-users	835	4,864	14 (2)	2.88
5-ASA	973	6,260	11 (1)	1.76
Thiopurines	314	1,924	2 (1)	1.04
Thiopurines & 5-ASA	456	3,242	1 (0)	0.31

5-ASA, 5-aminosalicylic acid

Figure 2. Kaplan-Meier curves comparing the development of advanced neoplasia in each treatment group (log-rank test p=0.04). Patients that develop advanced neoplasia are censored at the moment advanced neoplasia is detected. Vertical lines represent events of advanced neoplasia.



Numbers at risk	0	20	40	60	80	100
No medication	835	789	643	474	369	287
5-ASA	973	953	812	640	525	406
Thiopurines	314	310	253	181	153	119
5-ASA&thiopurines	456	452	419	355	296	235

Patients that had used ≥ 50 mg of thiopurines per day during at least 6 months had at a significantly decreased risk of developing advanced neoplasia (unadjusted HR 0.25, 95% CI 0.08-0.84) and this effect remained significant in the multivariate analysis (adjusted HR 0.10, 95% CI 0.01-0.75; Table 4).

5-ASA use of ≥ 1.2 grams per day during at least 6 months was also associated with a reduced risk of advanced neoplasia, but this did not reach statistical significance in the univariate analysis (unadjusted HR 0.52, 95% CI 0.24-1.09) and after adjustment for potential confounders (HR 0.56, 95% CI 0.22-1.40). In addition to the effects found in the multivariate model for exposure to 5-ASA and thiopurines, increasing age and disease involvement of

more than 50% of the colon were identified as risk factors for the development of advanced neoplasia (adjusted HR 1.07, 95% CI 1.03-1.10 and adjusted HR 5.32, 95% CI 1.79-15.8, respectively).

Table 4. Cox proportional hazard analysis of association between 5-ASA use and thiopurine use and the development of advanced neoplasia

	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
5-ASA use	0.52 (0.24-1.09)	0.56 (0.22-1.40)
Thiopurine use	0.25 (0.08-0.84)	0.10 (0.01-0.75)
Ulcerative colitis (vs. Crohn's disease)	1.72 (0.75-3.94)	0.97 (0.35-2.72)
Female gender	0.68 (0.33-1.43)	0.96 (0.39-2.37)
Increasing age (year)	1.05 (1.03-1.08)	1.07 (1.03-1.10)
Duration of IBD (year)	1.07 (0.97-1.17)	1.02 (0.92-1.14)
>50% colonic involvement	5.48 (2.05-14.7)	5.32 (1.79-15.8)
History of dysplasia	1.07 (0.15-7.88)	0.48 (0.06-3.83)
History of partial colon resection	2.27 (0.31-16.7)	3.54 (0.41-30.8)
Folic acid use	0.64 (0.15-2.68)	2.11 (0.45-9.94)
Calcium use	0.77 (0.35-1.71)	1.14 (0.44-2.95)

HR, hazard ratio; 95% CI, 95% confidence interval; adjusted HR, adjusted for covariates listed in the table; 5-ASA, 5-aminosalicylic acid

We also analyzed the association between different durations of 5-ASA or thiopurine use and the development of advanced neoplasia. The risk of advanced neoplasia decreased with increasing duration of 5-ASA use. (Figure 3) Patients who had used 5-ASA drugs for more than 2 years had a decreased risk of developing advanced neoplasia, but this was only borderline significant (HR 0.31, 95% CI 0.10-1.00). The preventive effect of thiopurine use sustained if used longer than 1 year (HR 0.12, 95% CI 0.02-0.87).

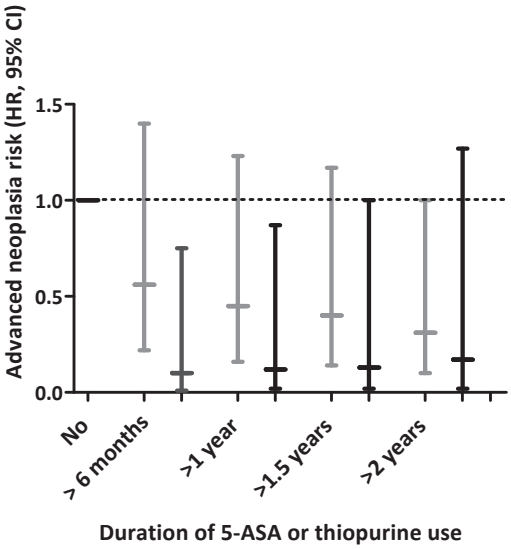
DISCUSSION

This study investigated the association between 5-ASA and thiopurine use and the development of advanced neoplasia in patients with ulcerative or Crohn's colitis. We found a significant chemopreventive effect of thiopurine use in patients with IBD. This effect was less pronounced in 5-ASA users.

Previous studies that investigated the effect of thiopurine use in patients with IBD mainly reported on the risk of extra-intestinal malignancies, including non-melanoma skin cancer and lymphoma.[12-14, 24-26] The development of these malignancies is usually attributed to the immunosuppressive properties of thiopurines. The present study is the first to report a significant chemopreventive effect of thiopurines. These data conflict with results from

previous studies.[8,15,16,18] This might be due to the methods of data acquisition in other studies, which might have resulted in an underestimation of the potential chemopreventive effect of thiopurines. Furthermore, different ways of analyzing thiopurine exposure have been applied. Two studies[16,18] compared patients who had ever used thiopurines with nonusers and found no effect of thiopurine use on the development of advanced neoplasia. By using a threshold of 25 mg thiopurines/day, no association between thiopurine use and advanced neoplasia was found by Matula et al. either.[18]

Figure 3. Hazard ratios (HR) and 95% confidence intervals (CI) for the development of advanced neoplasia categorized in different durations of 5-ASA (—) and thiopurine use (**—**)



Thiopurines act slowly with the full therapeutic effect reached approximately 17 weeks after initiation, and greater efficacy has been demonstrated for increasing doses.[27,28] Therefore, we used a stricter definition of thiopurine users, namely those patients who had used more than 50 mg of azathioprine or 6-mercaptopurine per day for at least 6 months. This might explain the clear chemopreventive effect of thiopurines found in our study. The effect sustained if thiopurines were used for a period of more than 1 year. Of all 770 patients that had used thiopurines for at least 6 months, 708 (92%) used thiopurines for 1 year or longer. In this study, we could not confirm a chemopreventive effect of 5-ASA in patients that had used the previously suggested chemopreventive daily dose of 1.2 grams per day[23] for at least 6 months. Patients who had used 5-ASA drugs for more than 2 years had a decreased risk of developing advanced neoplasia, but this was only borderline significant. Multiple studies have previously evaluated the association between 5-ASA therapy and colorectal neoplasia

and reported heterogeneous results. Although the magnitude of the effect is still uncertain, the present predominant assumption is that these drugs prevent against CRC.[29] Most of these studies, however, did not adjust for concurrent thiopurine use in their analyses, introducing a major source of bias.

Our study has several strengths. We used a large health claim database that was linked to the Dutch nationwide pathology database, providing high quality data on 5-ASA and thiopurine use in patients with a verified IBD diagnosis during 16,289 person-years of follow-up. Medication use was based on the number and dosages of drugs provided to each individual patient at the pharmacy. Previous studies that investigated the association between medication use and colorectal neoplasia risk in IBD mainly used patient charts to assess exposure to individual drugs. This method relies heavily on the accuracy of administrating medication use by the physician. The limited number of studies that consulted a drug database to evaluate the effect of medication use in IBD patients mainly used prescription data of individual drugs.[9,30] Since non-adherence to treatment is frequently encountered in IBD patients[31], a large discrepancy may exist between the physician's prescriptions and the actual used daily dose by individual patients. Therefore, data based on the administrative claim of the pharmacist, representing true-life amounts of drugs delivered to individual patients, probably better reflect the effective ingested dose of medication.

Besides the advantages of using both a health claim and pathology database, there is also a downside of exclusively using databases without consulting individual patient charts. Since our nationwide pathology database has only nationwide coverage since 1990, pathology reports originating from before 1990 could not be consulted. Disease duration might therefore be underestimated. Second, patients were only included in this study if the disease had been confirmed by histology. Although endoscopy and tissue sampling is widely performed in patients suspected of IBD, no histology was available from a significant number of patients for confirmation of the diagnosis. This might have introduced a bias as well, although it can be assumed that this would lead to inclusion of more patients with a mild IBD phenotype, who are not particularly prone to develop colitis associated CRC. Moreover, since CRC risk is lower in population-based studies[32] the chemopreventive effect found in the present population-based study is probably an underestimation of the actual effect. Third, only limited data on the IBD phenotype and no data on the use of anti-TNF compounds could be presented. The latter is due to a separate reimbursement system for these drugs in the Netherlands. For the same reason, the effect of surveillance colonoscopies could not be assessed. One might assume that patients on thiopurines have more severe disease, and that these patients are therefore more frequently enrolled in surveillance programmes. This could have resulted in an earlier detection and treatment of colorectal neoplasia, and thereby in an overestimation of the presumed chemopreventive effect of thiopurines. Finally, we combined UC and CD patients into one population. Whether the

strength of the chemopreventive effect of thiopurines is identical in each of the subtypes remains to be investigated, since our study allowed no further sub-analyses.

In conclusion, we report a significant chemopreventive effect of thiopurine use in patients with IBD. Thiopurine users demonstrate a 10-times lower risk of developing either HGD or CRC. The effect of 5-ASA therapy appeared to be less pronounced. The chemopreventive effect of thiopurines in IBD patients reported in the present study might provide an extra incentive to prescribe these drugs in IBD patients, especially in those with colonic disease.

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CHAPTER 9

The risk of non-melanoma skin cancer in patients with inflammatory bowel disease using thiopurines is not increased

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ABSTRACT

Background and aims

Transplant recipients who receive thiopurines (azathioprine and 6-mercaptopurine) are at an increased risk of developing non-melanoma skin cancer (NMSC). Patients with inflammatory bowel disease (IBD) are increasingly being treated with thiopurines. The risk of NMSC in this patient group is largely unknown. We investigated the association between thiopurine use and NMSC risk in a large cohort of IBD patients in the Netherlands.

Methods

Patients with IBD were identified in an anonymized computerized database of one of the Dutch health insurance companies, including 1.2 million policyholders. From this database, information regarding type of drugs and number of dosages provided to patients were collected between January, 2001 and December, 2009. Each IBD patient was linked to the Dutch nationwide pathology archive (PALGA) to verify the IBD diagnosis and to determine whether a patient had developed NMSC. Cox proportional hazard regression analysis was used to calculate the risk of NMSC in patients with and without thiopurine use, adjusted for type of IBD, gender, age and duration of IBD.

Results

A total of 2887 patients with a confirmed IBD diagnosis were included in this study. Of these, 819 patients (28%) used thiopurines. No statistically significant differences were found for type of IBD, gender, age and extent of IBD between thiopurine users and non-users. Disease duration was significantly shorter in users compared to non-users (34 months (±SD 45) versus 38 months (±SD 48), p=0.03). Eighty-six patients (3%) developed NMSC during 18,663 person years of follow-up. Of these patients, 24 (28%) had used thiopurines. Mean age at NMSC diagnosis was 64 years (±SD 12) in thiopurine users compared to 63 years (±SD 13) in non-users (p=0.68). Increasing age and duration of IBD were associated with a higher risk of developing NMSC (adjusted hazard ratio (HR) 1.06, 95% Confidence Interval (CI) 1.04-1.08 and adjusted HR 1.007, 95% CI 1.002-1.011, respectively). A diagnosis of ulcerative colitis and female gender were associated with a decreased risk of NMSC (adjusted HR 0.62, 95% CI 0.40-0.98) and adjusted HR 0.47, 95% CI 0.30-0.74). Thiopurine use was not associated with an increased risk of developing NMSC (adjusted HR 0.85, 95% CI 0.51-1.41).

Conclusion

In contrast to transplant recipients, thiopurine use in IBD patients is not associated with an increased risk of developing NMSC.

INTRODUCTION

Thiopurines, including azathioprine (AZA) and 6-mercaptopurine (6MP), are increasingly used for maintenance of remission in both ulcerative colitis (UC) patients and patients with Crohn's disease (CD). Thiopurines have cytotoxic and immunosuppressive properties. They act as anti-purines and inhibit the synthesis of proteins, DNA and RNA.[1,2] In organ transplant patients the use of thiopurines has been associated with an increased malignancy risk, including non-Hodgkin lymphoma and skin cancer. Already in 1973, Hoover et al. reported a 35 times higher risk of developing non-Hodgkin lymphoma and a 4 times higher risk of skin cancer in renal transplant recipients on immunosuppressive therapy compared to the general population.[3]

As a consequence of the reported malignancy risk associated with long-term thiopurine use in organ transplant patients, studies in IBD have focused on the pro-carcinogenic potential of thiopurines in these patients. As well as transplant recipients, IBD patients using thiopurines have been found to be at an increased risk of lymphoma. Recently, a large prospective observational cohort study in 19,486 IBD patients reported a 5 times higher risk of developing lymphoproliferative disorders in patients treated with thiopurines compared to patients who had never received these drugs.[4]

The risk of non-melanoma skin cancer (NMSC) in IBD patients using thiopurines, however, has been investigated less extensively. A recent study investigated this association in the United States using a large health claim database. Both recent and persistent thiopurine use were found to be associated with an approximately 4-fold increased risk of developing NMSC.[5] Until now no other study primarily investigated this association.

The aim of this study was therefore to investigate the association between thiopurine use and the risk of NMSC in a large cohort of Dutch patients with IBD, using a health claims database linked to a nationwide pathology network.

PATIENTS AND METHODS

Study population

For this study, IBD patients were identified in the Agis Health database, an anonymized computerized database of a large Dutch health insurance company covering 1.2 million Dutch inhabitants, which is approximately 8% of the Dutch population.[6] The database represents the urbanized area of the Netherlands regarding gender, age, ethnicity and socio-economic status, and contains data regarding diagnoses and medication use. Each drug delivered by a pharmacist to an insured individual is recorded in this database, including information about the type of drug according to the Anatomical Therapeutic Chemical (ATC) code, the number of dosages provided and the date of delivery.

Patients with prevalent or incident IBD between January 2006 and December 2007 were

identified, using the Dutch Diagnostic Treatment Combinations for IBD which are based on the International Classification of Disease, 9th Revision. Personal data of each IBD patient were linked to the Dutch nationwide pathology archive (PALGA), which contains all pathology reports from the Netherlands dating back to 1971. This allowed us to both verify the IBD diagnosis and to assess whether a patient had developed NMSC. Linkage was performed by a third trusted party in order to protect privacy during the linkage procedure. To ensure confidentiality, personal data were removed from the merged database before delivery to the researchers. Patients with a history of NMSC were excluded from this study.

Data collection

From the Agis database the number of daily defined doses (DDD) of thiopurines and demographic data of each patient including gender, date of birth and mortality were assessed. From the PALGA database the following data were extracted: date of IBD diagnosis, type and extent of IBD, development of NMSC, date of NMSC and type of NMSC. Disease extent was divided in more or less than 50% colonic involvement. A disease extent of more than 50% was defined as disease that extended beyond the splenic flexure in UC patients and as involvement of 3 or more anatomical parts of the colon in CD patients.

Exposure

Patients were included at the date of insurance in the Agis database, starting from January 1st, 2001. Whenever patients developed IBD after the date of insurance, patients were included at the moment of IBD diagnosis. In this study thiopurines included azathioprine (AZA) and 6-mercaptopurie (6MP). Patients were defined to be exposed to thiopurines if they had used at least 50 mg AZA and/or 6MP per day during 6 months.

Endpoints

Patients were followed over time until one of the following endpoints: 1. end of follow-up (December 31st, 2009); 2. death or 3. NMSC. Date of death was collected from the national death registry, which is linked to the Agis Health database. NMSC was defined as the finding of squamous cell carcinoma (SCC) or basal skin carcinoma (BCC) in a skin biopsy or resection specimen.

Statistical analysis

Baseline characteristics of included patients were analyzed with standard descriptive statistics, and compared between patients using thiopurines and patients using none of these drugs. Continuous variables were compared between these 2 groups using Student's t-test or Mann-Whitney U test, where appropriate. Categorical variables were analyzed using Pearson's chi-squared or Fisher's exact test, where appropriate.

Cox proportional hazard regression analysis was used to calculate hazard ratios (HR) and 95%

Confidence Intervals (CI) for the association between thiopurine use and the risk of NMSC in patients with IBD. In the multivariable model we adjusted for potential confounding of type of IBD, gender, age and duration of IBD. These procedures were repeated for different durations of thiopurine use to analyze whether the length of exposure influenced HRs. A p-value <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 15.0 for Windows.

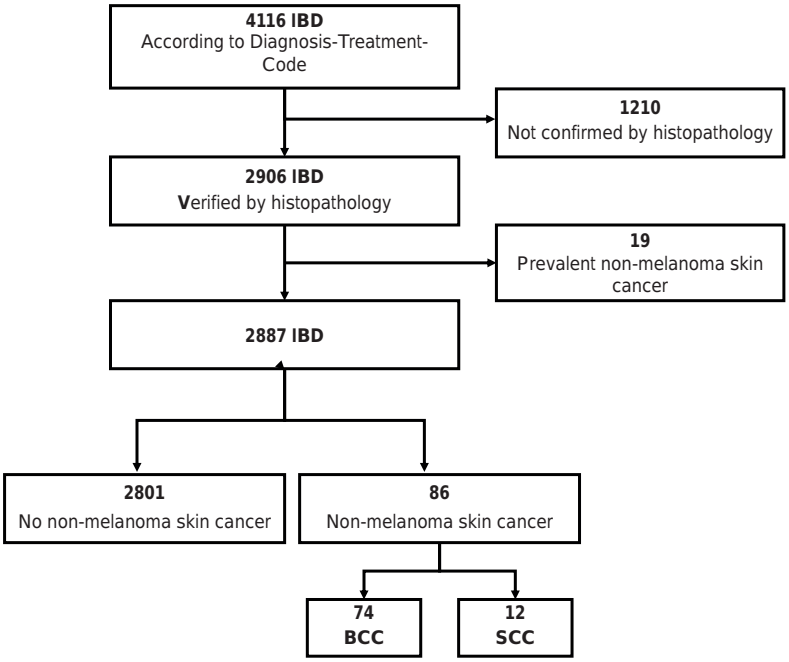
Ethical considerations

This study was carried out with the approval of and in attendance with the privacy and ethical guidelines of the privacy committee of PALGA and the research committee of the Agis Health Database.

RESULTS

We identified 4116 patients with a Diagnosis Treatment Combination for IBD. After linkage to the PALGA database and analysis of the pathology reports, 2906 patients with a verified IBD diagnosis were included. Application of the exclusion criteria excluded 19 patients, leaving 2887 IBD patients for further analysis (Figure 1).

Figure 1. Flowchart.



IBD, inflammatory bowel disease; BCC, basal cell carcinoma; SCC, squamous cell carcinoma

According to the predefined definition for thiopurine use, 819 patients (28%) were defined as thiopurine user. Baseline characteristics of each treatment group are presented in Table 1. No differences between treatment groups were found for gender, age, type of IBD and extent of IBD. Duration of IBD was significantly shorter in patients that had used thiopurines (34 months ± SD 45 versus 38 months ± SD 48 respectively, p=0.03).

Table 1. Baseline characteristics of thiopurine users and no medication users

	Non-user N= 2068	User N= 819	p-value
Type of IBD (%)			
Ulcerative colitis	893 (43)	344 (42)	0.84
Crohn's colitis	989 (48)	401 (49)	
IBD – unspecified	186 (9)	74 (9)	
Male (%)	851 (41)	356 (43)	0.26
Age, years (mean ± SD)	43 (17)	43 (17)	0.88
Duration IBD, months (mean ± SD)	38 (48)	34 (45)	0.03
Disease extension (%)			
> 50% of colon	610 (31)	220 (29)	0.07
< 50% of colon	775 (40)	339 (45)	
Unknown or no colonic involvement	563 (29)	200 (26)	

IBD, inflammatory bowel disease

Total duration of follow-up was 18,663 person years, wherein 86 patients (3%) developed NMSC. Of these, 74 (86%) developed BCC and 12 (14%) SCC. Among the 86 patients who developed NMSC, 24 (28%) patients had used thiopurines. The incidence rates are presented in Table 2.

Table 2. Incidence rates per 1000 person-years for non-melanoma skin cancer

	No. of patients	Person-years exposed	No. of events (% of patients)	Incidence rate (per 1000 person years)
Total population	2887	18,663	86 (3)	4.61
Non-users	2068	13,149	62 (3)	4.72
Thiopurine users	819	5,514	24 (3)	4.35

Thiopurine use of ≥50 mg per day during at least 6 months was not associated with an increased risk of NMSC in both the univariate analysis (unadjusted HR 0.94, 95% CI 0.58-1.50) and after adjustment for potential confounders (HR 0.85, 95% CI 0.51-1.41; Table 3). In the multivariate model increasing age and duration of IBD were identified as risk factors

for the development of NMSC (adjusted HR 1.06, 95% CI 1.04-1.08 and adjusted HR 1.00, 95% CI 1.00-1.01, respectively). A diagnosis of ulcerative colitis and female gender were associated with a decreased risk of NMSC (adjusted HR 0.62, 95% CI 0.40-0.98 and adjusted HR 0.47, 95% CI 0.30-0.74).

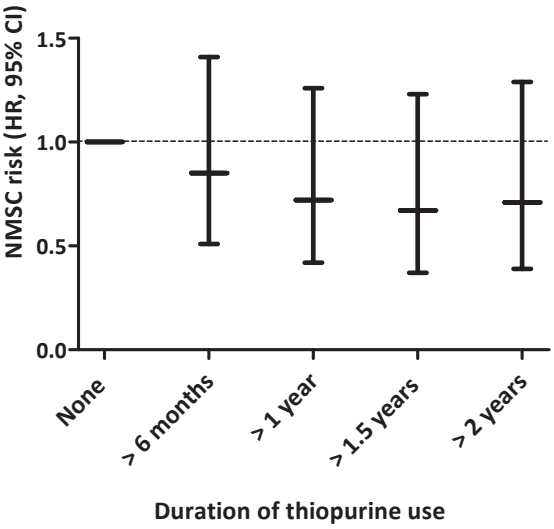
Table 3. Cox proportional hazard analysis of association between thiopurine use and development of non-melanoma skin cancer

	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
Thiopurine use	0.94 (0.58-1.50)	0.85 (0.51-1.41)
Presence of ulcerative colitis	1.08 (0.69-1.67)	0.62 (0.40-0.98)
Female gender	0.45 (0.29-0.69)	0.47 (0.30-0.74)
Increasing age (year)	1.06 (1.05-1.07)	1.06 (1.04-1.08)
Duration of IBD (month)	1.01 (1.00-1.01)	1.00 (1.00-1.01)

HR, hazard ratio; 95% CI, 95% confidence interval

In addition to the effect of thiopurine use for at least 6 months, we analyzed the association between thiopurine use for 1, 1.5 and 2 years and the development of NMSC. A longer duration of thiopurine use, however, was not found to influence NMSC risk. (Figure 2)

Figure 2. Hazard ratios (HR) and 95% confidence intervals (CI) for the development of non-melanoma skin cancer (NMSC) categorized in different durations of thiopurine use



DISCUSSION

This study investigated the association between thiopurine use and the development of NMSC in patients with IBD. Thiopurines did not increase the risk of NMSC in IBD patients, which was consistent over time. The risk for NMSC was associated with male gender, Crohn's disease (compared to ulcerative colitis), increasing age and increasing duration of IBD.

Our results were not in line with previous publications in organ transplantation studies and with the only competitive study in IBD.[3,5,7] Overall, these studies reported a four-fold increased risk for NMSC in thiopurine users, regardless of the treatment indication. Differences in outcomes may be related to differences in study design. Although Long et al. also used health claims data, they did not use pathology confirmed endpoints of NMSC, which can lead to overestimation of the incidence of their cases. The incidence rate for NMSC in IBD was 7.33 per 1000 person years of follow-up in their study, compared to only 4.61 per 1000 person years in the present study. Besides overestimation, regional differences should be taken into account.

Another issue is the definition of exposure. In order to emulate clinical practice, we defined IBD patients as a thiopurine user when they used at least 50 mg AZA or 6-MP per day during 6 months. This is strikingly different from the definitions used by Long and colleagues, as they already defined users if patients had received at least one thiopurine prescription during the 90 days before NMSC diagnosis. Persistent use was defined if patients met the first definition and received a prescription more than 365 days before the NMSC diagnosis. The exposure definition in controls was not mentioned, but we assume that definitions used were similar for cases and controls. By adapting the 6-month period of use in our study, we assured that patients had at least two prescriptions, whereas in the definition by Long et al. this might not be the case.

The present study is the first to report no increased risk of NMSC in IBD patients using thiopurines. It remains a matter of speculation whether this is influenced by publication bias of studies reporting a positive association between thiopurine use and NMSC risk.

In order to further elucidate the association between thiopurines and NMSC, a prospective randomized study stratified for geographical areas with predefined endpoints should be designed.

Our study has several strengths and some limitations. Using a large health claim database, we analyzed the effect of thiopurines provided to patients with IBD, in which the extent of thiopurine use was based on the number and dosages of thiopurines provided to each patient at the pharmacy. Studies that consult a drug database to study the association between medication use and a clinical endpoint mainly use prescription data of individual drugs. Since a large discrepancy may exist between the amount and dose of drugs prescribed by a physician and the truly used daily dose by individual patients, data based on the administrative claim of the pharmacist probably better represent the effective ingested dose

of medication. Moreover, linkage to our nationwide pathology database allowed us to verify the IBD diagnosis and to assess whether a patient had developed NMSC. However, we also faced a downside of exclusively using databases without consulting individual patient charts. Patients were only included in this study if the disease had been confirmed by histology. Although endoscopy and tissue sampling is widely performed in patients suspected of IBD, no histology was available from a significant number of patients for confirmation of the diagnosis. This might have introduced a bias, although this would lead to inclusion of more patients with a mild IBD phenotype which probably would not have used thiopurines. Moreover, since complete NMSC information was only available from 1999, we may have included some patients with a NMSC in history. This would, however, have lowered the number of cases and therefore also not have influenced the effect of thiopurines found in this study.

In conclusion, based on the results of this Dutch study, the risk of developing NMSC in IBD patients using thiopurines is less evident then previously reported. Therefore, it seems that thiopurines can safely be used in IBD patients with an indication for these drugs. To further elucidate the association between thiopurines and NMSC, a prospective randomized study stratified for geographic area with predefined end points should be designed.

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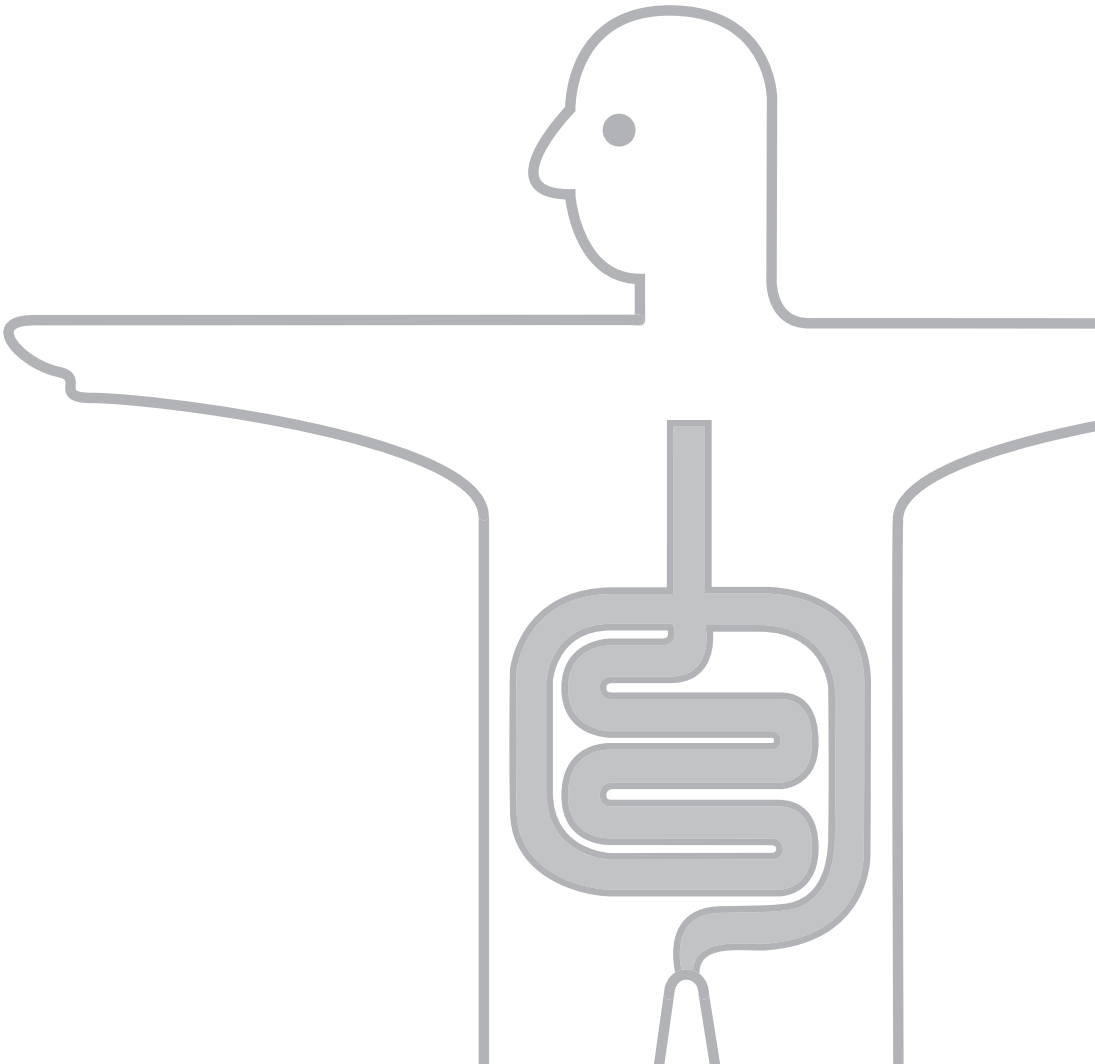
PART III

SUMMARY AND DISCUSSION



CHAPTER 10

Summary and discussion

Inflammatory bowel disease (IBD) comprises two main conditions: ulcerative colitis (UC) and Crohn's disease (CD). IBD is a serious health disorder characterized by periods of active intestinal inflammation alternated with periods of remission. Chronic inflammation renders the intestinal mucosa vulnerable to dysplastic changes, which may over time progress to colorectal cancer (CRC). Multiple factors have been implicated in the development of IBD and IBD-associated neoplasia. In this thesis, several novel issues regarding the development of IBD (PART I) as well as IBD-associated neoplasia (PART II) were addressed. The general aim of this thesis was to provide new tools for the prediction, treatment and risk stratification of inflammatory bowel disease and colitis-associated neoplasia. In this chapter we summarize our findings and give directions for future research.

PART I - NEW INSIGHTS INTO INFLAMMATORY BOWEL DISEASE

Numerous candidate genes have been associated with IBD, including genes involved in innate immunity and autophagy.[1] In mouse models of colitis, a novel gene, called nuclear farnesoid X receptor (FXR), was found to exert anti-inflammatory effects when stimulated by a synthetic FXR agonist.[2] Recent data have shown that patients with Crohn's colitis have an altered FXR expression.[3] In **Chapter 2** we investigated the effect of pharmacological activation of FXR with a synthetic FXR agonist in patients with Crohn's colitis. The main finding of this study was the feasibility to activate FXR by the FXR ligand chenodeoxycholic acid (CDCA) in these patients. Furthermore, we found that the increase of FGF19 after 7 days of CDCA ingestion was not 30% lower in CD patients. This may have resulted from the inability to recruit the estimated number of 12 CD patients. However, a post-sensitivity analysis revealed that inclusion of three more hypothetical CC patients assumed to have the lowest increase in plasma FGF19 level of the CC patient group, would not have changed our results. Although we cannot exclude a difference in FXR activation between CD patients and controls in this study, our findings strongly direct to an adequate FXR activation in Crohn's patients. Taken together, these results provide a rationale to further explore the potential therapeutic role of FXR agonists in this patient category. A randomized-controlled trial with CDCA (or its stronger variant, 6-ethyl-CDCA) against placebo may therefore be the next step to investigate the benefit of these ligands with regard to several clinical endpoints.

Various serological antibodies, including anti-neutrophil cytoplasmic antibodies and antimicrobial antibodies, have been detected in serum of patients with both Crohn's disease (CD) and ulcerative colitis (UC).[4] In **Chapter 3** we investigated whether these serological markers could predict the development of CD or UC in a large European cohort of apparently healthy individuals, which were followed over time. We analyzed serum of these cohorts, which was obtained at inclusion, for anti-neutrophil cytoplasmic antibodies (ANCAs), anti-Saccharomyces cerevisiae mannan antibodies (ASCA), antibodies against Escherichia coli

outer membrane porin C (OmpC) and against flagellin CBir1. We combined the antibody levels in two serological scores, one for CD and one for UC, and established cut-off values that had a high specificity for predicting IBD. A serological score of 1.46 as cut-off value for a CD diagnosis was associated with a specificity and positive predictive value of 90% and 64%, respectively. A serological score of 0.163 as cut-off value for a UC diagnosis was associated with a specificity and positive predictive value of 90% and 63%, respectively. We showed that a combination of serological markers is able to reliably predict the later occurrence of CD and UC in individuals from a low-risk population. This finding is of clinical importance for several reasons. It suggests that these serological markers can be used as a screening test to identify individuals at risk of developing IBD and therefore opens new avenues for pathogenetic studies in the 'prediagnostic IBD phase'. Moreover, the identification of individuals at risk of IBD offers a window of opportunity for early intervention. With this study, we were the first to illustrate the clinical applicability of a combination of serological markers for the prediction of IBD. However, although the proposed cut-off values for each serological score were associated with a high specificity and positive predictive value, sensitivity was relatively low, i.e. only 39 and 35% of incident CD and UC patients were seropositive for the combination of markers according to our proposed cut-off values. In the general population, the use of these combinations of markers may therefore be limited. However, since previous studies have reported increased antibody levels in healthy family members of CD and UC patients[5-7], it can be assumed that the proposed markers will perform better when tested in 'high-risk' family members. In our opinion, these serological tests should be used to identify individuals at an increased risk of IBD, i.e. seropositive healthy family members of IBD patients, to enable research in early intervention strategies. Moreover, the search for antigens that may be involved in the pathogenesis of IBD is rapidly emerging and the identification of new serological markers will definitely improve the role of serology as screening test for IBD in the future.

PART II: NEW INSIGHTS INTO COLITIS-ASSOCIATED NEOPLASIA

Dysplasia and cancer in IBD

In the second part of this thesis we explored several aspects of IBD-associated neoplasia. In **Chapter 4** we presented an overview of the endoscopic and pathological characteristics of colitis-associated neoplasia. In this chapter the importance of regular scheduled meetings between pathologists and endoscopists was emphasized, since identification and classification of colitis-associated dysplasia remains difficult for both endoscopists and pathologists.

In **Chapter 5** we investigated progression rates to advanced neoplasia (high-grade dysplasia (HGD) and CRC) in patients with flat low-grade dysplasia (LGD) and indefinite dysplasia (IND), before and after review of the histological diagnosis by a panel of three expert

gastrointestinal pathologists. After a thorough revision of the histological diagnosis, we found that the 5-year progression rate to advanced neoplasia was increased in patients with a confirmed LGD diagnosis, whereas it was decreased in patients with IND. With this study we clearly demonstrated that a diagnosis of LGD or IND in patients with IBD, and the associated prognosis with regard to progression to advanced neoplasia largely depends on the interpretation of the consulting pathologist.

Since in this study no clinical factors were found to be associated with the development of advanced neoplasia, we investigated in **Chapter 6** the value of a series of immunohistochemical markers in UC patients with flat LGD or IND for the prediction of neoplastic progression. We found that the co-expression of p53 and AMACR performed most optimal in this respect. More specific, when patients showed co-expression of p53 and AMACR, 6/7 patients (86%) developed advanced neoplasia, compared to 4/15 patients (27%) without p53/AMACR co-expression (p=0.02). In **Chapter 7** we studied the risk of advanced neoplasia, i.e. HGD or CRC, in IBD patients with adenomas. Adenomas in patients with IBD are a clinical challenge because their malignant potential has not clearly been delineated. We compared the risk of colorectal neoplasia in a large cohort of IBD patients with an adenoma with that in IBD subjects without adenomas as well as in non-IBD patients with adenomas. IBD patients with an adenoma were found to have the highest risk of developing advanced neoplasia followed by IBD patients without an adenoma and patients with an adenoma but without IBD.

The findings in Chapters 5,6 and 7 give us new tools to optimize the identification of IBD patients at increased risk of CRC. As discussed in chapter 5, the interobserver variability in the morphologic diagnosis of dysplasia is a serious problem and might cause a delay in the recognition of early dysplastic changes and thereby the prevention of progression to more advanced stages. However, although revision by a panel of expert pathologists may improve the precision of the diagnosis of dysplasia, previous studies have reported poor agreement even between expert gastrointestinal pathologists.[8,9] Moreover, revision of all histological slides by a panel of expert pathologists is laborious and time-consuming. More recent studies have therefore been focusing on the identification of biomarkers that aid in the differentiation between the different stages of dysplasia and may predict progression to advanced neoplasia. However, many of these studies investigated markers in different grades of dysplasia and at a single time point. No accurate conclusions can thus be made on the chronologic sequence of these markers. We studied various immunohistochemical markers throughout the IND/LGD-HGD-CRC sequence and found p53/AMACR co-expression to be useful as potential marker of neoplastic progression in patients with UC. In clinical practice, assessment of p53 and AMACR expression may be able to assist physicians in the identification of patients with dysplasia that have an increased risk of HGD or CRC. Another molecular marker that has been evaluated in this regard, and was demonstrated to be a marker of subsequent progression to (advanced) neoplasia, is DNA aneuploidy (abnormality

in cellular DNA content).[10,11] The identification of these and other new markers is of major importance for the early detection of individuals at risk of developing CRC. However, its incorporation into the management of IBD patients will need further studies, since advising patients to undergo a resection based on marker positivity only, are of course of great consequence. In chapter 7 adenomas themselves were identified as a factor associated with an increased risk of CRC in IBD patients. This finding underscores the importance of a stricter follow-up protocol in patients with adenomas and IBD compared to non-IBD patients with adenomas.

Future studies should focus on the possible implementation of these (predictive) factors in current surveillance strategies in order to enable a more personalized approach with regard to surveillance strategies and therapy. A prospective cohort study on established and new predictive factors for CRC in IBD currently being performed in our department is promising in this respect.

Thiopurines and IBD

Previous studies have reported a chemopreventive effect of 5-aminosalicylic acid (5-ASA) drugs in patients with longstanding colitis.[12] 5-ASA drugs are amongst the most commonly prescribed anti-inflammatory drugs in patients with Crohn's colitis and UC. Thiopurines, including azathioprine and 6-mercaptopurine, are systemic immunomodulators commonly used for maintenance of remission in both UC and CD. Since a possible chemopreventive effect had never been reported in patients using thiopurines, in **Chapter 9** we investigated the association between thiopurine use and the development of advanced neoplasia**.** Thiopurine-use was found to reduce the risk of developing advanced neoplasia with 90%, while 5-ASA therapy did not have a statistically significant chemoprotective effect. Although several plausible biological mechanisms have been reported for the chemopreventive effect of 5-ASA (as described in chapter 9), our and other population-based studies published during the last five years[13,14] rather convincingly demonstrate that 5-ASA drugs have no chemopreventive effect in patients with IBD. With regard to thiopurines, to date our study is the first that primarily investigated the chemopreventive effect of thiopurines and reported such an effect. Only one other study reported a similar effect for these drugs. In that study, thiopurines were part of an analysis for risk factors of IBD.[15] For clinical practice, the evidence for a chemopreventive effect of thiopurines is still limited and the use of these agents as primary chemoprophylactic medications should not widely be advocated. Moreover, thiopurines have cytotoxic and immunosuppressive properties and long-term thiopurine use has been reported to increase malignancy risk in organ transplant and IBD patients. In **Chapter 10** we evaluated the risk of developing non-melanoma skin cancer (NMSC) in IBD patients using thiopurines. In contrast to a study previously performed[16], we could not confirm this serious adverse effect of thiopurines. This may have resulted from a different

study design but also from geographic differences, as the other study was performed in the United States. We concluded that thiopurines can be used safely in IBD patients with an indication for these drugs. Nonetheless, just recently, a large study was published that reported a significantly increased risk of NMSC in IBD patients in France using thiopurines.[17] Likewise, a small study from South-Africa concluded that IBD patients on thiopurines are at an increased risk of NMSC .[18] Our current, general conclusion on the use of thiopurines in IBD is that patients with a clear indication for these drugs should have them prescribed and might benefit from a lower CRC risk, but patients should be informed that they are at an increased risk of extra-intestinal malignancies. Large prospective studies that stratify for disease and geographic characteristics are needed to ultimately conclude on the risks and benefits of thiopurine use in patients with IBD.

MAIN FINDINGS AND CONCLUSIONS

This thesis described new tools for the prediction, treatment and risk stratification of IBD and colitis-associated neoplasia. Our main findings were:

- A panel of serological markers is able to predict the development of CD and UC in individuals from a low-risk population
- Pharmacological activation of the farnesoid X receptor has a promise as therapeutic strategy in patients with Crohn's colitis
- A diagnosis of LGD or IND in patients with IBD largely depends on the interpretation of the consulting pathologist
- Patients with IBD and a diagnosis of flat LGD that is confirmed by a panel of expert gastro-intestinal pathologists are at a significant increased risk of developing HGD or CRC, while patients with a confirmed diagnosis of IND have a low risk of neoplastic progression
- A combination of p53 and AMACR expression can be used as marker of neoplastic progression in patients with UC
- Patients with IBD and an adenoma have an increased risk of developing advanced neoplasia compared to non-IBD patients with an adenoma and IBD patients without an adenoma
- Thiopurine use protects patients with IBD against the development of advanced neoplasia; the effect of 5-ASA appears to be less pronounced
- The risk of NMSC in Dutch patients with IBD using thiopurines seems not to be increased, but this association remains to be further investigated

The findings of this thesis can be used in future studies that aim to identify individuals at increased risk of IBD and colitis-associated CRC and may aid physicians in diagnosing and treating patients with IBD.

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CHAPTER 11

Summary in Dutch – Nederlandse samenvatting

Colitis ulcerosa (CU) en de ziekte van Crohn (ZvC) zijn chronische ontstekingsziekten van de darm ('inflammatory bowel diseases' ofwel 'IBD') waarvan het ziektebeloop gekenmerkt wordt door periodes van actieve ontsteking van de darm, afgewisseld door periodes van remissie. Chronische ontsteking maakt de darmmucosa kwetsbaar voor het ontstaan van dysplastische ofwel neoplastische veranderingen, die naar verloop van tijd kunnen leiden tot het ontstaan van colorectaal kanker. Multipele factoren zijn betrokken bij het ontstaan van IBD en de geassocieerde neoplasie van de darm. In dit proefschrift worden nieuwe inzichten beschreven in het ontstaan van IBD en ontstekingsgerelateerde neoplasie.

DEEL I: NIEUWE INZICHTEN IN INFLAMMATOIRE DARMZIEKTEN

Verschillende genen zijn geassocieerd met IBD, zoals genen betrokken bij de aangeboren immuniteit en genen betrokken bij autofagie. In muismodellen van ontsteking is ontdekt dat een nieuw gen, de 'farnesoid X receptor (FXR)' genoemd, ontstekingsremmende effecten teweegbrengt wanneer het gestimuleerd wordt door een synthetische FXR agonist. In eerder onderzoek is echter gebleken, dat in een ongestimuleerde toestand de expressie van dit gen in het ileum van patiënten met de ZvC lager is. In **Hoofdstuk 2** onderzochten wij daarom bij patiënten met de ZvC het effect van FXR-activatie door middel van een synthetische FXR agonist. De belangrijkste bevinding van dit onderzoek was, dat het mogelijk blijkt om FXR te activeren door middel van de FXR-agonist chenodeoxycholzuur. Deze bevinding suggereert dat FXR agonisten een potentiële therapeutische rol hebben binnen deze patiëntencategorie. In **Hoofdstuk 3** beschrijven wij de waarde van serum markers als voorspellers voor het ontstaan van IBD. In eerdere studies werd aangetoond dat verschillende antilichamen, waaronder anti-neutrofiele cytoplasmatische antilichamen en antimicrobiële antilichamen, frequent aanwezig zijn in het serum van patiënten die al langer bekend zijn met de diagnose ZvC of CU. In een groot Europees cohort onderzochten wij of deze serum markers het optreden van de ZvC en CU kunnen voorspellen. We concludeerden dat het mogelijk is om met een combinatie van serum markers het ontstaan van IBD te voorspellen bij individuen afkomstig uit een laagrisico populatie. Deze markers zouden daarom in de toekomst gebruikt kunnen worden als screeningstest om individuen met een verhoogd risico op IBD te identificeren. Dit maakt onderzoek in een vroege fase, voorafgaand aan de diagnose IBD, mogelijk en zou uiteindelijk kunnen leiden tot vroege interventie bij individuen met een verhoogd risico op het ontwikkelen van IBD.

DEEL II: NIEUWE INZICHTEN IN ONTSTEKINGSGERELATEERDE NEOPLASIE

In het tweede deel van dit proefschrift beschrijven wij nieuwe inzichten in de diagnose en preventie van ontstekingsgerelateerde neoplasie. In **Hoofdstuk 4** wordt een uiteenzetting gegeven over de endoscopische en histopathologische kenmerken van

ontstekingsgerelateerde neoplasie. In dit hoofdstuk benadrukken wij het belang van een regelmatig en structureel overleg tussen pathologen en MDL-artsen, aangezien het herkennen en classificeren van dysplasie een uitdaging blijft voor beide specialismen. Wij denken dat intensieve communicatie tussen beide disciplines noodzakelijk is om een gedegen beleid te kunnen opstellen voor individuele IBD patiënten. In **Hoofdstuk 5** onderzochten wij bij IBD patiënten de kans op het voortschrijden van laaggradige dysplasie (LGD) en indefinite (onzekere) dysplasie (IND) naar hooggradige dysplasie (HGD) en colorectaal kanker. Wij onderzochten dit op basis van de oorspronkelijke diagnose, maar ook na revisie van de histologische diagnose door een panel van drie expert pathologen. Na een zorgvuldige revisie van de histologische diagnose bleek dat de 5-jaars kans op progressie naar HGD en colorectaal kanker toenam bij patiënten met een bevestigde LGD diagnose, terwijl deze kans afnam bij patiënten met een bevestigde IND diagnose. Met deze studie toonden wij aan dat de diagnose LGD of IND, en daarmee de prognose met betrekking tot het ontstaan van HGD of colorectaal kanker, sterk afhankelijk is van de interpretatie door de patholoog die het weefsel beoordeelt. Deze bevindingen zijn voor specialisten belangrijk bij het nemen van klinische en therapeutische beslissingen bij patiënten met LGD of IND en benadrukt het belang van een zorgvuldige revisie door een expert patholoog. Aangezien in deze studie geen klinische factoren werden gevonden die geassocieerd zijn met het ontstaan van HGD of colorectaal kanker, onderzochten wij in **Hoofdstuk 6** bij patiënten met CU en LGD of IND de voorspellende waarde van een serie immunohistochemische markers voor het ontstaan van HGD of colorectaal kanker. Uit dit onderzoek kwam co-expressie van p53 en AMACR als beste voorspeller naar voren. Wij concludeerden dat de co-expressie van p53 en AMACR gebruikt zou kunnen worden als een marker voor neoplastische progressie bij patiënten met UC en een diagnose van LGD of IND. In de dagelijkse praktijk zou de bepaling van p53/AMACR co-expressie de specialist kunnen helpen bij het identificeren van patiënten met dysplasie en een verhoogd risico op HGD of CRC, wat zou kunnen leiden tot een meer op de persoon gespitste benadering met betrekking tot surveillance en behandeling.

In **Hoofdstuk 7** werd het risico van aanwezigheid van adenomen op ernstigere vormen van dysplasie of kanker onderzocht bij patiënten met IBD. Wij vergeleken het risico op colorectale neoplasie in een groot cohort IBD patiënten met een adenoom met twee andere cohorten, namelijk IBD patiënten zonder een adenoom, en patiënten met een adenoom maar zonder IBD. IBD patiënten met een adenoom bleken het hoogste risico te hebben op het ontwikkelen van HGD en colorectaal kanker vergeleken met beide andere cohorten. Op grond van deze bevindingen concludeerden wij dat adenomen bij een patiënt met IBD volledig verwijderd dienen te worden en dat deze patiënten door middel van surveillance nauwgezet vervolgd moeten worden.

Eerdere studies hebben een chemopreventief effect laten zien van aminosalicylaten (5-ASA) op het ontstaan van CRC bij patiënten met IBD. Aminosalicylaten zijn de meest voorgeschreven

ontstekingsremmende medicijnen bij patiënten met de ZvC of UC. Thiopurines, waaronder azathioprine en 6-mercaptopurine, zijn systemische immunomodulatoren die veel gebruikt worden als onderhoudstherapie bij patiënten met de ZvC of UC. Een chemopreventief effect is niet eerder beschreven bij patiënten die thiopurines gebruiken. In **Hoofdstuk 9** onderzochten we het verband tussen thiopurinegebruik en het ontwikkelen van HGD of CRC. Thiopurinegebruik bleek geassocieerd te zijn met een fors verlaagd risico op het ontstaan van HGD en colorectaal kanker, terwijl dit effect bij aminosalicylaten niet aanwezig was. Thiopurines hebben echter cytotoxische en immunosuppressieve eigenschappen, en in eerdere studies is bij zowel patiënten met een orgaantransplantatie als bij IBD patiënten een verband aangetoond tussen langdurig thiopurinegebruik en het ontstaan van maligniteiten. In **Hoofdstuk 10** hebben we daarom het risico op basaal- en plaveiselcelcarcinomen van de huid (ook wel 'non-melanoma skin cancer (NMSC)' genoemd) onderzocht bij IBD patiënten die thiopurines gebruiken. In tegenstelling tot eerdere studies, bleek thiopurinegebruik in ons onderzoek niet geassocieerd met een verhoogd risico op het ontwikkelen van NMSC. Wij concludeerden dat thiopurines veilig gebruikt kunnen worden bij IBD patiënten die een indicatie hebben voor het gebruik van deze medicatie. Een prospectieve studie met vooraf vastgestelde eindpunten, waarbij gestratificeerd wordt voor geografisch gebied, is echter nodig om uitsluitsel te kunnen geven over het daadwerkelijke verband tussen thiopurinegebruik en NMSC.

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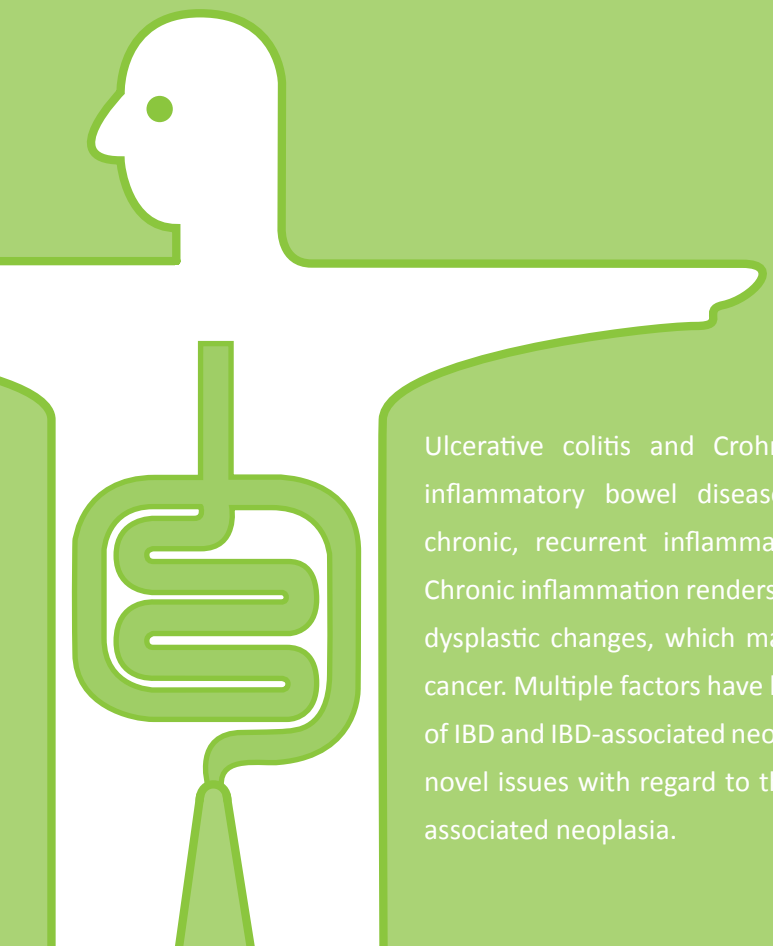
Curriculum Vitae

Fiona van Schaik werd geboren op 18 januari 1984 in Woerden. Na haar middelbare school periode (Calvijn College, Goes) begon zij in 2002 met de studie geneeskunde aan de Universiteit van Utrecht. In het 5e jaar van haar studie startte zij met wetenschappelijk onderzoek op de afdeling Maag-, Darm- en Leverziekten van het Diakonessenhuis Utrecht onder begeleiding van Dr. M.A.M.T. Verhagen en Dr. B. Oldenburg (UMC Utrecht). Dit resulteerde in haar eerste wetenschappelijke publicatie. In het laatste jaar van haar studie verrichte zij onderzoek op de afdeling Maag-, Darm- en Leverziekten onder begeleiding van Dr. B. Oldenburg naar de kans op hooggradige dysplasie en colorectaal kanker bij IBD patiënten met laaggradige dysplasie. Dit onderzoek werd na haar afstuderen in augustus 2008 voortgezet in een promotietraject onder begeleiding van Prof. dr. P.D. Siersema, Prof. dr. F.J.W. ten Kate en Dr. B. Oldenburg. In mei 2011 is zij begonnen met de opleiding tot Maag-, Darm- en Leverarts. Momenteel doorloopt zij de vooropleiding Interne Geneeskunde in het Universitair Medisch Centrum te Utrecht (opleider: Prof. Dr. M.M.E. Schneider).



Ulcerative colitis and Crohn's disease are two subtypes of inflammatory bowel disease (IBD). IBD is characterized by chronic, recurrent inflammation of the gastrointestinal tract. Chronic inflammation renders the intestinal mucosa vulnerable to dysplastic changes, which may over time progress to colorectal cancer. Multiple factors have been implicated in the development of IBD and IBD-associated neoplasia. This thesis addresses several novel issues with regard to the development of IBD and colitis-associated neoplasia.