Evaluation of confocal laser endomicroscopy for assessment and monitoring of therapeutic response in patients with inflammatory bowel disease

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THE THREE ORIGINAL PAPERS ARE

Study I
Karstensen JG, Săftoiu A, Brynskov J, Hendel J, Klausen P, Cârtână T, Riis LB and Vilmann P. Confocal Laser Endomicroscopy – a novel method for prediction of relapse in Crohn's Disease. Endoscopy. 2016 Apr;48(4):364-372 [1]

Study II
Karstensen JG, Săftoiu A, Brynskov J, Hendel J, Ciocalteu A, Klausen, P, Klausen TW, Riis LB and Vilmann P. Confocal laser endomicroscopy in ulcerative colitis: a longitudinal study of response to medical therapy. Gastrointest Endosc. 2016 Aug;84(2):279-286 [2]

Study III
Karstensen JG*, Klausen P*, Săftoiu A, Brynskov J, Hendel J, Cowland J, Riis LB and Vilmann P. Smad4 expression and evaluation of gut epithelial barrier function by E-cadherin and confocal laser endomicroscopy in patients with Crohn's disease
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Systematic Review
Karstensen JG, Klausen P, Săftoiu A and Vilmann P. Molecular confocal laser endomicroscopy: a novel technique for in vivo cellular characterization of gastrointestinal lesions, World J Gastroenterol. 2014 Jun 28;20(24):7794-7800 [3]

BACKGROUND

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two major chronic idiopathic inflammatory bowel diseases (IBD). While CD can affect any segment of the gastrointestinal tract, UC is considered to be limited to the colon. Both diseases typically have an early age of onset, and symptoms range from mild cases with bloody diarrhoea and abdominal pain to more severe cases, where intense immunosuppressive treatment and in some cases surgery is required. The incidence rates of CD and UC in Europe range from 0-11.5 and 2.4-31.5/100,000 person-years, respectively and the incidence seems to be rising [4, 5]. The cause of UC and CD remains an enigma. However, an inappropriate activation of the immune system in response to the commensal microbiota of the gut is considered to play an important role [6]. The layers of the intestinal mucosa serve as a barrier segregating the microbiota from the mucosa-associated immune system. Increased permeability of the mucosal barrier may lead to exposure of the mucosa-associated immune system to organisms from the gut microbiota and their products [7]. In this thesis, we wished primarily to explore the role of gastrointestinal endoscopy with confocal laser endomicroscopy (CLE) in the assessment of IBD and to study its ability to detect intestinal barrier loss and the consequences for the disease course. Furthermore, we wished also to correlate an impaired barrier function with possible molecular mechanisms in relation to the homeostasis of the epithelial lining.

Endoscopic assessment of inflammatory bowel disease

While earlier treatment goals in IBD comprised clinical response and normalisation of biochemical markers, recent studies evaluating the effect of novel biologic treatments have included endoscopic remission and more specifically mucosal healing as primary endpoints [8-10]. This is important since complete healing of the mucosal layer of the gut has been shown to predict steroid-free sustained clinical remission and reduced risk of surgery [10-12]. Furthermore, in an era of biological treatment identification of patients suitable for safe discontinuation of anti-tumour necrosis factor α therapy, such as infliximab, has moved to the frontline of clinical practice [13, 14]. In this context, achievement of mucosal healing constitutes an important target that may allow successful treatment cessation [15]. The significance of mucosal healing emphasises the importance of standardised and validated scoring systems for endoscopic assessment of CD and UC, respectively.

Crohn's disease

Ileocolonoscopy remains the mainstay to assess disease extent and severity of CD. The disease is characterised by segmental transmural inflammation with superficial aphthous lesions progressing to longitudinal snail-track like deep ulcers, cobblestone appearance, and bowel strictures [16]. Traditionally, the Crohn's Disease Endoscopic Index of Severity (CDEIS) has been applied to assess mucosal inflammation and is considered the gold standard (Table 1) [17]. However, CDEIS has its drawbacks as it focuses on the presence of ulcers, is very cumbersome to use, and has been shown to correlate poorly with clinical disease activity [18, 19]. To simplify endoscopic evaluation, Daperno et al proposed the Simply Endoscopic Score of Crohn's Disease (SES-CD), which grades inflammation by four variables in the terminal ileum and four predefined colorectal segments from 0-12 points [20]. The variables grade the size of ulcers, the extent of ulcerated surface, the extent of other lesions, and the presence of strictures. Finally, the scores are summed grading the severity and extent of CD from 0-60. Usually, a total score of 0-2 or 0-3 is accepted as endoscopic remission, but mucosal healing has not been defined [21-23]. Both the CDEIS and the SES-CD tend to underestimate ileal inflammation and overestimate colonic disease [21]. A third scoring system named the Rutgeerts score is used in a postsurgical setting only [24]. Deep remission constitutes a novel therapeutic target, which is defined as mucosal healing combined with absence of clinical symptoms [25]. Common for the above-mentioned scoring systems are that they are hampered by the fact that only macroscopic changes are recognised during endoscopy.

Table 1

Endoscopic activity indices for Crohn's disease

Index	Reference	Variables	Remarks
CDEIS	Mary et al, 1989	Deep and superficial ulcerations, ulcerated surface (cm), and affected surface (cm)	Considered gold standard, complicated to apply, overestimate colonic disease, and no definition of mucosal healing
SES-CD	Daperno et al, 2004	Size of ulcers, ulcerated surface (%), affected surface (%), and presence of narrowing	Easy to apply, one validation study only, overestimate colonic disease, and no definition of mucosal healing
Rutgeerts score	Rutgeerts et al, 1990	Number and distribution of aphthous lesions, mucosal inflammation, and nodules, ulcers, or narrowing	No use outside postoperative setting

CDEIS, Crohn's Disease Endoscopic Index of Severity; SES-CD, Simple Endoscopic Score for Crohn's Disease.

Ulcerative colitis

In contrast to CD, the endoscopic appearance in UC is characterised by uniform inflammation with continuous distribution extending proximally from the rectum and frequently with a well-defined demarcation line between inflamed and non-inflamed mucosa. The extension ranges from proctitis, which is limited to the rectum, to pancolitis involving the entire colon [16]. Several endoscopic indices have been proposed for UC, including the Baron score, the Powell-Tuck index, Sutherland index, the Rachmilewitz index, and the modified Baron score (Table 2) [26-32]. However, the most commonly used index is the endoscopic Mayo Clinic subscore, which is easy to apply in daily practice. It grades the severity of UC from 0-3, 0 being without signs of inflammation, 1 for mild disease (erythema, decreased vascular pattern, and mild friability), 2 moderate disease (marked erythema, absent vascular pattern, friability, and erosions), and 3 severe disease (spontaneous bleeding and ulceration) [32]. However, an inter-observer study has indicated that scoring consistency is suboptimal with kappa-values ranging from 0.53 in experts hands to 0.71 for non-experts [33]. Furthermore, Travis and colleagues found a concordance of only 27% for mild disease within a blinded expert panel [31]. Consequently, the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) was recently constructed and

validated to improve concordance. The UCEIS integrates the three variable; vascular pattern, bleeding, and erosions and ulcers. The initial study showed a concordance of 94% when evaluating the severity of UC [31]. Although the UCEIS seems to make up a more robust scale in evaluating disease activity in UC, there is currently no agreement on a threshold value for mucosal healing, and the results need to be reconfirmed in further studies. In addition to endoscopy, faecal calprotectin (FC) is an emerging non-invasive alternative to endoscopy. This surrogate marker accumulates in neutrophils at inflammatory sites in the gastrointestinal tract, which results in a release of calprotectin into faeces [34, 35]. The marker is significantly correlated with the endoscopic grading of inflammation and a normalised FC is a closely correlated with mucosal healing [21, 36]. Furthermore, an increased FC indicates a risk of relapse during clinical remission [37-39].

Table 2

Endoscopic activity indices for ulcerative colitis

Index	Reference	Variables	Remarks
Baron score	Baron et al, 1964	Vascular pattern and bleeding	Easy to apply, but no assessment of ulcers and no definition of mucosal healing
Powell-Tuck index (St Mark´s index)	Powell-Tuck et al, 1978	20-point scale with 2 additional points for endoscopic findings (bleeding)	Mainly based on clinical parameters
Mayo Clinic endoscopy subscore	Schroeder et al, 1987	Erythema, vascular pattern, friability, erosions, ulcers, and spontaneous bleeding	Easy to apply and commonly used, but no definition of mucosal healing
Sutherland index	Sutherland et al, 1987	Friability and bleeding	Easy to apply, but no definition of mucosal healing
Rachmilewitz index	Rachmilewitz et al, 1989	Granulation, vulnerability, vascular pattern, and mucosal damage	No definition of mucosal healing
Modified Baron score	Feagan et al, 2005	Vascular pattern, friability, bleeding, and ulcers	Easy to apply, but no definition of mucosal healing
UCEIS	Travis et al, 2012	Vascular pattern, bleeding, erosions and ulcers	Validated, easy to apply, but no definition of mucosal healing

UCEIS, Ulcerative Colitis Endoscopic Index of Severity.

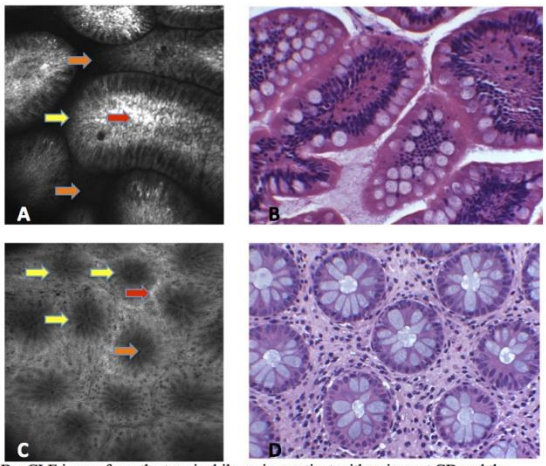
Histology

Histological assessment of biopsy specimens remains a key component of diagnosing and staging IBD patients. It is mandatory that the initial (and preferably repeat) ileocolonoscopies include multiple biopsies from the terminal ileum and each colonic segment. CD is microscopically characterised by focal crypt irregularity, patchy active chronic inflammation with lymphocytes and plasma cells, mucin preservation at active sites, and the variable presence of granulomas [40, 41]. In addition, irregular villous structure and aphthous ulcerations in the terminal ileum are useful diagnostic findings. UC is characterised by a widespread architectural distortion of the crypts, transmucosal inflammatory infiltrates with basal plasmacytosis, cryptitis or crypt abscesses, and mucin depletion [41].

Confocal laser endomicroscopy

All of the endoscopic indices mentioned above are limited by the fact that microscopic details and dynamic processes cannot be assessed with standard endoscopes. With the advent of the novel technology of CLE, real-time microscopy can now be performed in vivo during endoscopic examination. CLE uses a laser device, which is inserted or integrated into an endoscope, and illuminates an area of interest and at a precise depth. The systems, which are approved for clinical procedures, use blue light with a wavelength of 488 nm [42]. The tissue is made fluorescent with intravenously administered fluorescein, which has been approved as an off-label agent and is considered to be safe in humans [43]. Subsequently, reflection of the mucosa is then captured in a focused area and magnified up to 1000-fold providing a two-dimensional image of microscopic structures within the mucosa. In terms of magnification and resolution an image obtained using CLE is comparable with a histopathological prepared slide image (Figure 1).

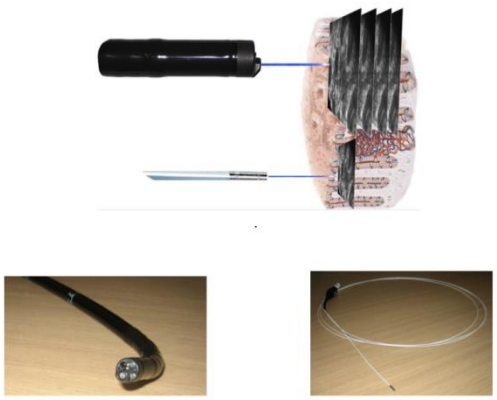
Figure 1 Confocal laser endomicroscopy images from mucosa appearing normal



A and B: eCLE image from the terminal ileum in a patient with quiescent CD and the corresponding histopathology (HE). There are no defects in the epithelial layer surrounding the intestinal villi (yellow arrow) and the lumen appear dark (orange arrows) as no fluorescein is leaking from the capillary vessels in the lamina propria (red arrow). C and D: eCLE image from colon in a patient with quiescent CD with corresponding histopathology (HE). The colonic crypts are normally distributed (yellow arrows) and the shape is round with a small dark opening as no fluorescein is leaking (orange arrow). The small vessels between the crypts appear bright (red arrow) as fluorescein is administered intravenously.

Currently, two CLE systems are clinically available (Figure 2). One is endoscope-based (eCLE), where a confocal microscope is integrated in the tip of a white-light colonoscope. The resolution is relatively high (1024 x 1024 pixels, with a lateral resolution of 0.7 um) and the depth adjustable from 0 to 250 µm. The second system is probe-based (pCLE) and has various miniprobes available that all can pass through the working channel of a standard endoscope. Here, the resolution is lower compared to the eCLE system and the depth is fixed at a predefined level, but a high frame rate makes movie acquisition possible (8 frames/sec). Only limited head-to-head data are available comparing the two systems, but in general the eCLE system has the advantage of a superior resolution and the variable depth-of-view, whereas the pCLE system can be used on demand and has applications for the common bile duct and pancreatic lesions as well [44-47].

Figure 2 Endoscope- and probe-based confocal laser endomicroscopy

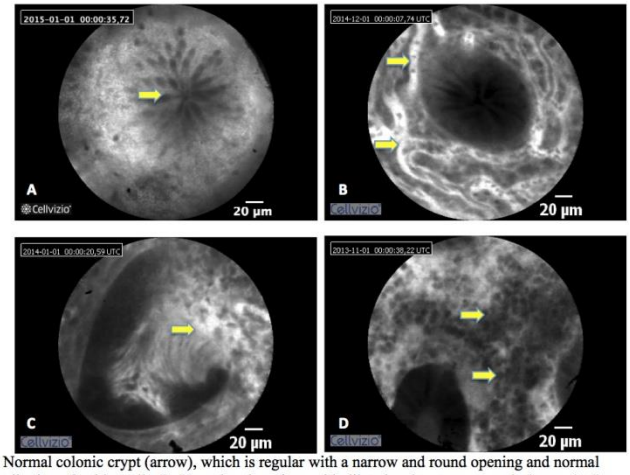


The principle behind CLE. A laser light with a defined wavelength excites the tissue, which creates an optical biopsy. Top and left: eCLE with possibility to produce images at different depths. Bottom and right: pCLE, which provides images with fixed depth, but with possibility to provide movie sequences.

Confocal laser endomicroscopy in inflammatory bowel disease

The use of CLE enables us to visualise structural mucosal changes (Figure 3), and Kiesslich et al described how vessel architecture, crypt shape and distribution, and the degree of cellular infiltrations correlate with activity of UC [48]. Furthermore, changes in crypt architecture and vascular alterations have been shown to assess the corresponding histopathological degree of UC more accurately than conventional white-light endoscopy (Table 3) [49]. Neumann et al introduced the Crohn's Disease Endomicroscopic Activity Score (CDEAS), which includes colonic parameters such as the number of goblet cells, crypt density, changes in crypt architecture, vascular changes, microerosions, and the presence of cellular infiltrates (Table 3) [50].

Figure 3 Structural changes in ulcerative colitis depicted by confocal laser endomicroscopy



A: Normal colonic crypt (arrow), which is regular with a narrow and round opening and normal distribution of goblet cells. B: Vascular alterations with dilated and tortuous vessels (arrows). C: Erosion of the epithelial layer of a colonic crypt (arrow). D: Intercryptal infiltrates, which probably consist of inflammatory cells (arrows). All images are obtained with pCLE equipment and are representative for patients with UC.

Table 3

Endomicroscopic indices for inflammatory bowel disease

Index	Reference	Disease	Variables	Remarks
Li Score	Li et al, 2010	UC	Crypt architecture in terms of shape, distorted openings, and decreased density	Assessment using eCLE, one validation study only, and no definition of mucosal healing
CDEAS	Neumann et al, 2012	CD	Colonic crypt number (increased or decreased), crypt distortion, microerosions, cellular infiltrate, vascularity, and number of goblet cells (increased or decreased)	Assessment using eCLE, one validation study only, and no definition of mucosal healing
Watson score	Kiesslich et al, 2012	CD and UC	Ileal fluorescein leakage and microerosions	Assessment using eCLE, one validation study only, mainly validated on UC patients, and no definition of mucosal healing
Buda score	Buda et al, 2013	UC	Quantitative estimation of pericrypt fluorescence and crypt diameter	Assessment using pCLE, one pilot study only, not validated, and no definition of mucosal healing

UC, Ulcerative Colitis; CD, Crohn's Disease; CDEAS, Crohn's Disease Endomicroscopic Activity Score; eCLE, endoscope-based Confocal Laser Endomicroscopy; pCLE, probe-based Confocal Laser Endomicroscopy.

In addition to in vivo assessment of structural changes in IBD, CLE has the unique ability to evaluate functional features, notably the integrity of the intestinal barrier (Figure 4). Using CLE it has been shown that the number of gaps between the cells in the epithelial lining of the terminal ileum is increased in UC and CD patients [51, 52]. As a result, fluorescein leaks from the vessels in lamina propria over the epithelial barrier to the intestinal lumen, which can be visualised with CLE as shown by Kiesslich et al [53]. Furthermore, in this pivotal study, which mainly included UC patients, is was demonstrated that a defective ileal barrier, as measured by fluorescein leakage and microerosions, could predict relapse in patients, who were otherwise in clinical and endoscopic remission (Table 3) [53]. Buda et al found similar results and showed that a composite score of the degree of colonic fluorescein leakage and crypt diameter was able to predict disease flare within a 12-month follow-up period (Table 3) [54]. While these studies described the integrity of the intestinal barrier in the lower part of the gastrointestinal tract, a recent study demonstrated that both CD and UC patients may also have a defective barrier function in the duodenum, which suggests that IBD is a systemic disease with subtle abnormalities extending to the entire gastrointestinal tract [55]. CLE is currently the only available endoscopic tool to visualise and evaluate whether the mucosal barrier function is intact. As a defect barrier function is found in a subgroup of IBD patients otherwise in endoscopic remission, we can possibly refine the definition of mucosal healing using CLE. On the other hand, it is currently unknown if the barrier function is normalised over time as a result of optimised medical treatment.

Figure 4 Confocal laser endomicroscopy images from patients with quiescent Crohn's disease

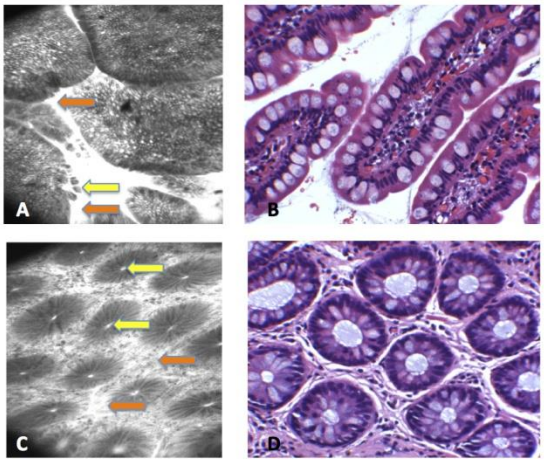


Figure 4 A and B: eCLE image from terminal ileum in a patient with quiescent CD with the corresponding histopathology (HE). The epithelial layer is defect due to a microerosion (yellow arrow) and consequently extensive fluorescein leakage is depicted with the intestinal lumen appearing bright (orange arrows). The histopathology appears normal. C and D: eCLE image from colon in a patient with quiescent CD with corresponding histopathology (HE). There is fluorescein leakage with the crypt openings (yellow arrows) and intestinal lumen (orange arrows) appearing bright. However, the colonic crypts are normally distributed and shaped and the histopathology appears normal.

Molecular parameters

By using CLE as an in vivo microscope, it seems possible to evaluate how immunological mediators correlate to an impaired intestinal barrier function. One of the interesting regulators in IBD is the immunosuppressive cytokine, transforming growth factor-β (TGF-β) (Figure 5). TGF-β is a key component in regulation of the immune system and the activity of TGF-β is decreased in CD [56]. Furthermore, TGF-β plays a prominent role in wound healing and mice whose gut epithelial cells are unable to respond to TGF-β develop gut inflammation [57, 58]. Consistent with this notion, it has also been demonstrated that bioactive TGF-β1 promotes epithelial restitution, an important element of mucosal healing, in a wounded epithelial cell monolayer model [59]. TGF-β signalling is mediated by activation of Small mothers against decapentaplegic (Smad) proteins [60]. When TGF-β binds to the receptor, it signals through phosphorylation and thus activation of a regulato-

ry Smad2/3 complex. Smad7 competes with binding of Smad2 and Smad3 to the TGF-β receptor 1 and thus prevents TGF-β signalling (Figure 5) [56]. Hence, an increased level of Smad7 has been shown to hamper appropriate immunosuppression, and a recent phase 2 trial evaluating a Smad7 neutralising antisense oligonucleotide (mongersen) showed convincing short-term effects in CD [61]. Another important and less studied factor in the Smad signalling circle is Smad4. Smad4 does not bind to the TGF-β receptor I itself, but complexes with the phosphorylated Smad2/3 to form a fully active transcription factor complex, which then translocates to the nuclei and regulates a variety of genes including repression of pro-inflammatory genes (Figure 5) [62]. Thus, a decreased level of Smad4 inhibits the immunosuppressive effect of TGF-β. A systematic investigation of Smad4 levels in CD patients has not yet been carried out. If disease severity and a defective barrier function as judged by CLE could be correlated with Smad4 expression, this would add to our understanding of the pathogenesis in IBD and ultimately serve as novel targets for medical treatment.

Figure 5 The classical TGF-β signalling pathway.

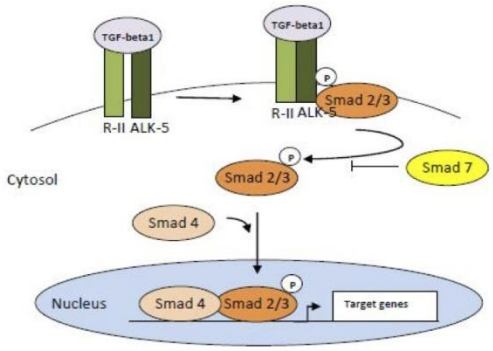


Figure 5 TGF-β functions by binding to its two receptors located on the cell surface, and this activates a signalling pathway inside the cell, which in the end leads to activation or repression of a variety of genes. The classical TGF-β pathway signals through phosphorylation and thus activation of a regulatory Smad2/3 complex. The phosphorylated Smad2/3 complex binds to cytoplasmatic Smad4, the only co-Smad expressed in mammals, and this multi-Smad complex acts as a transcription factor on several genes amongst others genes involved in immune-suppression. Smad7 inhibits the activation of the multi-Smad complex.

The intestinal epithelial cells (IEC's) are joined together by tight junctions and adherens junctions, which together form the apical junction complex [63]. As barrier dysfunction is closely linked to IBD, tight junction proteins such as claudins and factors that possibly alter expression of these have drawn considerable attention [64-66]. E-cadherin is a component of the adherens junctions, where it serves as the principle mediator of cell adhesions [67]. The locus (CDH1) that codes for E-cadherin is associated with UC [68]. In CD it has been shown that the architecture of E-cadherin is altered due to a single nucleotide polymorphism that causes cytoplasmatic mislocalisation of E-cadherin [67]. Moreover, it has previously been demonstrated that E-cadherin expression is decreased in colonic tissue from CD patients with active disease [69]. Consequently, it would be intriguing to demonstrate an altered E-cadherin expression in IBD patients with fluorescein leakage demonstrated with CLE, which would lead to a possible explanation of impaired ileal barrier function.

Aim of the study

The aim of this PhD-thesis was to investigate the clinical value of CLE for evaluation of IBD. We wished to assess whether CLE findings were reproducible, reversible, and if they could have clinical implications, which could benefit patient care. Both patients with CD and UC were included as well as control patients without IBD. Furthermore, to elucidate the molecular mechanisms of barrier function impairment, we hypothesised that Smad4 and E-cadherin were correlated with disease activity and the presence of a defect barrier function as measured by CLE. This was investigated in three studies with the following objectives:

Study I: Firstly, to describe and correlate CLE features with the macroscopic appearance of the mucosa and histopathology from patients with ileocolonic CD and to evaluate the inter- and intra-observer variations of the CLE parameters. Moreover, we wished to analyse if the CLE parameters could serve as predictors of relapse in patients with CD.
Study II: Secondly, to correlate colonic mucosal CLE features with disease activity and histopathology and evaluate the reproducibility of these findings in patients with UC. Further, we wished to examine how pCLE findings change after intensified medical treatment and correlate these with endoscopic and histopathological scores.
Study III: Thirdly, to examine mucosal Smad4 and E-cadherin protein expression levels in the terminal ileum of patients with CD and correlate these with disease activity, histopathology, and CLE features.

MATERIAL AND METHODS

Study design

Study I

This was a prospective observational study of patients with a known history of CD referred for ileocolonoscopy at Copenhagen University Hospital Herlev. To meet the inclusion criteria, patients had to have ileocecal involvement, be 18 years or more of age, no known allergy to fluorescein, no renal dysfunction, no pregnancy or breastfeeding, and they had to provide an informed written consent. Medical history, age, sex, and previous surgery were registered. Furthermore, during a follow-up period any medical treatment escalations or surgical interventions due to relapse in CD were registered. Treatment escalation was defined as instigation of medical treatment, intensification of current therapy, or initiation of concomitant medical treatment. The patients were compared to a control group consisting of asymptomatic patients referred for colonoscopy as part of an adenoma surveillance program.

During endoscopic procedures, the SES-CD was used to grade macroscopic appearance in the terminal ileum, right colon, transverse colon, left colon, and rectum [20]. The SES-CD comprises size of ulcers (0=none, 1=aphthous ulcers<0.5 cm, 2=large ulcers 0.5-2 cm, and 3=very large ulcers>2 cm), extent of ulcerated surface (0=none, 1<10%, 2=10-30%, and 3>30%), proportion affected with other lesions (0=unaffected segment, 1<50%, 2=50-75%, and 3>75%), and presence of a stenosis (0=none, 1=single and passable, 2=multiple and passable, and 3 impassable) [20]. The macroscopic assessment was performed by J.G.K and an SES-CD of two or lower was defined as endoscopic remission [23]. Corresponding biopsy specimens were formalin fixed and paraffin-embedded. From each biopsy 3 µm sections were cut and stained with haematoxylin and eosin. Afterwards, an expert pathologist (L.B.R.) carried out the histopathological assessment grading the severity of CD after a scale, which includes epithelial damage, architectural changes, infiltration of mononuclear cells in

lamina propria (LPMC's), infiltration of polymorphonuclear cells in the lamina propria, polymorphonuclear cells in the epithelium, presence of erosions and/or ulcers, and presence of granuloma according to procedures previously described [70].

Study II

This was a prospective longitudinal study of patients with a known history of UC, who were referred for either sigmoidoscopy or colonoscopy due to suspected relapse. In case of endoscopic signs of a flare, medical treatment was optimised, and the patients were subsequently offered a second endoscopic evaluation after 6-8 weeks. Patients referred with non-specific abdominal complaints and patients referred as part of an adenoma surveillance programme with no history of IBD or diarrhoea served as controls. All patients provided informed written consent. Patients with impaired renal function, pregnancy, breastfeeding, or allergy to fluorescein were excluded from the study. Medical history, sex, and age were registered.

In the patients referred for colonoscopy, macroscopic assessment was performed in the caecum, splenic flexure, left colon 40 cm from the anal verge, and in the rectum. The endoscopic Mayo Clinic subscore was used to grade the severity of UC from 0-3 [32]. An endoscopic Mayo Clinic subscore of 0-1 was defined as endoscopic inactive disease. At the same colonic sites biopsy specimens were obtained, which were formalin fixed and paraffin-embedded. From each biopsy 3 µm sections were cut and stained with haematoxylin and eosin using standard procedures. The histopathological assessment was performed in a blinded manner by L.B.R. according to the Geboes index, which incorporates structural changes, chronic inflammation, lamina propria neutrophils, neutrophils in the epithelium, crypt destruction, and erosions or ulcers into a 12-point-scale [71].

Study III

The study was an investigator-blinded study aimed at correlating the expression of Smad4 and E-cadherin with severity of CD and intestinal barrier dysfunction in the terminal ileum as judged by CLE. Patients from study I in whom the terminal ileum had been intubated and tissue acquisition was sufficient to perform supplementary staining were included. The biopsy specimens corresponding to the sites, where CLE was performed, were formalin-fixed and paraffin-embedded. The specimens were stained for Smad4 and E-cadherin protein expression according to standard immunohistochemistry procedures at the Department of Pathology, Copenhagen University Hospital Herlev. Afterwards, L.B.R. scored the slides in a blinded manner rating Smad4 expression on a four point scale, where 3=high, 2=moderate, 1=low, and 0=absent. Smad4 was assessed both at the luminal and basal site of the villous structure. The membranous E-cadherin expression was rated according to the same scale, where 3=high, 2=moderate, 1=low, and 0=absent.

Luminal and basal Smad4 expression as well as E-cadherin in CD were compared to controls and, within the patient group, to severity of CD (macroscopic and histopathological) and the presence of endomicroscopic changes such as ileal fluorescein leakage or microerosions. Furthermore, Smad4 and E-cadherin were correlated to the risk of relapse within the follow-up period.

Confocal laser endomicroscopy and procedures

Study I/III

Ileocolonoscopy was performed using the eCLE colonoscope (Pentax EC-3837CILK, Tokyo, Japan). When the terminal ileum was intubated, the mucosa was rinsed with water if necessary. Subsequently, 5 mL of fluorescein sodium 100 mg/mL was injected intravenously and CLE imaging initiated by gently angling the tip of the colonoscope towards the mucosa. In some cases light suction was used to maintain a stable position. After sufficient images had been obtained, the same area of the mucosa was biopsied. Afterwards predefined colonic sites were examined in a similar manner.

A.S. assessed the CLE images after all patient details (disease status, age, and sex) had been blinded. The ileal images were scored according to the Watson score, which includes fluorescein leakage and microerosions [53]. Fluorescein leakage was defined as fluorescein signal in the intestinal lumen with intensity equal to or brighter than the epithelium, or fluorescein plumps leaking over the epithelial layer into the lumen. Microerosions were defined as the lamina propria being exposed to the lumen with multiple cells being shed per site. The scale has three points where 0 is when no endomicroscopic changes are present, 1 is when fluorescein leakage is found, and 2 is when both fluorescein leakage and microerosions are present [53]. The colonic images were also assessed in a blinded manner by A.S. The presence of fluorescein leakage (free fluorescein in the crypt openings or intestinal lumen), microerosions (multiple cells being shed per site with the lamina propria being exposed), vascular alterations (tortuous or dilated vessels), and inflammatory infiltrates (bright or dark clusters of cells between in crypts) were registered. Furthermore, the cryptal architecture was assessed in terms of crypt tortuosity, distortion of the crypt openings, and number per field of view. Number of crypts per field of view were analysed as a mean for the colonic sites, the rest of the parameters were positive if present at one or more examined colonic sites. An inter- and intra-observer study was constructed by selecting two ileal and four colonic images representing all patients enrolled in the study. The images with the highest quality were chosen and the selection was performed by J.G.K. For the inter-observer study these images were assessed in a blinded manner by three investigators all having experience with interpretation of CLE (T.C., A.S., and J.G.K.). For the intra-observer study, the selected images were randomised and re-assessed after 48 hours by J.G.K.

Study II

Colonoscopy or sigmoidoscopy was supplemented with pCLE and biopsy specimens in four predefined colonic sites. Before CLE imaging was performed, the mucosa was properly rinsed with water and 5 mL of fluorescein sodium 100mg/mL was injected intravenously. After CLE, biopsy specimens were obtained from the same spot. In the patients, who were examined twice, the second procedure was performed after the same protocol.

The CLE movies were assessed in a blinded manner by A.S. The CLE parameters included were colonic fluorescein leakage, microerosions, vascular alterations, crypt tortuosity, distorted crypt openings, intercryptal inflammatory infiltrates, and crypt density. The parameters were defined according to Table 4. For the inter-observer study, all movies were assessed by A.S and A.C. After randomisation of the images, A.C. repeated her evaluation of the movies for the intra-observer study.

Table 4

Definitions used for CLE parameters in UC

CLE parameter	Definition
Fluorescein leakage	Fluorescein visible in the crypt lumen, which appears brighter than the surrounding epithelium or free luminal fluorescein
Microerosions	Multiple cells are shed uncovering the lamina propria
Crypt tortuosity	The crypts are tortuous or merged
Crypt openings	The crypt openings are distorted or dilated
Crypt density	One or less crypts per field of view
Vascular alterations	Vessels appear tortuous and dilated
Inflammatory infiltrates	Inter-cryptal collection of dark or bright cells

CLE, Confocal Laser Endomicroscopy; UC, Ulcerative Colitis.

Statistic

For all statistics, IBM SPSS Statistics 22 (SPSS, Chicago, IL, USA) was used. A two-sided p-value of less than 0.05 was considered significant.

Study I

Mean numbers and range representing the minimum and maximum value were presented. Parametric t test was used to determine differences between continuous variables, previously tested for normal distribution using Shapiro-Wilk, while the Jonckheere-Terpstra test was used to determine differences for continuous variables, which were not normally distributed. Jonckheere-Terpstra test is used when there is a priori ordering of the compared groups (active CD – inactive CD – controls [72, 73]. Chi-Square or when appropriate Fishers exact test was used for comparisons between categorical variables. For correlations, Spearman's rank was used. For the inter- and intra-observer study, κ-statistics were applied and interpreted according to Landis and Koch [74]. Kaplan Maier plot and log-rank were used for follow-up data.

Study II

The median and interquartile range (IQR) were presented. Mann-Whitney U test was used to compare continuous variables, while Chi-Square was used for categorical variables. Wilcoxon was used for paired independent variables. As three or four sites were examined in each individual, the observations were not considered independent. Consequently, a generalised estimated equation was applied when data including more than one site per individual were analysed [75, 76]. Generalised estimated equations are adjusting for the likelihood of clustering of observations within the same individual and is often used when more than one observation is conducted per individual [77, 78]. Kappa statistics were used in the inter- and intra-observer study with interpretation according to Landis and Koch, while Spearman's rank was used for other correlations [74].

Study III

The median and interquartile range were presented. Due to limited sample size, non-parametric statistics were used. Mann-Whitney U test was used to test for differences between continuous variables. When three groups with a priori ordering were compared (active CD – inactive CD – controls), the Jonckheere-Terpstra test was applied [72, 73]. Chi-Square was used to compare categorical variables. Spearman's rank was used for correlations.

Ethical considerations

All studies were approved by the Regional Ethics Committee (H-1-2012-089-94) and the Danish Data Protection Agency (2007-58-0015 / HEH.750.89-32). The studies were registered under clinicaltrial.gov (NCT01738529 and NCT01684514).

RESULTS

Study I

Participants

Fifty patients were enrolled in the study of whom 39 were CD patients and 11 adenoma surveillance patients. Within the group of CD patients, 20 were in endoscopic remission. Patient characteristics are summarised in Appendix A1. The groups were equally distributed in terms of sex, but controls were, as expected, significantly older (p=0.012). The majority of CD patients received medical treatment, and 23% had a surgically altered anatomy due to bowel resections.

Procedures

The ileal intubation rate was 92.0% and did not differ significantly for patients and controls (92.3% vs. 90.9%, p=0.64). Similarly, proper CLE imaging in the terminal ileum was comparable for the two groups (87% in CD vs. 91% in controls, p=1.0). Colorectal CLE imaging was performed in all patients. The mean time of the procedure was 46 and 47 minutes for CD patients and controls, respectively and did not differ significantly (p=0.69). Besides transient yellowing of the skin due to administration of fluorescein, no adverse advents were registered in relation to the procedures.

Confocal laser endomicroscopy

In the terminal ileum, fluorescein leakage and microerosions were more common in patients with active CD compared to inactive CD and controls (p=0.005 and p=0.006, respectively) (Figure 6). Consequently, the Watson score was significantly increased in CD patients compared to controls (Table 5).

Figure 6 Ileal fluorescein leakage and microerosions in terminal ileum

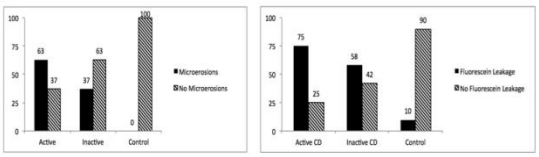


Figure 6: Ileal fluorescein leakage and microerosions were significantly correlated to CD (p=0.005 and p=0.006, respectively)

Table 5

Assessments of the terminal ileum according to endoscopic activity

	Active CD	Quiescent CD	Controls	p-value
No (%)	16 (36)	19 (42)	10 (22)	
Mean Watson score (range)	2.4 (1-3)	2.00 (1-3)	1.1 (1-2)	0.001
Mean macroscopic score (range)	6.63 (0-12)	0.13 (0-2)	0	<0.001
Mean histopathological score (range)	3.92 (0-9)	0.37 (0-5)	0	<0.001
Histopathological characterisation				
Normal (%)	3 (25)	17 (90)	10 (100)	
Chronic inactive inflammation (%)	2 (17)	1 (5)	0	
Chronic active inflammation (%)	7 (58)	1 (5)	0	<0.001

CD, Crohn's Disease.

In the colon, fluorescein leakage and vascular alterations were significantly more common in patients with active CD compared to patients with inactive CD and controls (p=0.043 and p=0.034, respectively) (Figure 7). No significant correlation was established for the presence of microerosions, crypts tortuosity, distortion of crypt openings, presence of inflammatory infiltrates, and the number of crypts per field of view (Table 6).

Table 6

Assessments of colon according to endoscopic activity

	Active CD	Quiescent CD	Controls	p-value
No (%)	19 (38)	20 (30)	11 (22)	
Microerosions (%)	7 (37)	5 (25)	1 (9)	0.24
Mean number of crypts per field of view (range)	10,9 (5-17)	13.0 (5-19)	11,5 (8-14)	0.27
Tortuosity of crypts (%)	14 (74)	17 (85)	8 (73)	0.62
Tortuosity of crypt openings (%)	17 (90)	17 (85)	8 (73)	0.48
Inflammatory infiltrates (%)	10 (53)	4 (20)	7 (64)	0.84
Mean macroscopic score of the colon (range)	1.00 (0-16)	0.13 (0-2)	0	<0.001
Mean histopathological score (range)	1.71 (0-11)	0.47 (0-4)	0	<0.001

Figure 7 Colonic fluorescein leakage and microerosions in terminal ileum

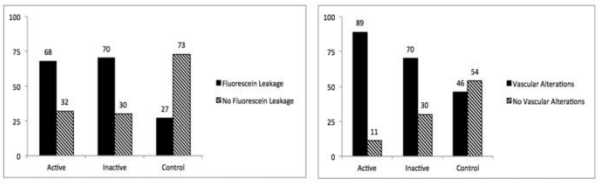
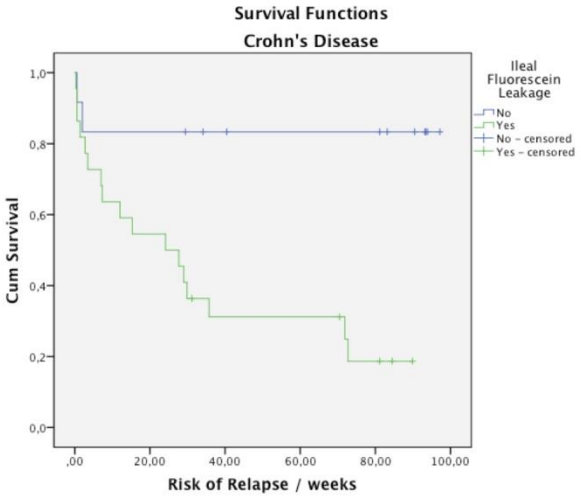


Figure 7: Colonic fluorescein leakage and vascular alterations were significantly more common in patients with active CD compared to patients with inactive CD and controls (p=0.043 and p=0.034, respectively).

Follow-up

No patients were lost to follow-up during a mean period of 68 weeks (range 29-98). Within the 39 CD patients included in the trial, 22 events were registered, which included medical treatment escalations in 19 patients and surgical interventions in three patients (Appendix A2). Ileal fluorescein leakage (p=0.003) and microerosions (p=0.017) as well as colonic inflammatory infiltrates (p=0.018) were all significant risk factors of relapse in CD (Figure 8). Accordingly, an increased Watson score of two or three was also significantly correlated to the risk of relapse (p=0.024). Except for the presence of colonic inflammatory infiltrates, none of the colonic CLE parameters were significant risk factors of relapse. A completely intact intestinal barrier with no fluorescein leakage in the terminal ileum or the colonic sites was found in eight CD patients. None of these patients relapsed during the follow-up period; hence, an intact ileocolic barrier function is a significant predictor of sustained remission without need for treatment escalation (p=0.002).

Figure 8 Risk factors for relapse in Crohn's disease



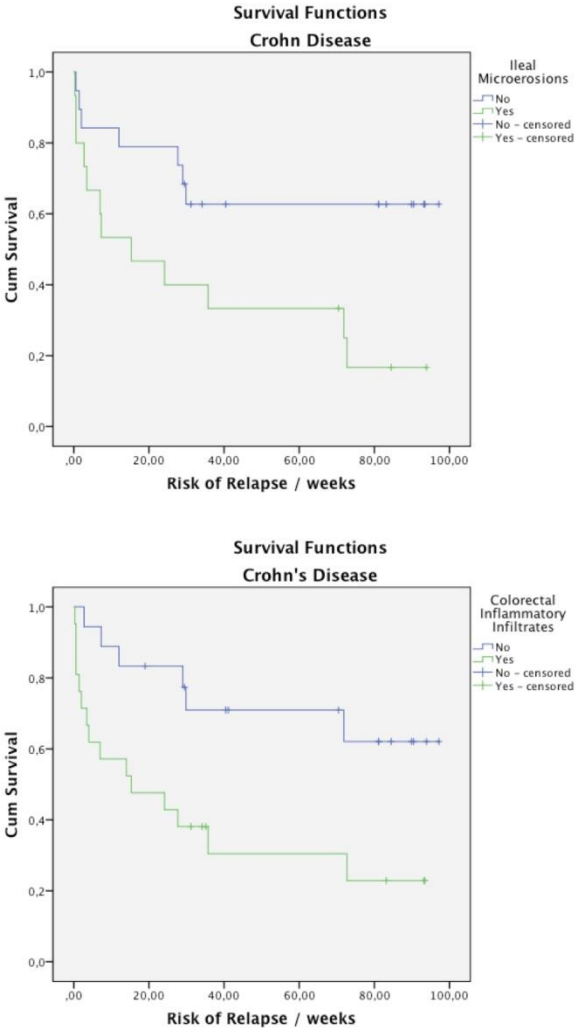


Figure 8: Kaplan-Meier plots presenting the risk of relapse for CD patients with ileal fluorescein leakage, ileal microerosions, and colonic inflammatory infiltrates.

A subgroup of CD patients was in endoscopic remission at the time of the ileocolonoscopy (n=20). During follow-up, eight of these patients relapsed, as they required medical treatment escalation. Ileal fluorescein (p=0.007) and microerosions (p=0.025) as well as colonic presence of inflammatory infiltrates (p=0.009) were significant risk factors of relapse in this group of CD patients (Figure 9). None of the patients in remission with a Watson score of one relapsed during the follow-up period and consequently, an increased Watson score of two or three was a significant risk factor of relapse (p=0.007).

Figure 9 Risk factors in Crohn's disease in endoscopic remission

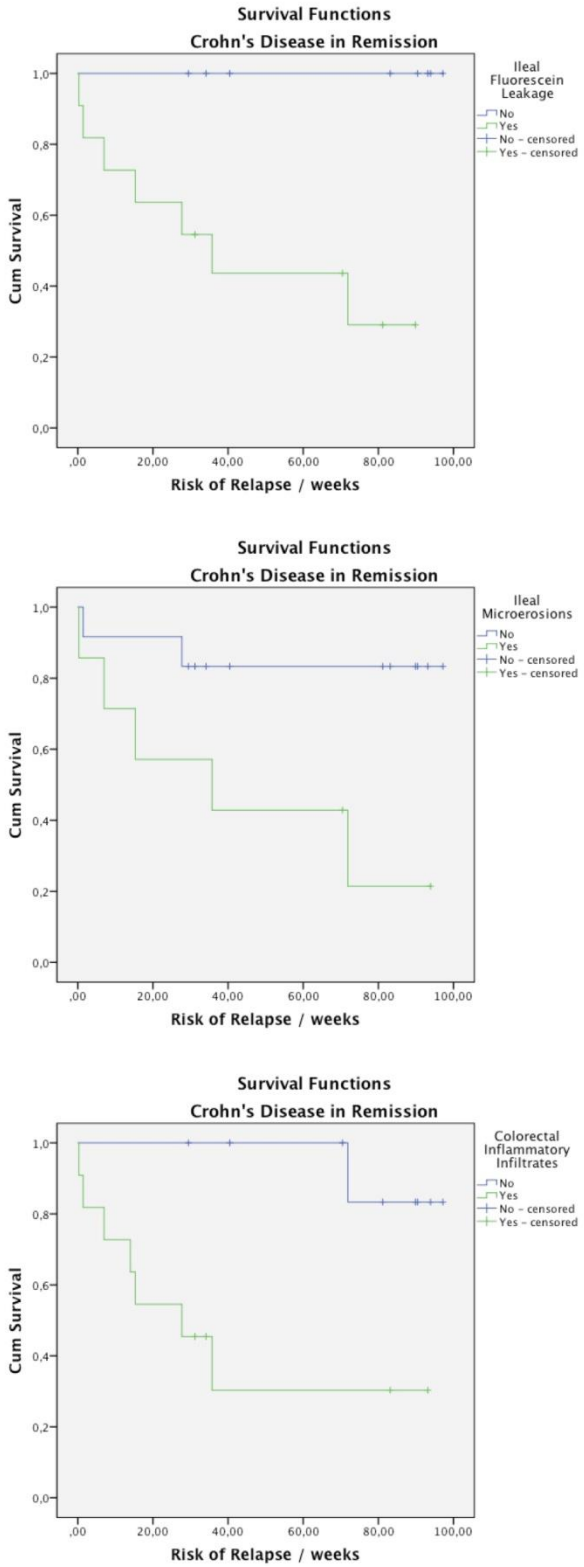


Figure 9: Kaplan-Meier plots presenting the risk of relapse for CD patients in endoscopic remission with ileal fluorescein leakage, ileal microerosions, and colorectal inflammatory infiltrates.

Intra- and inter-observer study

Three investigators assessed 88 ileal and 200 colonic images and one investigator assessed the images twice. As the image quality differed from one procedure to another, two ileal and four colo-

nic images from each patient were included in the image selection. The results are summarised in Table 7.

Table 7

Inter- and intra-observer study of ileocolonic CLE parameters

	Inter-observer variability	Intra-observer variability
Terminal ileum		
Fluorescein Leakage (κ)	0.86	0.91
Microerosions (κ)	0.78	0.89
Colon		
Fluorescein Leakage (κ)	0.82	0.91
Microerosions (κ)	0.46	0.76
Vascular alterations (κ)	0.64	0.70
Tortuosity of crypts (κ)	0.78	0.69
Distortions of crypt openings (κ)	0.66	0.76
Inflammatory infiltrates (κ)	0.44	0.76
Crypts per field of view (κ)	0.84	0.94

CLE, Confocal Laser Endomicroscopy.

Study II

Participants

Twenty-nine patients were included in the study (22 were UC patients referred with a suspicion of a flare and seven controls). A total of 80 sites were examined in UC patients (55 inactive) and 25 in the controls. Two of the UC patients did not have endoscopic signs of flare and only presented with inactive sites. Of the seven controls, two were subsequently diagnosed with irritable bowel syndrome (IBS), while the remaining five were asymptomatic patients referred as part of an adenoma surveillance program. After initiation or intensification of medical treatment (n=18), 14 patients with UC agreed to undergo a re-examination, which was performed after a median period of 7.0 weeks (IQR=6-8). The groups were equally distributed in terms of sex, but the controls were significantly older than the UC patients. During the study, a total of 27 colonoscopies and 16 sigmoidoscopies were performed. Besides transient yellowing of the skin due to administration of fluorescein, no adverse advents were registered in relation to the procedures. The patient characteristics are summarised in Appendix A3.

Confocal laser endomicroscopy

The CLE features were registered per site according to the definition of active or inactive UC, respectively. The frequencies of the CLE parameters are presented in Table 8. The frequencies of sites with fluorescein leakage, microerosions, tortuosity of the crypts, distortion of the crypts openings, presence of inflammatory infiltrates, and decreased crypt density were significantly higher in active UC compared to inactive UC and controls. However, no significant association was found for vascular alterations. When the CLE features were correlated with the Geboes index a significant correlation was found for fluorescein leakage rs=0.42 (p<0.001), microerosions rs=0.54

Table 8

Assessment of colon according to endoscopic activity

	Active UC	Inactive UC	Controls	p-value
Sites (%)	22 (21)	58 (55)	25 (24)	
Fluorescein Leakage (%)	18 (82)	13 (22)	9 (36)	<0.001
Microerosions (%)	11 (50)	7 (12)	0 (0)	<0.001
Vascular alterations (%)	16 (73)	37 (64)	12 (48)	0.46
Tortuosity of crypts (%)	18 (82)	15 (26)	1 (4)	0.001
Distorted crypt openings (%)	16 (73)	20 (35)	1 (4)	0.001
Inflammatory infiltrates (%)	22 (100)	38 (66)	14 (56)	<0.001
Decreased crypt density (%)	17 (77)	23 (40)	0 (0)	<0.001

UC, Ulcerative Colitis. Endoscopic inactive ulcerative colitis was defined as endoscopic Mayo Clinic subscore 0-1 and endoscopic active ulcerative colitis as endoscopic Mayo Clinic subscore 2-3.

(p<0.001), crypt tortuosity rs=0.66 (p<0.001), distortion of crypt opening rs=0.63 (p<0.001), inflammatory infiltrates rs=0.28 (p=0.004), and decreased crypt density rs=0.54 (p<0.001), while no significant correlation was found for vascular alterations rs=0.13 (p=0.20). The endoscopic Mayo Clinic subscore was significantly correlated with the Geboes index rs=0.84 (p<0.001).

Longitudinal study

All 14 patients, who accepted to undergo a re-examination, had received intensified medical treatment (Table 9). Subsequently, the endoscopic Mayo Clinic subscore (median 1.0 vs. 0.0, p=0.009) as well as the Geboes index (median 2.0 vs. 0.5, p=0.007) both decreased significantly. A significant correlation was found between changes in Geboes index and changes in CLE crypt features, that is crypt tortuosity rs=0.35 (p=0.016), distortion of crypt openings rs=0.30 (p=0.045), and decreased crypt density rs=0.33 (p=0.026), while only decreased crypt density was correlated to changes in endoscopic Mayo Clinic subscore rs=0.35 (p=0.016). No significant correlation could be established for fluorescein leakage, microerosions, vascular alterations, or inflammatory infiltrates.

Table 9

Medical treatment after escalation

No=14*	Before	After
Infliximab (%)	1 (7)	5 (36)
Vedolizumab (%)	0	1 (7)
Azathioprine (%)	4 (29)	5 (36)
Local corticosteroids (%)	2 (14)	2 (14)
Oral corticosteroids (%)	0	3 (21)
Local 5-ASA (%)	3 (21)	5 (36)
Oral 5-ASA (%)	13 (93)	14 (100)

*Some patients received multiple drugs. 5-ASA, 5-Aminosalicylic Acid.

Inter- and intra-observer study

All movies were assessed post-procedural by two blinded investigators before any editing was performed. The inter-observer analysis showed a substantial agreement for fluorescein leakage, microerosions, crypt tortuosity, distorted crypt openings, and decreased crypt density (Table 10). A moderate agreement was found for vascular alterations, while the agreement was fair for inflammatory infiltrates. The intra-observer variability was almost perfect for fluorescein leakage, microerosions, crypt tortuosity, distorted crypt openings, and decreased crypt density, while substantial for vascular alterations and inflammatory infiltrates.

Table 10

Inter- and intra-observer study

	Inter-observer variability	Intra-observer variability
Fluorescein Leakage (κ)	0.75	0.91
Microerosions (κ)	0.63	0.81
Vascular alterations (κ)	0.47	0.78
Tortuosity of crypts (κ)	0.63	0.90
Distortions of crypt openings (κ)	0.70	0.92
Inflammatory infiltrates (κ)	0.38	0.73
Decreased crypt density (κ)	0.79	0.92

CLE, Confocal Laser Endomicroscopy.

Study III

Participants

The study group consisted of 38 patients. Of those, 28 were CD patients (18 in endoscopic remission) and ten served as controls (adenoma surveillance programme). The groups were equally distributed in terms of sex, but the controls were, as expected, older than the CD patients (p=0.010). The characteristics of the CD patients are found in Appendix A4.

Smad4 protein expression in Crohn's disease

Smad4 expression in the ileal biopsy specimens was estimated both on the villi (luminal) and the crypts (basal) (Figure 10). The luminal expression was significantly decreased in patients with active CD compared to patients with inactive CD and controls (Table 11). Furthermore, luminal Smad4 expression levels were significantly correlated to the histopathological scores (rs=0.51, p<0.001), but not to ileal fluorescein leakage or microerosions. There was also a trend towards decreased basal Smad4 expression in active CD compared to inactive CD and controls (Table 11). No correlation could be established with histopathological or macroscopic severity of CD. During a mean follow-up of 71 weeks, 15 (54%) CD patients relapsed after a mean period of 18 weeks. While a defective barrier function in the terminal ileum as judged by CLE could predict relapse in CD

CD, Crohn's Disease. IQR, Interquartile Range.

Table 11

Ileal Smad4 expression in Crohn's disease

	Active CD	Quiescent CD	Controls	p-value
No (%)	10 (27)	18 (49)	10 (24)	
Age (IQR)	36 (31-61)	41 (24-53)	71 (51-76)	0.010
Sex / female (%)	5 (50)	10 (56)	3 (30)	0.42
Median luminal Smad4 (IQR)	0.5 (0-1.0)	1.0 (0-2.0)	3.0 (2.8-3.0)	<0.001
Median basal Smad4 (IQR)	2.0 (1.8-3.0)	2.0 (1.0-3.0)	3.0 (2.8-3.0)	0.075

patients, no correlation was found for luminal or basal Smad4 expression in neither risk of relapse nor time to relapse.

Figure 10 Ileal Smad4 expression

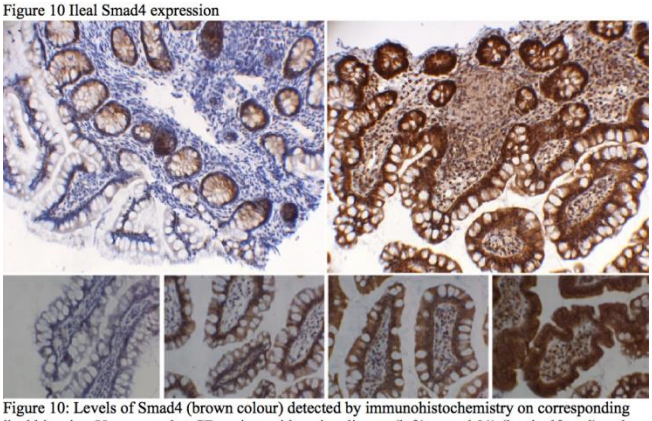


Figure 10: Levels of Smad4 (brown colour) detected by immunohistochemistry on corresponding ileal biopsies. Upper panel: a CD patient with active disease (left) scored 0/1 (luminal/basal) and a control (right) scored 3/3 (luminal/basal) are shown (original magnification x 200). Lower panel: Luminal Smad4 scores 0, 1, 2, and 3 are shown (original magnification x 400).

Seven patients with relapse and two in remission were re-examined with conventional ileocolonscopy and biopsies during the follow-up period. The luminal Smad4 expression in terminal ileum increased in the two patients who remained in remission, while luminal Smad4 decreased or remained under three in those who relapsed. Thus, Smad4 levels changes with time and data indicates that a high Smad4 level might protect patients against relapse.

E-cadherin expression in Crohn's disease

The luminal expression of E-cadherin in the biopsy specimens was not significantly associated with CD (p=0.26) (Figure 11). However, E-cadherin was significantly correlated with luminal Smad4 rs=0.36 (p=0.001) in the complete cohort as well as in CD patients rs=0.40 (p=0.04). We did not find any correlation to basal Smad4 or ileal fluorescein leakage and microerosions.

Figure 11 Ileal E-cadherin expression

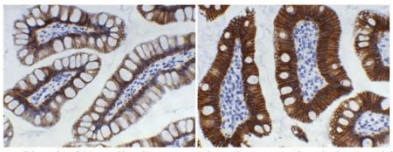


Figure 11: Examples of luminal E-cadherin expression (brown colour) detected by immunohistochemistry on corresponding ileal biopsies. Membranous scores 2 (left) and 3 (right) are shown (original magnification x 400).

DISCUSSION

The novel technology of CLE has enabled endoscopists to visualise not only structural microscopic changes, but also a novel set of functional features in the intestinal mucosa in vivo. This means that the epithelial barrier function can be assessed, and we have demonstrated that surrogate markers for a defect barrier function such as ileal fluorescein leakage and microerosions are more common in CD compared with controls. Moreover, they appear to be strongly associated with the risk of relapse during follow-up. Functional and structural CLE alterations in the colonic mucosa of UC patients were also found to be significantly associated with the severity of the disease, and augmented crypt tortuosity, distortion of crypt openings as well as decreased crypt density are reversible after medical treatment. Several molecular markers have been associated with the barrier function in IBD. In our study, we found that ileal Smad4, an activator of the anti-inflammatory cytokine TGF-β, was significantly reduced in active CD compared to controls, but did not correlate with fluorescein leakage and microerosions.

The Intestinal barrier

Fluorescein leakage in the terminal ileum

During recent years, endoscopic imaging has improved significantly with the introduction of high definition technology and virtual chromoendoscopy. These methods have impacted the quality of the endoscopic procedures and to some degree enabled in vivo diagnosis of distinct lesions and disorders [79-81]. Nevertheless, none of these have the ability to recognise microscopic conditions such as the intestinal barrier function. The barrier function is dependent on a proper homeostasis of the epithelial lining, which consists of IECs connected by tight junctions and adherens junction [82, 83]. As a physiological mechanism, the IEC's are continuously shed from the epithelial layer, while new cells migrate from the basal layers in crypts. Animal studies have shown that the discontinuity created when IECs are shed, resolves rapidly within 10 minutes [84]. In the meantime, a gap in the lining is present, which in humans can only be visualised with CLE [51, 52]. Studies have shown that cell shedding is abnormal in IBD with an increased number of gaps in the epithelial layer, which possibly gives rise to increased permeability [52]. Fluorescein leakage is considered a surrogate marker for increased permeability, and in our study we found a significant association between ileal fluorescein leakage and CD compared to asymptomatic controls. Additionally, both ileal fluorescein leakage and microerosions were significant risk factors of relapse not only in the complete group of CD patients, but also in a interesting subgroup of patients in remission without any macroscopic signs of active disease. This confirms and extends the results of Kiesslich et al, who exclusively included IBD patients in clinical and endoscopic remission [53, 85, 86]. While the definition of a flare in this study was a Crohn's disease activity index >150 or a clinical activity index >3, we defined a flare as the need for treatment escalation. Hence, a proper validation using clinical indices, and at best endoscopy to confirm relapse, would have strengthened our study considerably. Patients in clinical and endoscopic remission constitute the most interesting group of IBD patients to examine with CLE as it enables us to detect subtle changes unrecognisable with other endoscopic modalities or histopathology. However, whether these findings can be incorporated into an algorithm for safe discontinuation of for instance biological therapy, needs to be answered in an interventional study including a homogenous group of patients in terms of previous disease extension, phenotype, and medical treatment.

Validation of endomicroscopic features

Our CLE study in CD patients as well as the study by Kiesslich et al relied solely on subjective assessments of the barrier function [53]. An objective estimation would be preferable and increase the usefulness of CLE for less experienced endoscopists. A semi-quantitative measurement was proposed by Liu et al to analyse the gap density in the terminal ileum of IBD patients. Disruptions in the epithelial lining of representative pCLE images from endoscopically normal mucosa were counted manually, and presented as gaps/1000 cells [52]. A significantly higher gap density was found in IBD compared to controls, and the increased gap density was correlated with increased risk of hospitalisation and surgery [52, 87]. However, in a clinical setting it is unrealistic to manually count the number of gaps per 1000 cells in the epithelial barrier, and if the parameter should be useful an automatic estimation is required. In a recent pilot study, colonic CLE parameters such as microvascular fluorescein leakage and cryptal architecture were automatically quantified using a software program (Image J) [88]. Thus, an objective estimation of CLE parameters is feasible, which in addition allows a quantification of parameters; hence, the barrier function is probably more accurately described by a continuous variable such as most physiological parameters.

Distribution of intestinal barrier impairments

Interestingly, in the study presented by Kiesslich et al the majority of participants were UC patients and CLE was performed in the terminal ileum [53]. Thus, the intestinal barrier function was assessed at a location, which is usually considered healthy in UC patients. However, a defective barrier with a Watson score of 1 or 2 was found in 15 of 58 IBD patients included. Consequently, the classical definition of UC with inflammation limited to the colon may have to be redefined. Consistent with this notion, a recent study of CLE in the duodenum of UC patients found a significantly higher number of gaps and Watson score compared to controls. Obviously, no macroscopic or histopathological manifestations of UC were found [55]. Further studies are needed to confirm these results, and it would be interesting to investigate whether a defective barrier in the upper gastrointestinal tract is correlated with disease severity, and if so, reversible with optimised medical treatment.

Is fluorescein leakage a surrogate marker for impaired barrier function?

Extensive research has been conducted to exploit intestinal barrier function and permeability. The latter is defined as the functional features of the intestinal barrier at given sites as measured by analysing flux rates across the intestinal wall as a whole or across wall components of defined molecules that are largely inert during the process and that can adequately be measured in these settings [89]. An impaired intestinal permeability is defined as being non-transiently changed compared to normal permeabil-

ity leading to a loss of intestinal homeostasis, functional impairments, and disease [89]. Numerous disorders have been linked to an impaired permeability including celiac disease, IBS, and IBD [90]. I our studies we interpreted ileal fluorescein leakage as a sign of increased intestinal permeability. However, no human studies have tested this marker against a gold standard. Several studies in IBD, where inert probes have been administered orally, have found increased small bowel permeability compared to controls. Impaired permeability has also been shown to correlate with risk of relapse in otherwise quiescent CD [91-94]. One of the most frequently used test is the lactulose/mannitol test, where these substances are measured in the urine after ingestion [95]. Mannitol, a relatively small monosaccharide, is believed to pass the intestinal barrier in the small bowel via a transcellular route, while lactulose, a somewhat larger disaccharide, is believed to be transported along a paracellular route. Subsequently, a ratio between these two substances is widely accepted as a surrogate marker of impaired small bowel permeability [96, 97]. However, test conditions may impact this test [98, 99]. On the other hand, as lactulose/mannitol is considered gold standard, it would be interesting to compare the methods for estimation of small bowel permeability even though fluorescein is detected by efflux and lactulose/mannitol by influx over the intestinal barrier [95]. FC is another marker used to discriminate organic diseases such as IBD from functional disorders, and a low value is considered a valuable surrogate marker of mucosal healing in IBD [100, 101]. To our knowledge, no data are available on the correlation between FC and CLE features and further studies are warranted to clarify this.

Confocal laser endomicroscopy in the colon

In our study in UC, we found significant associations between functional as well as structural CLE parameters and the severity of the disease according to endoscopic Mayo Clinic subscore. Furthermore, we found a significant correlation between the parameters and the histopathological Geboes index, which suggests that CLE parameters can supplement an endoscopic evaluation with potentially important information on histological alterations and functional defects of the bowel wall. Currently, the macroscopic appearance of UC is the reference for determination of remission as mucosal healing predicts sustained clinical remission and decreased risk of surgery [102, 103]. However, data on the prognostic value of histological remission are limited [104]. In a study with UC patients in clinical and endoscopic remission (endoscopic Mayo Clinic subscore=0), microscopic active inflammation (Geboes index>3.0) was surprisingly found in 40% of patients. Additionally, active inflammation and basal plasmacytosis were significant risk factors of relapse [105]. Moreover, in a study comparing the histological and macroscopic scores it was demonstrated that an increased microscopic score was an independent risk factor of relapse and the correlation with macroscopic score was poor when patients had mild disease. In other words, subtle inflammatory signs are likely to be missed by standard endoscopy [106]. The authors of this paper suggest that histological remission should be used in addition to endoscopy in clinical trials and that histopathological remission should be the ultimate outcome in UC. Histological features in UC that predict relapse are basal plasmacytosis, increased transmucosal cellularity, high number of neutrophils and eosinophils, crypt abscesses, mucin depletion and damage of the surface epithelium [41]. With CLE we are not able to detect all of these features, but relapse in quiescent UC has been correlated with increased crypt diameter, crypt disruption, and erosions [54, 107]. Although definitions differ from one study to another, we found that structural parameters such as crypt tortuosity, distortion of crypt openings, and decreased crypt density were significantly correlated to progress in histopathological score after medical treatment escalation.

Fluorescein leakage is a functional parameter, which is not visible with conventional histopathology, has been incorporated into the CLE indices used for prediction of relapse in UC [54, 107]. It is plausible that the absence of fluorescein leakage is a sign of complete restoration of the intestinal barrier and could serve as an ultimate treatment goal. Nevertheless, the studies from Buda et al and Li et al included a limited number of patients and further validation is needed to confirm the results. Although our follow-up study was in CD, we found that the presence of colonic inflammatory infiltrates was strongly associated with the risk of relapse, but no correlation was found with the functional parameter of colonic fluorescein leakage. Moreover, during a seven-week follow-up period in UC patients after treatment escalation, there was no significant correlation between the presence of colonic fluorescein leakage and progress in Geboes index. Consequently, we have not been able to show that colonic fluorescein leakage is a risk factor of relapse in IBD in the short term. Hence, in the longitudinal study, the follow-up was limited and it would have been interesting to perform a longer follow-up. Second, we performed CLE examinations at multiple sites both in Study I and II. The kinetics of intravenously administered fluorescein in the gastrointestinal tract is not fully understood. Thus, the interpretation of fluorescein leakage in the colon, as measured in our studies, is difficult to interpret and may be hindered by the differences in time intervals from injection to sampling [108]. Third, the assessment was performed qualitatively, and fluorescein leakage was not quantified. However, even in trials implementing a quantitative assessment of colonic fluorescein leakage, there are conflicting data as to whether the parameter is an appropriate surrogate marker of an impaired colonic barrier [49, 54].

Endoscope-based vs. probe-based confocal laser endomicroscopy

Despite obvious differences in terms of design and imaging, pCLE and eCLE are often understood as one modality. Only a limited number of studies have made head-to-head comparisons. Gorespe et al compared the two platforms for detection of dysplasia on fresh endoscopic mucosa resections from Barrett's oesophagus and found an insignificant trend towards higher accuracy of eCLE compared to pCLE [44]. A recent trial that randomised patients with Barrett's oesophagus to either in vivo pCLE or eCLE found that the eCLE system provided significantly better image quality. However, the procedural time was significantly longer for eCLE compared to pCLE (18.13 vs. 16.78, p=0.027) [47]. In the colon, procedural time was also longer using eCLE (39.89 vs. 32.48 minutes, p<0.001) and the rate of incomplete colonoscopies seemed higher with eCLE compared to pCLE, although there was no significant difference [47]. There is no literature available comparing the systems in IBD and we have made no head-to-head comparisons, but in our experience the results of the studies mentioned in the above are similar to our impression. The image resolution is higher with eCLE, but handling of the endoscope is somewhat more challenging. Furthermore, the eCLE endoscope is fragile and the system may seem less reliable. On the other hand, the mini-probes for the pCLE system are limited to 20 procedures.

Reproducibility of endomicroscopic findings in inflammatory bowel disease

When a novel modality such as CLE is introduced, it is crucial that the reproducibility of the findings is thoroughly investigated. We have presented the inter- and intra-observer variability for eCLE findings in CD as well as pCLE findings in UC. In general, the colonic parameters, which were almost similar in both studies, had an acceptable inter- and intra-observer agreement. The κ-values were comparable between Study I, where eCLE was used for confocal imaging and Study II, where pCLE was applied. However, in both studies inflammatory infiltrates had an unacceptable reproducibility, which conflicts with previous studies in CD patients, where the inter- and intra-observer agreement was κ=0.830 and κ=0.607, respectively [50]. Moreover, we find inflammatory infiltrates in our control patients in both Study I and II. We reviewed a selection of CLE images together with an experienced pathologist and even with patient information at hand, we could not explain the cellular infiltrates found in controls. Possibly, it is endothelial cells or tangentially imaged crypts. In conclusion, we are currently not able to differentiate inter-cryptal cells from each other, thus the definition of the parameter needs further refinement before it can be applied. Our inter- and intra-observer variability on the ileal CLE parameters were almost perfect or substantial. This confirms the results from Kiesslich et al, who found a Cohen's κ coefficient of 0.87 [53]. Hence, probably due to the vertical view of the villous structures in the terminal ileum, the CLE parameters are highly reproducible.

Molecular marker expression to support endomicroscopic findings

As CLE provides new information on subtle inflammatory changes and the integrity of the barrier function of the intestinal wall, it is of interest to link molecular parameters to these novel findings. The luminal expression of Smad4 was significantly downregulated in CD compared to controls and it was correlated with the severity of the disease. However, it correlated neither to endomicroscopic signs of an impaired barrier function nor to risk of relapse. Despite the fact that Smad4 expression was downregulated in the same cells that form the epithelial lining and are involved in maintaining an appropriate barrier function, no correlation could be established. A defective barrier as measured by CLE predicts relapse, but we do not know in detail how it affects the LPMC's. However, in our study patients with a defective barrier function relapsed thought-out the follow-up period; hence, some were in remission several months before they experienced a flare. It seems likely that patients with a defect barrier function develop influx of commensal bacteria from the intestinal lumen, which over time will instigate an aberrant immunoreaction if the immune system is dysfunctional. However, to thoroughly investigate the relationship between TGF-β signalling and intestinal barrier impairments, it would be interesting to study both the complete Smad signalling circle (Smad7, Smad2, Smad3, and TGF-β receptor I and II) as well as factors, which regulate Smad4 translation [109].

One of the most promising drugs in the pipeline for treatment of IBD is an oral Smad7 antisense oligonucleotide, [61, 110]. Smad7 has mainly been investigated in the LPMC's, where it is upregulated, and to lesser extend in the IEC's [56, 111]. We aimed our study at another factor in the TGF-β signalling pathway, Smad4, which was significantly downregulated in IEC's from CD patients compared to controls. Thus, our findings suggest an immune defect in CD patients and indicate that Smad4 could serve as a novel target for medical treatment. However, our findings need to be confirmed in isolated IEC's from CD patients. Furthermore, Smad4 is regulated by a variety of micro-RiboNucleic Acids (miRNA), which consist of single-stranded RNA that is able to inhibit translation of their target messenger-RNA [109, 112]. It would be interesting to examine whether an inhibition of these miRNAs would lead to proper TGF-β signalling, thereby providing a novel treatment principle in IBD. In addition to the correlation between Smad4 expression in CD and asymptomatic controls, we conducted a pilot study analysing Smad4 in ileal biopsy specimens from eight IBS patients. Surprisingly, we found that expression of both luminal and basal Smad4 was significantly decreased in the IBS patients compared to controls ($p<0.001$ and $p=0.001$, respectively). While several studies have demonstrated an increased number of immune cells and a low-grade activation of T-cells in IBS, an aberrant TGF-β signalling has not been reported in IBS [113-118]. As the number of patients in our study is limited, these results need to be interpreted with great caution. Nonetheless, it would be interesting if our finding could be confirmed in a larger setting and additionally underlines the importance of a comparison with a control group consisting of healthy asymptomatic individuals.

E-cadherin is a key component of the adherens junctions and it has been demonstrated that E-cadherin is downregulated in colonic IECs located next to inflammatory sites [69]. However, colonic tight junctions architecture is only affected in CD patients with active disease [69, 119]. We did not find a correlation between ileal E-cadherin expression and the severity of CD. Further, no correlation to intestinal barrier impairments was shown. The conflicting data in relation to E-cadherin expression is likely to be caused by measurements from different locations; hence, the data in our study originate in the terminal ileum, while the data mentioned above are from colonic tissue. In future studies with CLE in the small bowel, it would be interesting to correlate our finding with the expression of claudins, and in addition, proinflammatory cytokines such as interleukin 9, which in animal studies have been shown to alter expression of tight junction proteins [64, 65].

Strengths and limitations

Study limitations not mentioned above include the fact that endoscopic images obtained by the eCLE system are standard white light and not high definition. Further, as the distal five cm of the CLE colonoscope is rigid, manoeuvrability is compromised compared to standard endoscopes, which may increase the procedural time and challenge intubation of the terminal ileum. No update is expected for this platform in relation to neither the endoscopic imaging nor the integrated CLE technology, and future CLE studies will probably rely on pCLE equipment. Besides a limited resolution of the CLE images, the use of pCLE mini-probes carries continuous costs. As long as no reimbursement is available, research groups and clinicians will depend on external funding as the cost per pCLE procedure is around 400 Euros and increasing. However, both in the US and France, reimbursement is now granted for CLE in Barrett's oesophagus and indeterminate strictures of the common bile duct.

Study III was a spin-off from study I, thus it would be wrong to categorise it as prospective. Nonetheless, the findings should be easily reproducible in future prospective trials. Another limitation is that a single individual assessed endoscopy and histopathology. The endoscopic grading of the severity of CD and UC in Study I and II, respectively, was solely based on the endoscopist (J.G.K). A blinded assessment would have been preferable. Despite having an experienced pathologist to assess the histopathological speci-

mens in a blinded manner, it would have strengthened the reliability if a second pathologist had assessed the slides as well.

When we introduced CLE in Denmark and initiated the research leading to this thesis, only one of the authors was familiar with CLE (A.S.). However, during the introduction period, he was monitoring the procedures and moreover, we received proper training by the manufactures and visited other more experienced centres. Thus, we find that the learning curve was steep, and all procedures included in these studies have been performed as described in the protocol.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, we have conducted three studies presenting original data regarding CLE and inflammation in IBD. It was demonstrated that CLE is able to visualise subtle inflammatory changes such as augmented crypt architecture and impairments of the intestinal barrier. The latter, was a significant risk factor predicting relapse in CD patients otherwise in endoscopic remission. Moreover, when UC patients with signs of relapse were included in a longitudinal study, we demonstrated that colonic structural crypt changes in UC detected by CLE are reversible after medical treatment and correlated with changes in the histopathological severity of the disease. Finally, we have shown that Smad4 expression was significantly decreased in CD and correlated with the severity of the disease. This mechanism may contribute to perpetuation of chronic inflammation by hampering the function of the natural anti-inflammatory cytokine TGF-β that is involved in epithelial cell restitution and wound healing [59].

The perspectives of CLE are substantial. In IBD the technology may contribute to a refinement of the definition of mucosal healing and serve as an ultimate therapeutic goal when new drugs are studied. In CD, a definition of endomicroscopic indices has been proposed for the terminal ileum and colon, respectively [50, 53]. However, both of these need further validation to be implemented fully in clinical decision making and randomised controlled trials investigating the effect of new drugs. In our opinion, further validation studies are at best conducted in a multicentre setting with a central assessment of CLE and endoscopic images with blinding of patient details.

The intestinal permeability has interested researchers in IBD for several years. So far, all studies have been carried out with inert probes or ex vivo. With CLE, we are able to demonstrate impairments of the intestinal barrier in vivo. These studies will not only enable an interesting stratification of IBD patients, it will also facilitate basic studies of molecular candidates involved in the defective barrier function and lack of mucosal healing and thus identification of possible novel targets of medical treatment. Recent studies suggest that intestinal permeability is also altered in IBS patients [114, 120, 121]. In study III, we additionally included eight IBS patients in a pilot study assessing the barrier function in the terminal ileum. Interestingly, we found an impaired barrier in 50% of these patients. This extends the data from Turcotte et al who compared the gap density in the terminal ileum of IBS patients with asymptomatic controls and found that cell shedding in IBS patients is significantly increased causing an increased number of gaps in the epithelial lining [122]. Furthermore, in an elegantly conducted study by Fritscher-Ravens et al, CLE was used to demonstrate widened intercryptal space and formation of epithelial leaks after administration of food allergens to the duodenal mucosa in patients suspected of food-dependent IBS [123]. Based on the results, the patients received an exclusion diet, which was associated with a decrease in symptoms during a 12-month follow-up period. Hence, the results of our pilot study add to evidence of an organic component in IBS with a compromised barrier function and altered small bowel permeability. However, one thing we have to keep in mind is the possibility of ubiquity as some of the features demonstrated by CLE may be found in a proportion of healthy patients as well. Furthermore, the controls were significantly older than the IBD patients in our studies. Although no studies have reported that the intestinal barrier function should be related to age, we cannot reject the possibly of an age related bias. Thus, solid case series from healthy individuals of different age describing CLE features including fluorescein kinetics in different bowel segments are needed to assure us what is normal.

Finally, there is a completely novel field of research termed molecular CLE [3]. In the future, molecular probes with conjugated fluorescein that are administered either topical or intravenously may be detected with CLE and possibly turn into a new endoscopic discipline. Recently, Atreya et al published a ground-breaking paper describing how adalimumab (a therapeutic anti-TNF-α antibody) conjugated with fluorescein was administered topically in CD patients with active disease. With the use of CLE for detection of cells binding adalimumab, the affinity of the drug was estimated. Next, the patients were treated with adalimumab and after a follow-up period, a significant correlation was found between the affinity of the drug and the effect of the treatment [124]. Additional studies mainly in cancer patients have also shown promising results although the majority of studies have been carried out in animals or ex vivo [125-129]. While these studies characterised tumours and estimated the affinity of a given drug, Sturm et al increased the diagnostic value of surveillance endoscopy in Barrett's oesophagus by the use of fluorescein-labelled peptides specific for oesophageal neoplasia [130]. No adverse advents were registered and the sensitivity and specificity was 75% and 97%, respectively. Hsiung et al, topically applied fluorescein-labelled septapeptides to the colonic mucosa and found a strong affinity to dysplastic tissue compared to normal mucosa [131]. Obviously, these methods and results have to be reproduced, but potentially molecular CLE can improve surveillance colonoscopy in IBD, stratify patients more accurately, and personalise medical treatment.

ABBREVIATIONS

CD	Crohn's Disease
UC	Ulcerative Colitis
IBD	Inflammatory Bowel Disease
CLE	Confocal Laser Endomicroscopy
CDEIS	Crohn's Disease Endoscopic Index of Severity
SES-CD	Simply Endoscopic Score of Crohn's Disease
CDEAS	Crohn's Disease Endomicroscopic Activity Score
FC	Faeces Calprotectin
eCLE	endoscope-based Confocal Laser Endomicroscopy
pCLE	probe-based Confocal Laser Endomicroscopy
UCEIS	Ulcerative Colitis Endoscopic Index of Severity
TGF-β	Transforming Growth Factor-β
Smad	Small mothers against decapentaplegic
IEC's	Intestinal Epithelial Cells

LPMC´s	Mononuclear Cells in Lamina Propria
IBS	Irritable Bowel Syndrome
IQR	InterQuartile Range
5-ASA	5-Aminosalicylic Acid
RNA	RiboNucleic Acid

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SUMMARY

Background: Crohn's disease (CD) and ulcerative colitis (UC) have been associated with altered intestinal barrier function. Moreover, it has been proposed that a defective barrier function is related to risk of relapse in patients with quiescent CD. Fluorescein-aided confocal laser endomicroscopy (CLE) is a novel endoscopic method, which enables real-time in vivo microscopy. Hence, the intestinal barrier function can be assessed as part of endoscopic evaluation of patients with inflammatory bowel disease (IBD) by measuring microerosions and fluorescein leakage into the intestinal lumen. Furthermore, barrier dysfunction can be correlated with biomarkers associated with intestinal barrier impairments. E-cadherin is a key factor for the adherence of epithelial cells and Smad4 is a cofactor in TGF-β signalling, which is compromised in IBD.

Aim: To correlate ileal and colonic CLE parameters with endoscopy and histopathology in IBD. Further, we wanted to correlate these features with risk of relapse and evaluate whether they were reproducible and reversible after intensified medical treatment. We also wanted to analyse, whether Smad4 and E-cadherin mucosal protein expression levels were associated with impairments of intestinal barrier function.

Methods: CLE was performed and correlated to histopathology and endoscopic appearance in two prospective studies in CD (n=39, controls=11) and UC patients (n=22, controls=7), respectively. In the first study, results were correlated to risk of relapse, whereas the latter assessed the reversibility of CLE features in a longitudinal setting. κ-statistics were used in both studies to assess reproducibility of the CLE findings. Furthermore, ileal biopsy specimens from CD patients and controls were stained by immunohistochemistry for Smad4 and E-cadherin and subsequently correlated to the severity of CD and intestinal barrier impairments.

Results: We found that fluorescein leakage and microerosions in the terminal ileum were significantly associated with CD compared to controls (p=0.005 and p=0.006, respectively) and that ileal fluorescein leakage and microerosions could predict relapse (log-rank p=0.003 and p=0.017, respectively). In UC patients with clinical relapse, an augmented crypt architecture and colonic fluorescein leakage were significantly correlated to the severity of the disease (p=0.001 and p<0.001, respectively). After intensified medical treatment, a correlation was found between histopathological progress and improvement of abnormal colonic crypt architecture (rs=0.35, p=0.016), but we did not observe a resolution of the intestinal barrier dysfunction (rs=0.09, p=0.56). The inter-observer variability of CLE parameters ranged from fair to substantial, while the intra-observer variability was somewhat higher. Smad4 expression (rs=0.56, p=0.002), but not E-cadherin (rs=0.01, p=0.95), was correlated with the severity of the disease; however, Smad4 expression did not correlate with a defect barrier function.

Conclusions: CLE can visualise crypt alteration and barrier impairments in both CD and UC, which are otherwise undetectable. Further studies are warranted to incorporate CLE in the endoscopic and therapeutic management algorithm for CD and UC possibly refining the definition of mucosal healing. Smad4 expression was correlated with CD as well as disease severity and may serve as a novel treatment target.

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APPENDIX

A1

Patient characteristics in study I

	Endoscopically Active CD	Endoscopically Quiescent CD	Controls
No (%)	19 (38)	20 (40)	11 (22)
Age / years (range)	44.5 (20-69)	42.7 (20-72)	65.5 (38-78) (p=0.012)
Sex/females (%)	8 (42)	12 (60)	3 (27)
Phenotype of CD			
Luminal (%)	16 (84)	14 (70)	0
Fistulising	0	0	0
Both luminal and fistulising	3 (16)	6 (30)	0
Previous maximal extent of CD			
Small bowel (%)	4 (21)	3 (15)	0
Colorectal (%)	8 (42)	14 (70)	0
Small bowel and colon (%)	7 (37)	3 (15)	0
Perianal involvement (%)	3 (16)	6 (30)	0
Disease localization of active luminal CD (SES-CD>2)			
Small bowel (%)	10 (53)	0	0
Colorectal (%)	6 (32)	0	0
Small bowel and colon (%)	3 (16)	0	0
Previous intestinal resection related to CD (%)	6 (32)	3 (15)	0
Small bowel (%)	0 (0)	0 (0)	0 (0)
Colorectal (%)	1 (5)	1 (5)	0 (0)
Small bowel and colon (%)	5 (26)	2 (10)	0 (0)
On-going medical treatment (%)	13 (68)	17 (85)	0
Infliximab (%)	1 (5)	8 (40)	0
Adalimumab (%)	2 (11)	2 (10)	0
Golimumab (%)	0 (0)	1 (5)	0
Azathioprine (%)	7 (36)	10 (50)	0
Methotrexate (%)	2 (11)	1 (5)	0
Corticosteroids (%)	2 (11)	3 (15)	0

CD, Crohn's Disease; SES-CD, Simplified Endoscopic Score for Crohn's Disease.

A2

Clinical features of patients with a clinical relapse in Study I

	Active CD	Quiescent CD
No	19	20
Relapse (%)	14 (74)	8 (40)
Medical treatment (%)	11 (84)	8 (100)
Infliximab (%)	2 (14)	2 (25)
Adalimumab (%)	2 (14)	1 (13)
Azathioprine (%)	3 (16)	0
Salazopyrine (%)	1 (5)	0
Corticosteroids (%)	3 (16)	5 (63)
Surgical resection (%)	3 (16)	0
Ileocecal (%)	1 (5)	0
Ileocecal and sigmoid (%)	1 (5)	0
Colectomy (%)	1 (5)	0

CD, Crohn's Disease.

A3

Baseline Patient characteristics in Study II

	UC	Controls	p-value
No (%)	22 (76)	7 (24)	
Median Age/years (IQR)	32.7 (20)	67.4 (23)	0.006
Sex /female (%)	9 (41)	6 (86)	0.08
Extent of UC			
Extensive (%)	9 (41)		
Left-sided (%)	4 (18)		
Proctitis (%)	7 (32)		
No active inflammation (%)	2 (9)		
Maximal Endoscopic Mayo Clinic subscore			
3 (%)	1 (5)		
2 (%)	14 (64)		
1 (%)	5 (23)		
0 (%)	2 (9)		
On-going medical treatment (%)*	21 (95)		
Infliximab	1 (5)		
Azathioprine (%)	6 (27)		
Local corticosteroids (%)	2 (9)		
Local 5-ASA (%)	5 (23)		
Oral 5-ASA (%)	21 (95)		

*Some patients received multiple drugs. UC, Ulcerative Colitis. IQR, interquartile range. 5-ASA, 5-Aminosalicylic Acid.

A4

Patient characteristics in Study III

	Endoscopically Active CD	Endoscopically Quiescent CD	Controls
No (%)	10 (27)	18 (49)	10 (24)
Phenotype of CD			
Luminal (%)	8 (80)	13 (72)	0
Fistulising (%)	0	0	0
Both luminal and fistulising (%)	2 (20)	5 (28)	0
Previous maximal extent of CD			
Small bowel (%)	1 (10)	3 (17)	0
Colorectal (%)	4 (40)	12 (67)	0
Small bowel and colon (%)	5 (50)	3 (17)	0
Disease localization of active luminal CD (SES-CD>2)			
Small bowel (%)	4 (40)	0	0
Colorectal (%)	4 (40)	0	0
Small bowel and colon (%)	2 (20)	0	0
Previous intestinal resection related to CD (%)	3 (30)	2 (11)	0
Small bowel (%)	0	0	0
Colorectal (%)	0	0	0
Small bowel and colon (%)	3 (30)	2 (11)	0
On-going medical treatment (%)	7 (70)	15 (83)	0
Infliximab (%)	1 (10)	7 (39)	0
Adalimumab (%)	1 (10)	1 (6)	0
Azathioprine (%)	4 (40)	9 (50)	0
Methotrexate (%)	1 (10)	1 (6)	0
Corticosteroids (%)	1 (10)	3 (17)	0

CD, Crohn's Disease; SES-CD, Simplified Endoscopic Score for Crohn's disease.