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## Collagenous Colitis

*A Study of Inflammatory Mediators and Growth  
Factors Based on Segmental Colorectal Perfusion  
and Immunohistochemistry*

YESUF AHMED TAHA



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#### **Abstract**

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Collagenous colitis (CC) is an inflammatory bowel disease of unknown etiology. It is characterized by watery diarrhoea without blood, normal endoscopic findings but microscopically colonic mucosal inflammation and increased thickness of the subepithelial collagen band, the latter being a pathognomonic sign. The inflammatory infiltrate in the mucosa of CC contains lymphocytes, plasma cells, eosinophils, mast cells but few neutrophils. The pathophysiological roles of the thickened collagen band and the inflammatory infiltrate in CC are not fully understood. The aims of the present study were to develop a colonoscope based segmental perfusions technique and to analyze local intestinal secretion of inflammatory mediators: Eosinophilic Cationic Protein (ECP), Myeloperoxidase (MPO), Basic Fibroblast Growth Factor (bFGF), Vascular Endothelial Growth Factor (VEGF) and permeability marker albumin in CC patients without medication and also during steroid treatment. Furthermore, the colonic mucosal distribution of bFGF and VEGF were studied by immunohistochemical methods.

Colonoscope-based segmental perfusions were performed in totally 22 patients and the success rate was 76% in both rectal and descending colon segments. The analysis showed high intraluminal concentrations of ECP, bFGF, VEGF and albumin in ten CC patients compared to 10 control patients. Further, albumin had correlations with ECP and VEGF. However, elevated concentrations of MPO, an important feature of ulcerative colitis, were only observed in a few CC patients. Immunohistochemistry visualized bFGF and VEGF in the colonic epithelium but also deeper in the lamina propria. The steroid treatment study (including 12 patients) showed that the perfusate concentrations of ECP, bFGF and VEGF declined significantly in parallel with decreased frequency of diarrhoea.

In conclusion, a safe colonoscope-based, segmental perfusion technique was developed and perfusions of the rectum and descending colon were performed. CC patients had elevated perfusate concentrations of ECP, VEGF and bFGF. There was a marked reduction of these mediators during steroid treatment supporting the hypothesis that these inflammatory mediators separately or synergistically participate in the inflammatory reaction and tissue remodelling in CC patients. The finding of correlations between albumin and ECP or VEGF implies that permeability is increased in CC and may be triggered by ECP and VEGF.

*Keywords:* Collagenous, Collagenous colitis, inflammatory mediators, Steroid treatment, Eosinophil Cationic Protein, Basic Fibroblast Growth Factor (bFGF), Vascular Endothelial Growth Factor (VEGF), albumin

*Yesuf Ahmed Taha, Department of Medical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden*

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*To my late beloved mother Kedja  
Abdurohman and father Ahmed Taha.*

“We are for difference,  
for allowing difference,  
for learning to understand difference,  
and for respecting difference  
until difference doesn’t make any more difference.”

**Johnnetta B. Cole, Ph.D.**  
Past President of Spelman College

## List of Papers

- I Yesuf Taha, Marie Carlson, Magnus Thörn, Lars Lööf, Yngve Raab. Evidence of local eosinophil activation and altered mucosal permeability in collagenous colitis. *Dig Dis Sci.*, 2001.888-97.
- II Yesuf Taha, Yngve Raab, Anders Larsson, Marie Carlson, Lars Lööf, Bengt Gerdin, Magnus Thörn. Mucosal secretion and expression of basic fibroblast growth factor in patients with collagenous colitis. *Am J Gastroenterol.* 2003. 2011-7.
- III Yesuf Taha, Yngve Raab, Anders Larsson, Marie Carlson, Lars Lööf, Bengt Gerdin, Magnus Thörn. Vascular Endothelial Growth Factor (VEGF) - A possible mediator of inflammation and mucosal permeability in patients with collagenous colitis. *Dig Dis Sci.*, 2004. 109-115
- IV Yesuf Taha, Yngve Raab, Anders Larsson, Mikael Lördal, Marie Carlson, Lars Lööf, Magnus Thörn. Steroid Treatment Reduces local Inflammatory Mediator Secretion and Mucosal Permeability in Collagenous Colitis Patients. Submitted

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## Abbreviations

bFGF	Basic fibroblast Growth Factor
CC	Collagenous Colitis
CTGF	Connective Tissue Growth Factor
ECM	Extra cellular matrix
ECP	Eosinophilic Cationic Protein
EPO	Eosinophil Peroxidase
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IELs	Intraepithelial Lymphocytes
iNOS	Inducible Nitric Oxide Synthetase
LC	Lymphocytic Colitis
MBP	Major Basic Protein
MC	Microscopic Colitis
MMP-1	Matrix Metalloproteinase-1
MPO	Myeloperoxidase
NOS	Nitric Oxide Synthetase
$\alpha$ -SMA	$\alpha$ -Smooth Muscle Actin
TGF- $\beta$	Transforming Growth Factor
TIMP-1	Tissue Inhibitor Metalloproteinase-1
VEGF	Vascular Endothelial Growth Factor
UC	Ulcerative Colitis



## Introduction

Collagenous colitis (CC) is a disease of unknown etiology and its pathophysiology is poorly understood. The disease was for the first time described by the Swedish pathologist Lindström<sup>1</sup>. He presented a case of CC in a woman who suffered of watery diarrhoea and had pathological subepithelial collagen accumulation in her colonic mucosa. CC is now considered a chronic inflammatory bowel disease (IBD) and is classified, together with lymphocytic colitis (LC), as microscopic colitis (MC)<sup>2,3</sup>. In LC, the intra-epithelial inflammatory cells infiltration is dominated by lymphocytes<sup>4,5</sup> and in 1989 consensus was reached to name all colitis with normal macroscopic features as MC<sup>2,6</sup>. MC includes all colitis with macroscopically normal colonic mucosa, predominately CC and LC. The clinical and macroscopic features of CC and LC do not differ greatly from each other but the presence of increased subepithelial collagen deposition in CC distinguishes CC from LC<sup>7-9</sup>.

The reported incidence and prevalence figures of CC varies between 0.6 – 5 /100 000 and 10-36/ 100 000 respectively<sup>7, 10-13</sup>. CC is nine to ten times more common among women than among men<sup>14-17</sup>. The incidence of CC peaks between the 6<sup>th</sup> and 7<sup>th</sup> decades where it reaches up to 15/ 100 000<sup>18</sup>. This means that CC is predominately a disease of elderly female patients and its incidence and prevalence reaches that of UC in this group of patients<sup>11</sup>. Thus, it is important to consider the diagnosis of CC among patients suffering from watery non-bloody diarrhoeas, especially in elderly women.

## Clinical features

The clinical characteristics of CC are continuous or intermittent watery diarrhoea without blood and non-specific abdominal symptoms similar to that of irritable bowel syndrome (IBS)<sup>17, 19</sup>. The frequency of diarrhoea varies widely between mild and very severe cases. In mild cases, low frequency diarrhoea is the major symptom, but in severe cases frequent intestinal evacuations in combination with abdominal pain, weight loss, fatigue and nocturnal diarrhoea are common.

The final diagnosis of CC is based on histopathology, clinical symptoms and normal macroscopic colonic mucosa<sup>8, 20, 21</sup>.

## Criteria for histopathological diagnosis of CC

The histopathological diagnosis of CC is based on the following criteria<sup>3, 8, 22</sup>.

- increased thickness of subepithelial collagen - more than 10  $\mu\text{m}$  beneath the basal membrane.
- increased number of chronic inflammatory cells in the surface epithelium and lamina propria
- epithelial detachment and loss

The histopathological changes observed in CC and LC are remarkably similar, with the exception of collagen deposition immediately beneath the epithelial basement membrane, which is a pathognomonic feature of CC. The inflammatory infiltrate in the colonic mucosa of CC contains several types of inflammatory cells such as lymphocytes, plasma cells, eosinophils and mast cells but few neutrophils<sup>5, 23-25</sup>. The relative contribution of these cell types to the pathophysiology in CC is not known. The Intraepithelial Lymphocytes (IELs) in CC are CD8<sup>+</sup> type T-cells which are the same types as in LC, but the number of IELs in CC is less marked compared to in LC<sup>5, 23, 25</sup>. The lymphocytes in the lamina propria of CC patients are mainly composed of CD4<sup>+</sup> T-cells similar to LC<sup>26</sup>. The composition of the inflammatory cells differs between the two diseases in some respects. Eosinophilic infiltration of crypt and surface epithelium is more common in CC than LC<sup>5, 24, 27</sup>. Neutrophilic granulocytes are rare but their mucosal infiltration, including crypt abscesses, have been reported in CC<sup>28</sup> and may represent an early stage or acute exacerbation of the illness<sup>29-31</sup>. Infiltration of mast cells may be localized in the upper part of lamina propria in CC patients. There are suggestions that mast cells contribute to the clinical picture of CC<sup>32-34</sup>. It must be emphasized here that crypt architecture deformation, which is a characteristic of both UC and Crohn's diseases, are not typical features of neither CC nor LC<sup>5, 8, 9, 35</sup>. Mucosal depletion and flattening of the epithelial cells seen in surface epithelium are common in both CC and LC, but the separation of strips of surface epithelium from subepithelial collagen band is more typical in CC than in LC<sup>14</sup>.

The collagen deposition that is the histopathological diagnostic criteria of CC is highly variable in thickness ( $10 \geq 100 \mu\text{m}$ ) and the distribution is patchy. It is less marked in caecum, sigmoid colon and rectum than in the rest of the colon and is located between two crypts in the so called intercryptal region<sup>5, 15, 33, 36-38</sup>. Haematoxylin and eosin do not stain the collagen layer selectively; therefore the borders are ill-defined. The Masson trichrome staining clearly highlights the collagen deposition and provides the opportunity to estimate the thickness of band more accurately<sup>21</sup>. As mentioned above, the thickness must be greater than 10 $\mu\text{m}$  in order for a diagnosis of

CC to be considered. Contrary to this, opinions are aired suggesting that assessing the qualitative enlargement of the collagen band should be the basis of CC diagnosis rather than simply appraising the quantitative value of the collagen thickness<sup>21</sup>. Measuring the thickness of the collagen deposition may be inaccurate in most cases, according to these authors, because the lower edge of the collagen layer is usually irregular or may be diffuse<sup>21</sup>. Furthermore, the collagen deposition is neither homogenic nor hypocellular but composed of inflammatory cells, capillaries and stellate cells entrapped within the collagen layer<sup>39</sup>. The satellite cells are synthetically active myofibroblasts, that produce collagen in the colonic subepithelial region as shown by ultrastructural studies<sup>40, 41</sup>. Electron microscopic, immunohistochemical and in situ hybridization studies have shown that the collagen composition is dominated by type VI, even though collagen types III and I are present as well<sup>15, 42, 43</sup>. Besides collagen, the abnormally thickened subepithelial layer contains a glycoprotein called Tenascin<sup>43-48</sup>. Tenascin is a marker of tissue remodelling found in immature Extra Cellular Matrix (ECM) during inflammation or reparation of injured tissue. Recent studies have shown that Tenascin specific staining is more sensitive than collagen staining in the diagnosis of CC, especially in cases where collagen accumulation is minimal. Tenascin staining may be the first alternative histopathological method in the diagnosis of CC in the future. Another possible method for assessing the thickness of the collagen band in CC is fluorescent microscopy<sup>28</sup>.

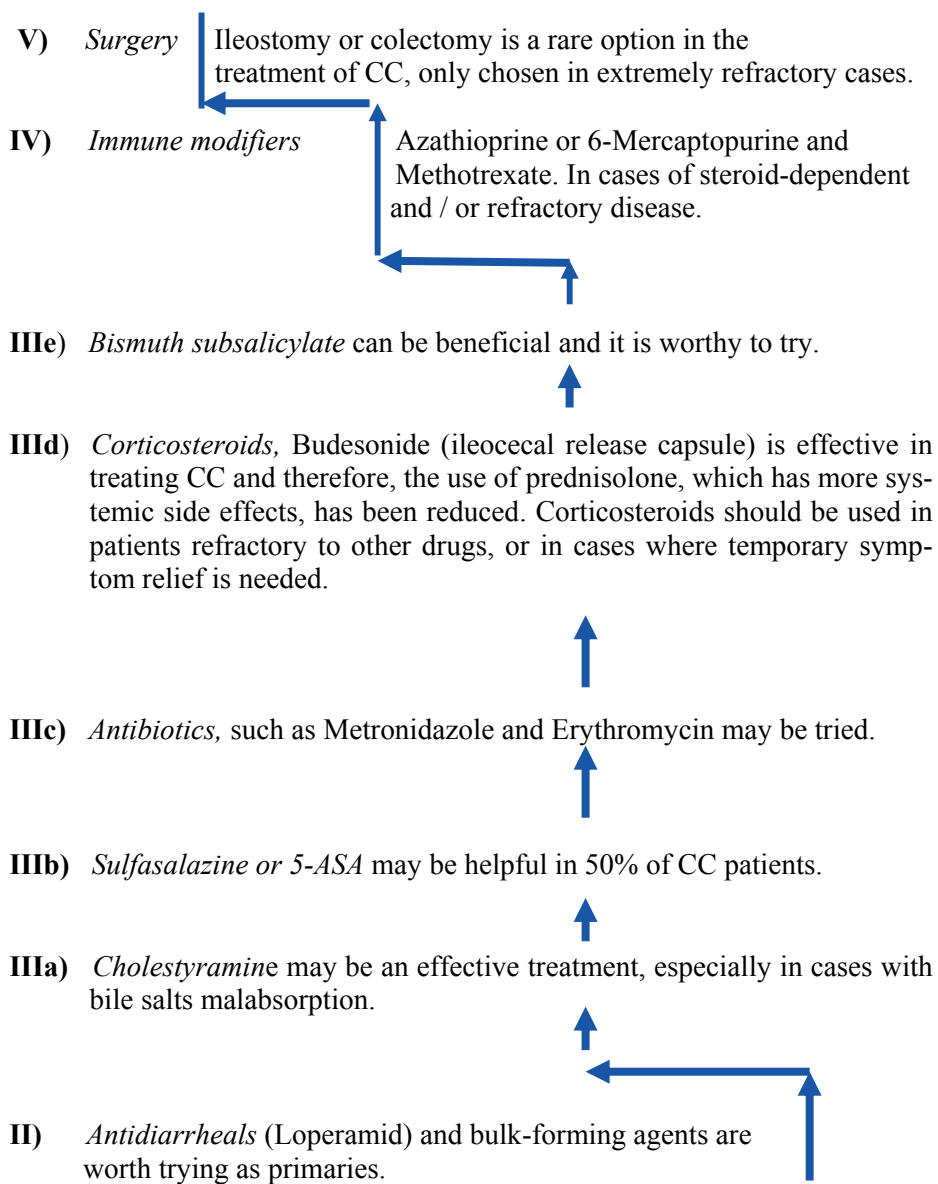
### Pitfalls in diagnosis

In the histopathological diagnosis of CC, the following pitfalls must be avoided.

- Collagen thickness should be measured in a vertically well-oriented part of the biopsies in order to avoid the over-interpretation of the basement membrane as collagen deposition in tangentially oriented biopsy sections.
- Because the collagen deposition has a patchy distribution several biopsies should be taken from different parts of the colon and collagen staining should be applied frequently, especially in suspected cases.
- Histological features overlap between CC and LC, therefore it is important to exclude CC before the diagnosis of LC can be established.
- Congo staining, which is specific to amyloid, is negative in CC.

## Treatment

There is no specific treatment available for CC today <sup>49, 50</sup>. Suggested therapies include bulking and anti-diarrhoeal agents, 5-ASA compounds, antibiotics, bile binding pharmaceuticals, steroids, and azathioprine/6-mercaptopurine. Those are based mainly on anecdotal evidence or uncontrolled trials with small case series <sup>49, 50</sup>. In randomized controlled trials, budesonide was shown to be effective in symptom relief, but the symptoms recurred in most cases when medication ceased <sup>49</sup>. Bismuth studies have indicated therapeutic benefits, but the studies need confirmation <sup>49-51</sup> through additional randomized controlled trials. The clinical outcome of drug therapy, however, is often unpredictable in CC and the mechanisms of different medications are mostly unknown. The following steps can simplify the approach to treating CC patients (figure 1).



*Verify* the diagnosis of CC and exclude infectious colitis, other IBD and other potential causes of diarrhoea. Discontinue NSAID and other medications, including coffee, that have known associations with pathogenesis of CC.

Figure1. Treatment algorithm for collagenous colitis

It is generally recommended that the drugs chosen to treat CC patients should be used at correct doses for a sufficient period of time before concluding that the tested drug is not effective.

The treatment algorithm, as outlined above, is a summary of the CC treatment accepted by most gastroenterologists. The step between III and IV is substantial and the different medical treatments mentioned in stage III should all be considered before stepping up to stage IV. The order of treatment proposed at stage III is not mandatory.

## Pathogenesis and pathophysiology in CC

The causes and mechanisms of collagen accumulation and chronic inflammation in CC are not fully understood. Several hypotheses are put forward concerning the pathogenesis of CC, including a) autoimmunity, b) luminal agents like bacteria, toxins or even ingested food agents leading to pathological processes c) abnormalities of collagen synthesis d) malfunction of mast cells e) plasmatic vasculosis.

### Autoimmunity

Autoimmunity has been suggested as one potential mechanism to explain the pathophysiology of CC. The association between CC and different autoimmune conditions such as rheumatoid arthritis, celiac diseases and hypo/hyperthyroidism support this mechanism<sup>5, 14, 19, 52-56</sup>. In addition, the female predominance and the remission of symptoms induced by steroid therapy, also favour the assumption that CC is an autoimmune disease. However, the absence of an identified, specific auto-antigen so far makes the hypothesis unproved<sup>9, 20</sup>.

### Luminal agents

The effect of antibiotics leading to remission in some CC patients has raised the possibility of an infectious cause behind CC<sup>7</sup>. Furthermore, supporting an infectious etiology or intraluminal factor triggering the disease process, are the observations that faecal steam diversion reverses the histopathological changes in the colonic mucosa of CC patients<sup>23, 57, 58</sup>. However, the effects of antibiotics are unpredictable and as long as infectious agents or an intraluminal factor are not identified, the hypothesis remains unproved.

### Abnormalities of collagen synthesis

In normal colon, the thickness of the subepithelial collagen band is less than 7  $\mu\text{m}$  and the band consists mainly of collagen type I and IV<sup>15, 25, 33, 36, 59</sup>.



The abnormal, subepithelial collagen composition in CC differs both qualitatively and quantitatively from normal colon and it is dominated by collagen type I and III<sup>3, 25, 42, 60, 61</sup> or VI collagen<sup>43, 62</sup>. Notably, the collagen composition of basal membrane in CC is identical to that of a normal colon and based on collagen type IV<sup>60</sup>.

In normal colonic mucosa, the subepithelial basal membrane closely surrounds the crypts and this membrane is intimately associated with the pericryptal fibroblast sheath<sup>63, 64</sup>. The pericryptal cells, which are myofibroblastic in nature originate from the deepest third of the crypts and mature while migrating towards the mouth of the crypts. These cells produce collagen and maintain the normal function and structure of the overlying colon epithelium.

In CC the thickened subepithelial collagen layer is located in the intracryptal region without engaging the crypts. Electron microscopic studies of the pericryptal myofibroblasts in CC patients have shown a clear separation of pericryptal fibroblasts from epithelial cells. The appearance and orientation of these cells suggest that myofibroblasts are synthetically active<sup>41, 60, 65, 66</sup>. Based on these findings, early studies suggested that the pathological accumulation of collagen in CC depends on enhanced synthesis. In addition, the presence of an increased amount of reparative collagen type III in the pericryptal sheath favours the theory of increased synthesis of this particular collagen. Contradicting this, other authors have recently argued that slowed degradation is the primary reason behind abnormal subepithelial collagen deposition<sup>43, 45</sup>. According to an in situ hybridization study, type VI collagen mRNA was not significantly elevated in colonic mucosa of CC patients compared to normal mucosa<sup>43</sup>, supporting that the pathological accumulation of collagen depends on decreased degradation of collagen rather than increased synthesis. This hypothesis is further supported by the relatively low expression of collagen degrading enzymes in colonic mucosa of CC patients<sup>45</sup>.

### The possible role of inflammatory mediators in CC

There is no consensus among researchers about the mechanisms of the subepithelial pathological deposition of collagen in CC. ECP, bFGF and VEGF, besides acting as pro-inflammatory factors, separately or by interacting with one another, stimulate proliferation of myofibroblasts<sup>67, 68</sup>. It is possible that these mediators directly or indirectly activate fibroblasts/ myofibroblasts of the pericryptal sheath causing the collagen deposition in collagenous colitis. Subepithelial myofibroblasts, besides producing several extracellular components, participate in different functions of epithelial cells, such as growth and differentiation. In CC, the numbers of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) expressing myofibroblasts are large in quantity compared to normal colonic mucosa, suggesting that there are many synthetically active

myofibroblasts. This observation also supports that intestinal subepithelial myofibroblasts are involved in the tissue remodelling process of CC<sup>47</sup>.

VEGF is a heparin-binding glycoprotein with numerous biological effects<sup>68-70</sup>. VEGF is a potent angiogenic, mitogenic, inflammation, permeability and fibrosis enhancing peptide<sup>69, 71</sup>. In active IBD, it may enhance fibrosis, vascular permeability, angiogenesis, mitogenic stimulation of endothelial cells and inflammation<sup>70-72</sup>, leading to alteration of ECM. It has been demonstrated that VEGF levels are significantly increased in intestinal mucosa of active UC and Crohn's disease<sup>73, 74</sup> but not in non-active disease states, suggesting that VEGF has an important role to play in the inflammatory processes of active IBD. A recent study on the pathophysiological role of VEGF in CC showed that VEGF, by changing the local Matrix Metalloproteinase-1 and Tissue Inhibitor Metalloproteinase-1 (MMP-1/TIMP-1) balance, causes the accumulation of immature subepithelial ECM in CC<sup>48</sup>.

### Plasmatic vasculosis

Plasmatic vasculosis is a phenomenon where leakage of plasma proteins in the region leads to arteriosclerosis as observed in diabetes and hypertension<sup>29</sup>. The pathological deposition of collagen and entrapped capillaries within the collagen layer in the subepithelial region of the colonic mucosa, has led to the speculation that plasmatic vasculosis may have a place in the pathogenesis of CC<sup>29, 40, 60, 75</sup>. Because of their biological effects, both bFGF and VEGF may be important mediators in the development of plasmatic vasculosis and CC.

### Mechanisms of diarrhoea

Lindström suggested that in CC, the thickened collagen layer in the subepithelial matrix blocks the reabsorption of water and electrolytes from the lumen of the colon, causing diarrhoea<sup>1</sup>. This hypothesis is also supported by the collagen band being located in the intercryptal spaces of the mucosa<sup>9, 54, 76</sup> where absorption of water occurs. Furthermore, there are studies that show that stool frequency is correlated with the thickness of the collagen band<sup>59, 77, 78</sup>. This band has been implicated in causing the decrease of net fluxes of sodium and chloride absorption, leading to the watery diarrhoeas observed in CC<sup>79</sup>. However, the hypothesis of Lindström could not be confirmed by other investigators<sup>35, 80, 81</sup>. The finding of a correlation between mucosal cellularity and stool weight, but not between stool weight and collagen thickness, undermines the importance of collagen band in the pathophysiology of CC diarrhoea.

Instead, secretory and osmotic mechanisms probably play a role in the cause of diarrhoea among CC patients. An osmotic mechanism is supported

by decreasing frequency induced by fasting, while the secretory mechanism is favoured by the observation of increased secretory factors such as prostaglandin E. <sup>82</sup>. A study of the activity of Nitric Oxide Synthetase (NOS) in the colonic mucosa of CC patients, showed that the up-regulation of NOS and its inducible form (iNOS) activity may contribute to the epithelial secretion leading to watery diarrhoea in CC <sup>83</sup>.

A recent study of the mechanisms of diarrhoea, with special emphasis on electrolyte and water transport in CC, showed that reduced net Na<sup>+</sup> and Cl<sup>-</sup> absorption are the main causes of diarrhoea, accompanied by a secretory component of active electrogenic chloride secretion <sup>79</sup>.

## Aims of the present studies:

- To develop a colonoscope-based segmental perfusions technique in order to study local inflammatory mechanisms in collagenous colitis.
- To qualitatively and quantitatively estimate secretion of inflammatory mediators (ECP and MPO), angiopeptides (bFGF and VEGF) and permeability marker albumin in the gut lumen of CC patients.
- To study mucosal contents and distribution of bFGF and VEGF by immunohistochemical methods in the colonic mucosa of CC patients.
- To qualitatively and quantitatively describe secretion of inflammatory mediators (ECP and MPO), angiopeptides (bFGF and VEGF) and permeability marker albumin in the gut lumen before and during steroid treatment of CC patients.

## Patients and Methods

### Patients

In the first part of paper one, 11 CC and 12 control patients underwent perfusions in rectum and descending colon. The CC patients were 9 women and 2 men; mean age 53 years (range 36-85 years), who fulfilled the criteria of CC<sup>8</sup>. The 12 controls (one healthy individual and 11 non-CC patients) were 10 women and 2 men with a median age of 56 years (range 45-71 years). Inclusion criteria for the control patients were normal bowel habits (one patient had loose, non-watery stools), macroscopically normal colon at endoscopy and normal histopathological findings. The study population in paper 1 (second part), and in paper II and III were 10 CC patients (mean age 56 years, range 36-85 years) and 10 control patients (55 years, range 45-61 years) (table 1).

Clinical characteristics in patients with collagenous colitis and controls at the time of investigation are shown in tables 2 & 3 respectively.

Twelve patients (10 women, 2 men; mean age 52 years; range 34 - 66 years) who fulfilled the diagnostic criteria of CC<sup>8</sup> were recruited for steroid treatment study (paper IV) between November 2000 and November 2002 at

the Department of Medicine, University Hospital, Uppsala, Sweden. Paper IV.

The clinical data of these patients are shown in table 6.

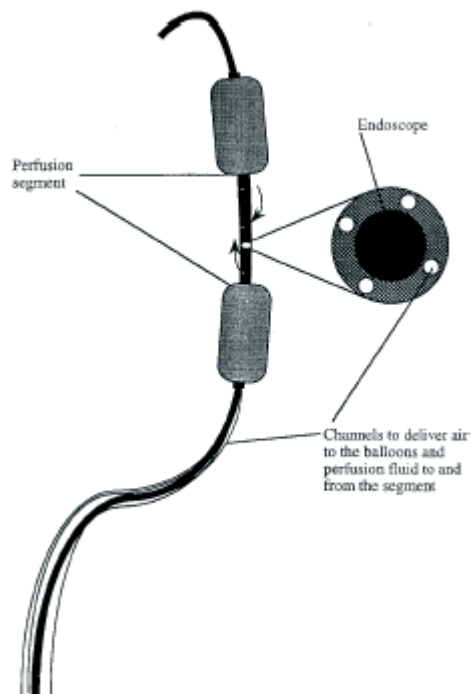
## Ethics

All subjects gave informed consent to participation. All studies were conducted according to the Declaration of Helsinki. The Ethics Committee of the Medical Faculty, Uppsala, approved the study.

## Methods

### Perfusions technique

The technique of intestinal perfusion using a closed segment has been previously described for the jejunum, colon and rectum<sup>84-86</sup>. In paper I, we developed a new technique whereby the perfusion segment was attached to a colonoscope (Olympus, CF-100HL or Pentax, EC-38406). The segment was fixed close to the flexible tip of the instrument, thereby making optimal positioning of the segment in the colon and rectum possible. The segment was made of a polyvinyl tube with a length of 26 cm (outer diameter 19 mm, inner diameter 13mm). Two latex balloons (length 5 cm) were attached to the tube creating a closed segment (8 cm in length) for the perfusion. Four small catheters were fused to the outer aspect of the tube wall and loosely attached to the whole length of the endoscope. The channels were used to deliver air to the balloons and perfusion fluid to and from the segment (Fig. 2). This technique is described in detail in paper I and was used for sampling of perfusion fluid from descending colon and rectum in papers I- III and only from rectum in paper IV.



*Figure 2.* A schematic drawing of the colonoscope with the perfusion segment attached close to the flexible tip of the instrument and positioned in the descending colon. Small channels loosely attached to the colonoscope end in the perfusion segment to deliver and recover perfusion fluid and air to the balloons.

### Perfusions technique and its procedure

The subjects were prepared as for a routine colonoscopy and were given 4 litres of polyethylene glycol (Laxabon<sup>R</sup>, Tika) the day before the investigation. Intravenous injections of meperidine 25 - 50mg (Petidin<sup>R</sup>, Pharmacia & Upjohn) and diazepam 5-10 mg (Stesolid<sup>R</sup>, Dumex-Alpha) were used as routine premedication, and additional small doses were given during the procedure on patient demand. The procedure started with the patient lying on their left side as for routine colonoscopy. The rectal perfusion was carried out first to avoid possible injury of the mucosa in the rectum while advancing proximally with the endoscope. The endoscopic inspection was done first and was followed by positioning of the perfusion segment in the rectum. The correct position was ascertained by fluoroscopy (fig. 3). The distal balloon was inflated followed by infusion of perfusion fluid into the segment while the patient was lying in a slightly upright position. When the perfusion seg-

ment was considered full, the proximal balloon was filled with air to create a closed segment.



*Figure 3.* The position of the perfusion segment in the descending colon ascertained by fluoroscopy.

The perfusions were carried out with the patient in a supine position, and the colonoscope was fixed externally during the perfusion to avoid possible displacement of the perfusion segment. When the rectal perfusion had been completed, the same procedure was repeated in the middle of the descending colon (Fig 3).

A thorough inspection of the colorectal mucosa was done to document possible injuries caused by the perfusion catheter and/or the colonoscope.

The degree of technical difficulties with respect to positioning of the segment/perfusion procedure were classified by the endoscopist as easy, difficult or very difficult for the rectal and colonic parts of the examination.

### Phenol red to check contamination

Phenol red solution (50 mg/L in physiological saline) was intermittently infused (5 mL every 20 minutes) above the proximal balloon to rule out contamination of the segment by intestinal contents from the colonic part located above the proximal balloon.

The concentration of phenol red was assessed, after alkalization of the perfusate samples to pH 11, by a spectrophotometric method at 520 nm.

### Biopsy samples (Paper II & III)

Total colonoscopy was performed in all CC and control patients with exception of the healthy volunteer. A minimum of 8 biopsies was obtained from the rectum, sigmoid, descending, and ascending colon of each patient and was fixed in formalin after performing perfusions in rectum and descending colon.

### Perfusion fluid

The perfusion fluid was a buffer consisting of 120 mM NaCl, 5.4 mM KCl, 2 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM glucose, 35 mM mannitol, and 1g/L polyethylene glycol (PEG; MW 4kD) at a pH of 8.20-8.48 and osmolality 290 mosm/L. The solution was kept at 37<sup>0</sup>C and was infused at a speed of 3 mL/minute for 60 minutes. The perfusates were collected at 20-minute intervals, the first 20 minutes being a rinsing period. Ten mL of aprotinin (Trasylo<sup>R</sup> Bayer 10.000 KIU/mL) were added to every litre of perfusion buffer to inhibit proteolytic activity. The protease inhibitor phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical Co., St Louis, MO, USA) was added to a final concentration of 2 mM immediately before analysis to counteract the effect of even small amounts of proteases in the perfusion fluid.



## Serum samples

Serum samples were drawn simultaneously when the perfusions were performed and frozen at  $-70^{\circ}\text{C}$  for subsequent analysis.

## Analysis of ECP and MPO (Paper I & IV)

The concentration of ECP and MPO in perfusion fluids and serum samples were analyzed by means of a specific RIA (Pharmacia & Upjohn, Diagnostic AB, Uppsala, Sweden). The inter- and intra-assay coefficients of variations were  $<10\%$  for both tests. The lower detection limits of ECP and MPO in perfusion fluid were  $2\ \mu\text{g/L}$  and  $8\ \mu\text{g/L}$ , respectively. The reference interval for ECP and MPO in serum was  $2.3\text{-}16\ \mu\text{g/L}$  and  $170\text{-}478\ \mu\text{g/L}$ , respectively.

## Albumin analysis in perfusions fluid and serum

Rate Nephelometry on a Beckman Array protein system (Beckman Instruments, Brea, California; USA) was used to analyse albumin in the perfusion fluids and a photometric assay method (Boehringer Mannheim, Mannheim, Germany) was applied to quantify albumin in the serum. The lower detection limit in perfusion fluids of albumin was  $3\ \text{mg/L}$ . The reference interval of s-albumin was  $40\text{-}51\ \text{g/L}$  ( $<50$  years) and  $37\text{-}48\ \text{g/L}$  ( $>50$  years), respectively.

## Quantification of bFGF (Paper II & IV)

An ELISA technique was used to measure bFGF (Human bFGF, Quantikine High Sensitivity, R&D Systems, Minneapolis, MN, USA). Monoclonal antibodies, specific for bFGF, were coated onto microtiter plates. Standards and samples were pipetted into the wells and any bFGF present was bound to the immobilized antibodies. After washing, an alkaline phosphatase conjugated polyclonal anti-bFGF antibody was added to the wells. After incubation and washing, a substrate solution was added. After incubation for 60 minutes, an amplifier solution was added. The development was stopped after 30 minutes and colour intensity was measured at  $490\ \text{nm}$  in a spectrophotometer. The bFGF concentrations in the samples were determined by comparing the optical density of the samples to the standard curve. The manufacturer has verified the specificity of the assay with many different human recombinants and mouse cytokines. The lowest detectable value for bFGF was  $0.50$  and  $0.25\ \text{pg/ml}$  in paper II and IV respectively. Reference intervals of S-bFGF were  $<4.0\ \text{ng/L}$  (males) and  $<10.8\ \text{ng/L}$  (females).

### Quantification of VEGF (Paper III & IV)

Samples were analysed using commercially available ELISA kits for VEGF (DVE00, R&D Systems, Minneapolis, MN, USA). Briefly, the micro titre plates had been coated with monoclonal antibodies specific for VEGF, and the first step was to add standards and samples to the wells. During the following incubation period, VEGF present in standards and samples was bound to the immobilized antibody. After a thorough wash, an enzyme-linked polyclonal antibody, specific for VEGF was pipetted into the wells and, following a second incubation-and wash step, a substrate solution was added and a colour developed in proportion to the amount of VEGF bound. The colour development was subsequently stopped and the intensity of the colour was measured by photospectrometry. Calculation of the results was performed according to the recommendations of the manufacturer. The lowest detectable value for VEGF was 9 pg/ml in perfusion fluid and the reference interval of S-VEGF was <500 ng/L.

### Thickness of the subepithelial collagen band (Paper II & III)

The thickness of the subepithelial collagen band was measured on Haematoxylin-Eosin-stained slides under an Olympus BH-2 microscope with 400x magnification. Measurements were done by the use of an ocular micrometer in vertically oriented parts of the biopsies. Means of five measurements were used for each biopsy.

Furthermore, the number of inflammatory cells per 100 colon epithelial cells in surface epithelium, was counted in CC patients and controls

### Immunohistochemistry (Paper II & III)

The immunohistochemistry in this study was performed using the Biotin-Avidin-complex technique (ABCComplex staining procedure (Dakopatts)). B-FGF was detected using goat polyclonal IgG (FGF-2 (147)-G, Santa Cruz Biotechnology) as primary antibody, and rabbit antigoat antibody as secondary antibody. VEGF was detected using rabbit polyclonal IgG (VEGF (147), Santa Cruz Biotechnology) as primary antibody. Secondary antibody was antirabbit of goat antibody. Depending on the intensity of the bFGF and VEGF expression in the surface epithelium and lamina propria of the colon mucosa, all samples were subjectively arranged from lowest expression to highest<sup>87</sup>. The subjective arrangement of the samples was done blindly and separately for surface epithelium and lamina propria.

## Statistics

The Mann-Whitney U-test was used to compare differences between groups, the Wilcoxon signed rank test for differences within groups, the Friedman's ANOVA to analyze the changes of values over time, and the Spearman rank correlation test to study co-variation within groups. All statistical calculations were performed on a Macintosh computer by means of a statistical package, Stat View 4,51 (Abacus, Concepta Inc.). If concentrations of the granule proteins and albumin were below the detection limit of the assay, the value of the detection limit was used in the calculations. A p-value of <0.05 was considered significant.

## Results

### Perfusion technique (Paper I, II & III)

Out of twenty-five perfusion attempts on 23 patients (two patients went through perfusion twice), the number of successful attempts in both descending colon and rectum were 19 (76 %). The perfusion attempts were unsuccessful in both descending colon and rectum, only in descending colon and only in rectum in two, three and one cases respectively. Two attempts were excluded, one due to pain (patient 5 at attempt 20) and the other one because of impaired perfusion fluid outflow (patient 18) (Table 1).

Table 1. Description of the studied individuals in relation to successful and unsuccessful perfusion attempts.

Patients	Perfusion attempts	Types of successful perfusions in two segments (both) or in one of them	
1	1	Both	
2	2	Both	
3	3	Rectum	
4	4	Both	
5 = 20	5	Both	
<b>6*</b>	<b>6</b>	<b>No biopsy</b>	<b>Both</b>
7	7	Both	
8	8	Both	
9	9	Both	
10	10	Rectum	
11	11	Both	
12	12	Both	
13	13	Both	
<b>14 *=15*</b>	<b>14</b>	<b>Steroid treatment</b>	<b>Descending colon</b>
<b>15*!=14*</b>	<b>15</b>	<b>Steroid treatment</b>	<b>Both</b>
16	16	Both	
17	17	Rectum	
<b>18*</b>	<b>18</b>	<b>Unsuccessful</b>	
19	19	Both	
<b>20=5</b>	<b>20</b>	<b>Unsuccessful</b>	
21	21	Both	
22	22	Both	
23	23	Both	
24	24	Both	
25	25	Both	
Sum	23	25	23

\*Excluded patients from mediators' analysis studies.

The collected perfusate volumes from the rectal segment were stable over time and did not differ between CC patients and controls. But in the descending colon segment, the volumes in CC patients were significantly increased at first and decreased in the second collection periods (40 min,  $p < 0.05$ ) and 60min,  $p < 0.05$ ) in CC patients compared with controls. In the patients included in the inflammatory mediators and albumin analysis (paper I, II and III), no difference was found in volumes collected at 60 min period ( $p > 0.05$ ) between CC patients and controls.

The endoscopic inspections of the colonic mucosa before and after the perfusions in both rectum and descending colon did not reveal abnormalities with the exception of one control patient in whom multiple mucosal tears were observed in the descending colon. The median time consumed to perform the whole perfusion procedure was 3.5 hr (range 1.5-4.3) in CC patients and 3.4 (range 1.5-5.0) in controls and the difference was not significant ( $p > 0.05$ ).

## No contamination by the proximal intestinal contents

The median concentrations of phenol red in the rectal and descending colon perfusates were 0.6 % (range 0.1-3.6) in CC patients and 0.5% (range 0.2-2.7) in controls. These values were within the acceptable range, which is less than 5%.

## Patients included in inflammatory mediators and albumin analysis

In 20 individuals (10 CC patients and 10 controls) analyses of ECP, MPO, bFGF, VEGF and albumin in perfusates and serum were performed. CC patient 14 and control patient 6 were excluded from the mediator part of papers I (table I), II and III. The reasons for the exclusion were that patient 14 was under steroid treatment and that no biopsy samples were available from patient 6. Furthermore, patient 18 was not included because of unsuccessful perfusions procedures in both descending colon and rectum segment. All analyzed perfusates were collected in the second perfusion period at 60 minutes.

## ECP, MPO and albumin (Paper I)

The median concentration of ECP in the perfusates from the rectum in CC was 3.7  $\mu\text{g/L}$  (range 1.9-25.6  $\mu\text{g/L}$ ), which was significantly increased compared with 1.9  $\mu\text{g/L}$  (1.9-5.3  $\mu\text{g/L}$ ) in the controls ( $p < 0.05$ ). The concentrations were below the detection limit in two CC patients and in seven of the controls. There was also a difference in ECP concentrations in the perfusion fluids from the descending colon in CC patients ( $n=9$ ; median 3.9  $\mu\text{g/L}$ , range 1.9-18.4  $\mu\text{g/L}$ ) compared with controls ( $n=7$ ; 1.9  $\mu\text{g/L}$ , 1.9-5.9  $\mu\text{g/L}$ ) (table 2 and 3) (Fig. 4), but this difference did not reach statistical significance ( $p > 0.05$ ). There were no differences in median serum concentrations of ECP between CC and control patients ( $p > 0.05$ ).

The MPO concentrations in the perfusates from both rectum and colon were only above the detection limit in three patients and two controls and the median value did not differ between the groups. The serum concentration of MPO was only increased in one CC patient and within reference limits in all other CC patients and controls (table 2 and 3).

The median concentration of albumin in the rectal perfusates in CC patients (median 12.0 mg/L; range 3.4-40.0 mg/L) was significantly increased ( $p < 0.05$ ) compared with controls (5.3 mg/L; 2.9-12.0 mg/L). In the descending colon there were also higher albumin concentrations in CC patients ( $n=9$ ; median 8.0 mg/L; range 2.9-91 mg/L) compared with the controls ( $n=7$ ; 2.9 mg/L; 2.9-12 mg/L) but this difference did not reach statistical significance ( $p > 0.05$ ) (table 2 and 3) (Fig. 5).

There was a significant correlation ( $r=0.72$ ;  $p < 0.050$ ), between ECP and albumin concentrations in the rectal perfusion fluids but not in the descending colon perfusates ( $r=0.48$ ;  $p > 0.05$ ). Serum albumin was analyzed in nine CC patients and four controls (Table 2 and 3). The values were within the reference intervals with no significant difference between the groups ( $p > 0.05$ ).

Table 2. Clinical characteristics in patients with collagenous colitis at the time of the investigation. Concentrations of ECP, MPO and albumin in serum and perfusion fluids from rectum and descending colon are also shown.

Patients	Age (years)	Gender	Disease duration (years)	Symptom duration (year)	Bowel movements (per day)	Current treatment (dose per day)	Rectum			Descending colon			Serum		
							ECP (µg/L)	MPO (µg/L)	Albumin (mg/L)	ECP (µg/L)	MPO (µg/L)	Albumin (mg/L)	ECP (µg/L)	MPO (µg/L)	Albumin (g/L)
1	54	female	2	3	0	losartan 50mg metoprolol	3.7	<8	3.4	3.3	<8	<3	8.9	146.0	48.0
2	61	female	1	1	3-5	ispaghula citalopram 10mg	11.8	<8	40.0	7.8	<8	8.0	15.3	375.0	46.0
3	36	female	2	12	8-10	none	4.1	<8	25.0	-	-	-	15.2	526.0	43.0
4	36	male	2	7	3	mesalazine 1g	3.6	<8	16.0	13.7	<8	7.9	-	131.0	46.0
5	49	female	2	3	15-20	none	25.6	157	26.0	18.4	88	44.0	9.3	317.0	34.0
7	76	female	2	3	10	omeprazole 20mg	3.5	<8	12.0	3.9	<8	<3	5.4	413.0	40.0
11	58	female	2.5	7	5-6	ispaghula	2.5	<8	6.7	15.3	38	91.0	3.0	98.0	37.5
12	53	female	2	20	2	mesalazine 1,2 g	8.0	<8	12.0	3.8	<8	51.0	16.1	442.0	42.0
19	85	female	2	2	4-5	none	<2	<8	6.5	<2	<8	33.0	8.3	3.3	39.0
21	51	female	3	35	6-7	none	<2	<8	3.7	<2	<8	7.5	5.8	7.5	-

The detection limits in perfusion fluids of ECP, MPO and albumin were 2 µg/L, 8 µg/L and 3 mg/L. The reference values in serum of ECP and MPO and albumin were 2.3-16 µg/L, 170-478 µg/L and 40-51 g/L (37 - 48g/L > 50 years), respectively. - = analysis not performed.

Table 3. Clinical characteristics in controls at the time of the investigation. Concentrations of ECP, MPO and albumin in serum and perfusion fluids from rectum and descending colon are also shown.

Controls	Age (years)	Gender	Indications for colonoscopy	Current treatment (dose per day)	Rectum			Descending colon			Serum		
					ECP (µg/L)	MPO (µg/L)	Albumin (mg/L)	ECP (µg/L)	MPO (µg/L)	Albumin (mg/L)	ECP (µg/L)	MPO (µg/L)	Albumin (g/L)
8	56	male	GI-bleeding	none	5.3	<8	5.3	4.4	<8	<3	5.0	174	-
9	53	female	polyp	none	3.3	<8	<3	5.9	12	<3	10.3	316	-
10	61	female	GI-bleeding	levothyroxinnatr 0.1mg cyanocobalamin 1mg	2.0	<8	11	-	-	-	9.3	215	-
13	53	male	polyp	none	3.4	<8	6.1	3.2	<8	<3	5.9	265	-
16	45	female	polyp	citalopram 20mg	<2	8	<3	-	-	-	-	-	47
17	60	female	GI-bleeding	none	<2	<8	<3	-	-	-	7.0	177	41
22	53	female	polyp	none	<2	<8	6.2	<2	<8	<3	7.0	165	-
23	47	female	IBS	none	<2	<8	9.1	<2	<8	5.7	3.2	113	43
24	71	female	(healthy volunteer)	none	<2	<8	4.4	<2	<8	12	10	283	-
25	50	Female	polyp	citalopram 20mg	<2	<8	12	<2	<8	4.4	4.0	118	44

The detection limits in perfusion fluids of ECP, MPO and albumin were 2 µg/L, 8 µg/L and 3 mg/L. The reference values in serum of ECP and MPO and albumin were 2.3-16 µg/L, 170-478 µg/L and 40-51 g/L (37 - 48g/L > 50 years), respectively. - = analysis not performed.



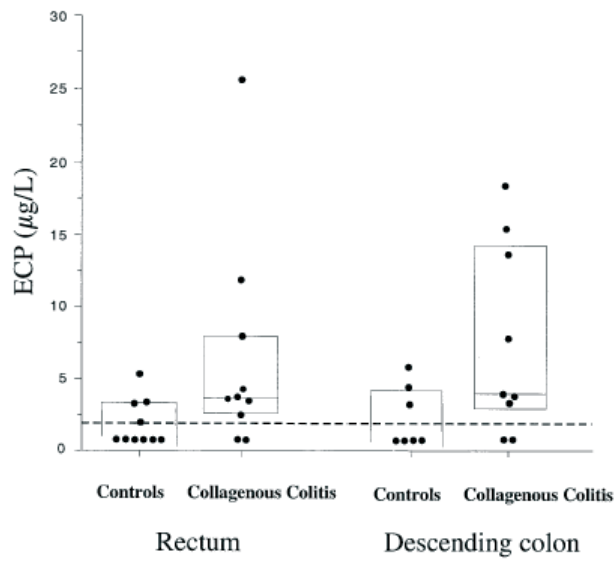


Figure 4. The individual values of eosinophil cationic protein concentrations in perfusion fluids from rectal and colonic segments in collagenous colitis and controls are shown. The boxes represent the interquartile ranges and the line in the boxes represents the median values. The interrupted line indicates the detection limit of the assay (2µg/L).

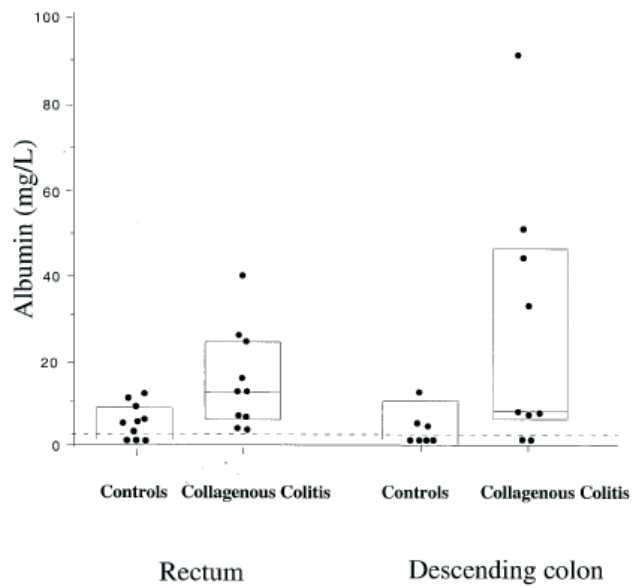


Figure 5. The individual values of albumin concentrations in perfusion fluids from rectal and colonic segments in collagenous colitis and controls are shown. The boxes indicate the interquartile ranges and the line in the boxes indicate median values. The interrupted line indicates the detection limit of the assay (3 mg/L).

### Basic fibroblast growth factor (Paper II)

The median concentration of bFGF in rectal perfusion fluid from CC patients was 1.92 pg/mL (range 0.49-25.13 pg/mL), which was significantly increased ( $P < 0.01$ ) compared to the median concentration of controls, which was 0.49 pg/mL (range 0.49-1.38 pg/mL) (Fig. 6). In descending colon the corresponding median concentration of bFGF in perfusion fluid was 1.97 pg/mL (range 0.49-19.22) in CC patients and 0.49 pg/mL (0.49-3.66) in the controls (Table 4) (Fig. 6)). This difference is also statistically significant ( $P < 0.05$ ). When the median concentrations of bFGF in rectal and descending colon perfusates were compared, no difference was found neither among CC patients nor control patients ( $p > 0.05$ ).

Table 4: The concentration of bFGF (pg/mL) in perfusion fluid (rectum and descending colon) and serum in patients with collagenous colitis and controls.

	<i>Case no</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>
Collagen colitis	Rectum	0.65	1.82	3.60	<0.50	25.13	2.09	0.80	2.03	1.41	6.69
	Colon	0.65	1.67	-	2.47	7.37	<0.50	2.65	1.97	1.94	19.22
	Serum	1.62	4.37	4.04	1.31	3.90	2.09	2.10	1.03	<0.50	4.90
	<i>Control no</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>
Controls	Rectum	1.38	<0.50	<0.50	0.86	<0.50	0.68	<0.50	<0.50	<0.50	<0.50
	Colon	0.77	3.66	-	<0.50	-	-	<0.50	0.65	<0.50	0.50
	Serum	2,20	5.83	1.27	3.77	-	0.94	3.59	6.00	4.94	-

*The detection limit (in perfusion fluids) of bFGF was 0.50 pg/mL. The reference value in serum of bFGF are below 7 pg/mL, - = analysis not performed.*

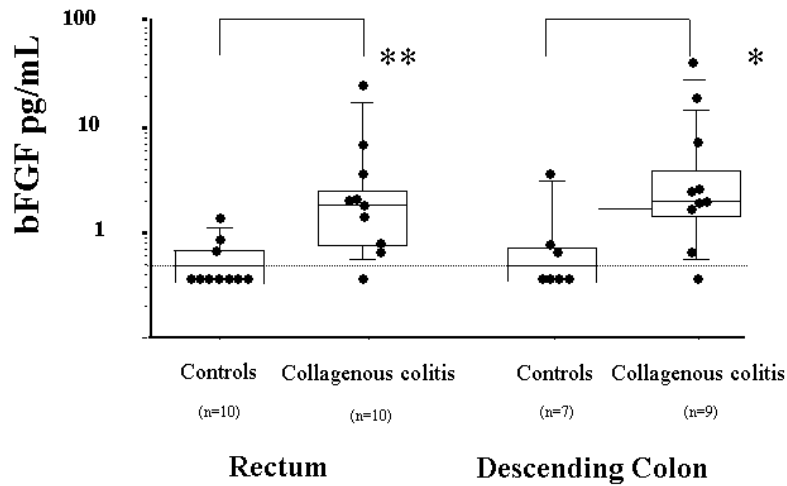


Figure 6. Individual values of bFGF concentrations in perfusion fluids from rectal and colonic segments in patients with collagenous colitis and controls. The boxes indicate the interquartile ranges and the line in the boxes indicates median values. The dotted line indicates the detection limit for the assay (0.50 pg/mL).

### Vascular Endothelial Growth Factor (Paper III)

The VEGF median concentration in rectal perfusion fluid of CC patients was 82.9 pg/mL (range 54.1-145.7) and the corresponding values for controls were 30.7 pg/mL (5.1-158.9) meaning that VEGF concentration in CC patients is significantly increased ( $p < 0.01$ ) compared to controls. In descending colon, the corresponding values of VEGF concentration in perfusion fluid were 126.3 pg/mL (30-524.8) in CC patients and 30.7 pg/mL (6.8-82.4) in the controls, i.e. a four-fold difference ( $p < 0.01$ ) (Table 5)(Fig. 7)). The median of VEGF concentrations in the descending colon perfusion fluids was higher compared to rectum ( $p = 0.04$ ). Furthermore, a positive correlation ( $p = 0.03$ ) was found between the concentration of VEGF and albumin in perfusates of descending colon. The difference between the VEGF median serum concentrations of CC patients and controls was not significant. Finally, no correlations were found between the concentrations of VEGF in perfusates and the thickness of the subepithelial collagen band or the number of mucosal inflammatory cells of CC patients.

Table 5. The concentrations of VEGF (pg/mL) in perfusion fluid (rectum and descending colon) and serum, in patients with collagenous colitis and control patients.

	Case no	1	2	3	4	5	6	7	8	9	10
CC patients	Rectum	55.9	102.8	165.7	95.3	87.9	76.9	54.1	73.2	115.0	77.8
	Colon	30.7	168.6	-	126.3	157.9	70.5	524.8	103.7	154.1	77.8
	Serum	159.5	134.0	389.0	473.0	328.9	503.2	503.2	106.5	158.6	298.0
Control patients	Case no	1	2	3	4	5	6	7	8	9	10
	Rectum	57.7	20.9	85.2	17.4	5.1	20.9	158.9	41.5	28.9	32.5
	Colon	6.8	13.0	-	30.7	-	-	45.1	45.1	77.8	82.4
	Serum	304.6	221.1	185.2	272.0	-	122.1	123.0	126.7	232.1	342.0

- = Analysis not performed

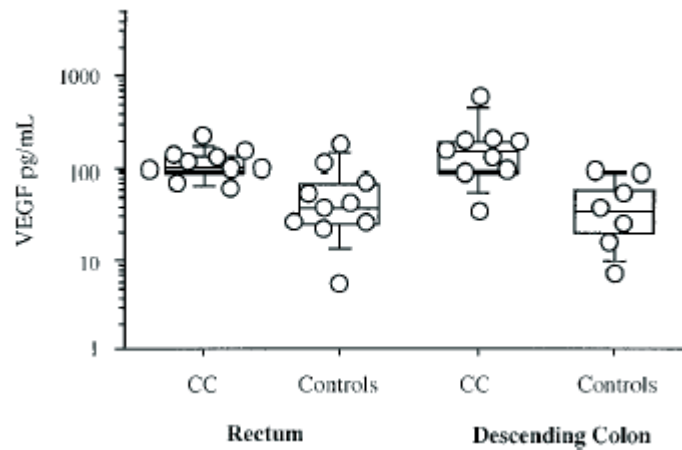


Figure 7. VEGF concentrations in rectal and colonic perfusions fluid of CC patients and controls are shown as individual values. The interquartile ranges are represented by boxes and the line within boxes is the median value.

### The subepithelial collagen band (paper II & III)

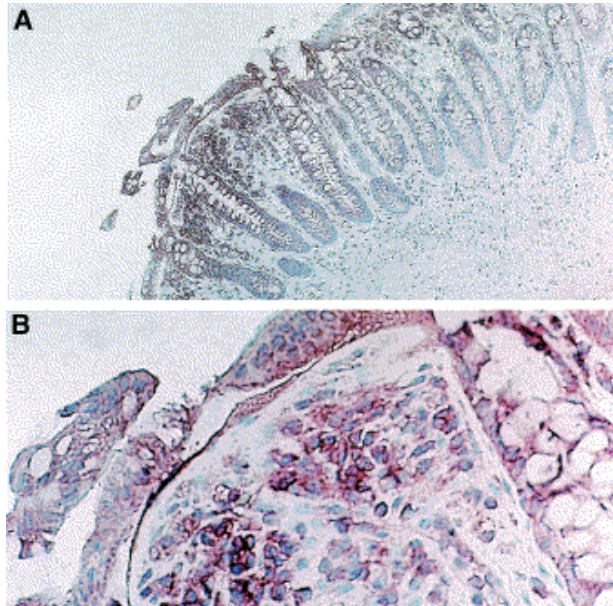
Fifty-five biopsy samples were available from all CC and control patients who underwent perfusions in both rectum and descending colon or in only one of them. In one sample, surface epithelium was of poor quality; all other samples consisted of vertical sections with good quality. Biopsies were obtained from ascending colon (n=3), descending colon (9), sigmoid colon (5) and rectum (10) in CC patients. Among controls the corresponding numbers of biopsies were 3, 8, 7, and 10, respectively.

The median value of subepithelial collagen band thickness in CC patients from descending colon was 17  $\mu\text{m}$  (range 0.5-28.0) and from rectum 9.5 $\mu\text{m}$  (3.0-21.0). In control patients the corresponding values were 2.0  $\mu\text{m}$  (range 0.5-6.0) and 5.5 $\mu\text{m}$  (0-8.0), respectively. As expected, the differences were significant in both segments. In CC patients, the thickness of the subepithelial collagen layer was more marked in the descending colon than in the rectum ( $p < 0.05$ ). The median numbers of inflammatory cells per 100 colon epithelial cells were 10 in descending colon from CC patients and 12 in control patients. The corresponding numbers in rectum were 21 and 15, respectively.

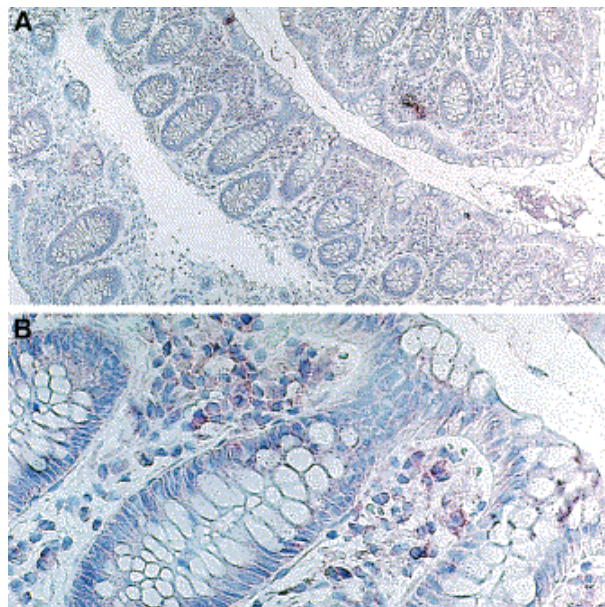
### Immunohistochemical analysis of bFGF (Paper II)

Expression of bFGF was seen both in the surface epithelium and in the lamina propria of colon mucosa of patients with CC (Fig. 8) and a similar, but sometimes weaker, staining pattern was detected in control patients (Fig. 9).

The immunohistochemical staining of bFGF in the surface epithelium was located within the epithelial cells, usually between the crypts and in exudates on top of epithelial cells towards the lumen. In lamina propria, inflammatory cells and fibroblasts contained bFGF. Expression was most prominent in the immediate subepithelial region. The intensity of the staining differed in the samples but this difference was not significant between CC patients and controls, neither in the surface epithelium nor in lamina propria (data not shown). There was no correlation between the subepithelial collagen thickness and the intensity of the staining, neither in surface epithelium nor in lamina propria (data not shown).



*Figure 8.* Immunohistochemical staining of bFGF in a specimen from rectal colon in a patient with collagenous colitis is shown. Magnification x100 (A) and x400 (B). Immunohistochemical localization was done with specific antibody labelling using an avidin-biotin peroxidase technique.



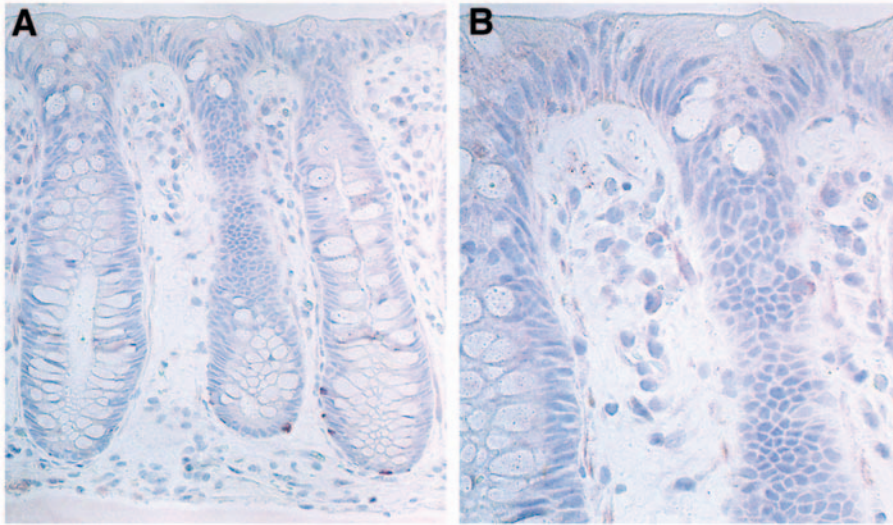
*Figure 9.* Immunohistochemical staining of bFGF in a specimen from sigmoid colon in a control patient is shown. Magnification x100 (A) and x400 (B). Immunohistochemical localization was done with specific antibody labelling using an avidin-biotin peroxidase technique.



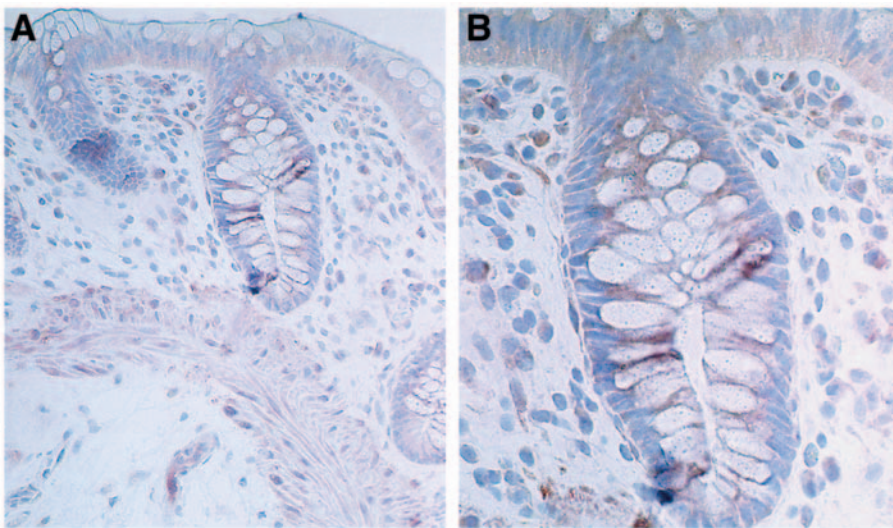
### Immunohistochemical analysis of VEGF (Paper III)

The total amount of samples obtained from both CC patients and controls for immunohistochemical analysis of VEGF in surface epithelium and lamina propria of the colon, were 51 (5 samples were not included because the surface epithelium found was of poor quality) and 56, respectively. The samples of colonic surface epithelium analysed from ascending, descending and sigmoid colon and rectum were 2, 8, 5 and 10 in CC patients, and the corresponding values in control patients were 3, 8, 6 and 9. Lamina propria samples obtained from ascending, descending, sigmoid colon and rectum were 3, 10, 5 and 10 in CC patients, while 3, 8, 7 and 10 were samples obtained from corresponding regions of the colon in controls.

The expression of VEGF was located mainly within inflammatory cells and in extracellular space of lamina propria and surface epithelium (Fig. 10 and 11). The intensity of the VEGF expression varied greatly, both between the samples of each individual participant and also between the samples within each group of CC patients or control patients. The median of the relative VEGF staining intensity in the rectal surface epithelium was 19 (2-37) in CC patients and 25 (1-54) in the controls. The difference was not significant ( $p=0.06$ ). The corresponding comparison in the descending colon did not differ ( $p=0.17$ ). The relative intensity in lamina propria was significantly ( $p<0.05$ ) higher among controls compared to CC patients; in rectum, values were 39.0 (range 4-58) versus 18.5 (5-41) and in descending colon, they were 41.5 (2-55) versus 18 (6-47).



*Figure 10.* A specimen from rectal colon in a patient with collagenous colitis is immunohistochemically stained by antibodies towards VEGF, and is shown with magnification x200 (A) and x400 (B). A specific antibody labelling, based on an avidin-biotin peroxidase technique, is used to visualize antibodies bound to VEGF.



*Figure 11.* A specimen from descending colon in a control patient without collagenous colitis is immunohistochemically stained by antibodies towards VEGF, and is shown with magnification x200 (A) and x400 (B). A specific antibody labelling, based on an avidin-biotin peroxidase technique, is used to visualize antibodies bound to VEGF.

## Steroid Treatment and local inflammatory mediators (Paper IV)

### **Perfusions and clinical outcome**

Out of Twelve CC patients studied, eleven successfully completed steroid treatment and all three perfusions according to the protocol. The excluded patient stopped taking steroids because of side effects. The number of bowel movements declined from median 5 before start of treatment, to 0-1 after one week, and 0-1 after four weeks, respectively. These differences were significant ( $p < 0.01$ ) (Table 6). All patients reported that their sense of general well-being was better after the start of steroid treatment, compared to the period before.

### **Analysis of ECP, MPO, Albumin, bFGF and VEGF in perfusate**

The concentrations of ECP, MPO, albumin, bFGF and VEGF in perfusates are shown in table six.

The median values of ECP concentrations in the rectal perfusates at week zero, one and four were 2.3  $\mu\text{g/L}$  (range 1.9-6.5), 1.9 (1.9-7.0) and 1.9 (1.9-1.9), respectively. The decline over time of ECP concentrations, assessed by Friedman's ANOVA, was close to significant ( $p = 0.0608$ ). Further comparisons of ECP perfusate concentrations were done between weeks zero, one and four by Wilcoxon signed rank test, and the difference was significant between week one and four ( $p < 0.05$ ).

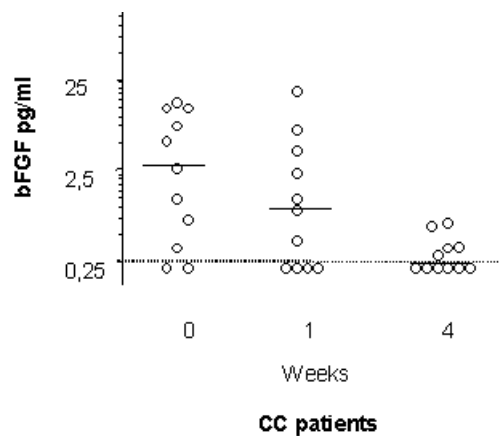
Three and one patients at week zero and one, respectively, had perfusate concentrations of MPO above the detection level (Table 6).

The median values of albumin concentrations in perfusates were 20.3  $\text{mg/L}$  (2.7-116), 10.7 (2.13-57.7) and 3.4 (2.13-17.5) at weeks zero, one and four, respectively. The decline over time of albumin perfusate concentrations was without significant when analyzed by Friedman's ANOVA.

The median values of bFGF concentrations in rectal perfusion fluids were 2.7  $\text{pg/ml}$  (range 0.2-14.0), 0.9 (0.2-18.3) and 0.2 (0.2-0.7) at week zero, one and four, respectively. A significant decline over-time in the concentrations of bFGF was found during the four-week study period. ( $p < 0.05$ ) (Fig 12).

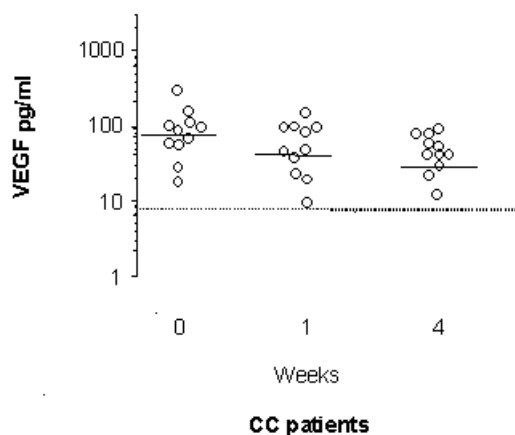
Table 6. The clinical data and the individual concentrations of ECP, MPO, bFGF, VEGF and albumin in rectal perfusion fluid of CC patients before and after one and four weeks of treatment with steroids.

Patients	Demography		Bowel movements			Mediators analyzed in Perfusion fluid														
	Age (years)	Gender	per day			ECP ( $\mu\text{g/L}$ )			MPO ( $\mu\text{g/L}$ )			bFGF (pg/mL)			VEGF (pg/mL)			Albumin (mg/L)		
			0	1	4	0	1	4	0	1	4	0	1	4	0	1	4	0	1	4
1	34	Female	3-4	0-1	0-1	3.7	<2	<2	29.1	14.9	<8	14.03	18.26	0.65	299.7	92.8	41.0	20.3	44.6	<2.1
2	57	Female	4	0-1	0-1	2.6	<2	<2	12.5	<8	<8	7.85	7.11	0.37	54.7	97.0	40.2	95.8	57.7	<2.1
3	59	Female	10-12	0-1	0-1	3.6	7.0	<2	<8	<8	<8	1.21	4.10	<0.25	113.1	146.7	81.1	20.3	10.7	3.4
4	43	Female	4-6	3-4	0-1	<2	<2	<2	<8	<8	<8	0.72	0.41	<0.25	28.4	22.9	40.2	2.8	10.9	17.5
5	62	Female	3-4	0-1	0-1	<2	<2	<2	<8	<8	11.3	0.35	<0.25	0.29	66.2	46.6	53.1	2.7	10.7	5.5
7	66	Female	4-5	0-1	0-1	<2	<2	<2	<8	<8	<8	<0.25	<0.25	<0.25	58.8	19.1	22.2	3.9	6.5	3.2
8	59	Female	5-10	2	0-1	6.5	<2	<2	<8	<8	<8	5.28	<0.25	0.35	86.1	37.8	58.8	17.1	3.1	2.3
9	64	Female	3	0-1	0-1	<2	<2	<2	<8	<8	<8	<0.25	<0.25	<0.25	17.6	9.6	12.3	5.0	<2.1	7.0
10	39	Male	7-10	2-3	0-1	<2	<2	<2	<8	<8	<8	2.70	1.21	<0.25	100.4	94.5	90.3	43.0	13.7	11.7
11	34	Female	5-6	2	0-1	3.1	<2	<2	18.3	<8	<8	11.99	235	<0.25	153.6	84.4	80.2	116.0	22.1	10.7
12	53	Male	6-7	2-3	0-1	23	<2	<2	<8	<8	<8	12.29	0.91	0.61	95.3	48.2	29.1	21.5	52	<2.1



*Figure 12.* The individual values of bFGF concentrations in perfusion fluids from rectum of CC patients before and after 1 and 4 weeks of steroid treatment are shown. The bars indicate median values. The dotted line indicates the detection limit for the assay (0.25pg/ml).

The median values of VEGF concentrations in perfusates of these individuals at week zero, one and four were 86.1 pg/ml (17.6-299.7), 48.2 (9.6-146.7), and 41.0 (12.3-90.3), respectively. The decrease of VEGF concentrations over time between weeks zero and four was significant (Fig. 13). Furthermore, there was a significant correlation between VEGF and ECP at week zero and one ( $p < 0.05$ ,  $R = 0,627$  and  $0,625$ ), but not at week four. Similar significant correlations were present between VEGF and bFGF concentrations at week zero and one ( $p < 0.05$ ,  $R = 0.68$  and  $p < 0.01$ ,  $R = 0.85$ ), but not at week four.



*Figure 13.* The individual values of VEGF concentrations in perfusion fluids from rectum of CC patients before and after 1 and 4 weeks of steroid treatment are shown. The bars indicate median values. The dotted line indicates the detection limit for the assay (VEGF = 9 pg/ml).

Furthermore, the following significant correlations were found in perfusates between:

- VEGF and ECP at week zero and one ( $p < 0.05$ ).
- VEGF and bFGF concentrations at week one and four ( $p < 0.01$  and  $p < 0.05$ ).
- bFGF and ECP at week zero, but not at week one and four.
- bFGF and albumin in perfusates remained significant throughout the study period ( $p < 0.05$ ).

The median serum concentrations of ECP, bFGF, VEGF and albumin at weeks zero, one and four were 11.3  $\mu\text{g/L}$  (1.9-29), 6.6 (1.9-18.8) and 6.35 (1.9-18.9); 9.1  $\text{ng/L}$  (1.7-44), 2.9 (0.9-17.6) and 3.6 (0.3-20.9); 842  $\text{ng/L}$  (622-1517), 775 (385-1215) and 811 (574-1114); and 42  $\text{g/L}$  (38-45), 43 (34-48) and 42 (36-46), respectively. No significant decline in the serum concentrations of these mediators was found when analyzed by Friedman's ANOVA.

## Discussion

The fixed catheter technique for perfusion of the rectum and sigmoid colon<sup>85</sup> was modified and a colonoscope-based segmental perfusion technique was used in all studies<sup>88</sup>. The results showed that the technique is safe to perform in the rectum and descending colon, and provides an opportunity to investigate an optional segment of the colon up to the splenic flexure. If the technique is further developed and refined, perfusion may be carried out in any segment of the colon, including the ileocaecal region.

The perfusate concentrations of ECP, bFGF, VEGF and albumin were increased in CC patients compared to control patients (paper I, II and III), and a decrease of these inflammatory mediators and albumin concentrations during oral steroid treatment was observed, even if the changes over time in albumin concentrations didn't reach statistical significance.

Additionally, significant correlations were found between the increased concentrations of albumin and ECP or VEGF in the CC patients included in paper I and II, which suggests a possible role for these mediators in the permeability mechanisms of CC. The disappearance of initially existing correlations between ECP, bFGF and VEGF concentrations at end of the steroid treatment study, implies that corticosteroids normalize the secretion of these mediators. Decreased bowel movement frequency and improved general health during corticosteroid treatment may also be explained with normalized secretion of mediators.

The ECP, bFGF, VEGF and albumin serum concentrations of CC patients were not different compared with controls. The changes in the serum concentrations of these inflammatory mediators and albumin during corticosteroid treatment period were not significant, indicating that the pathophysiological process in CC is better studied locally with perfusion techniques.

In previous perfusion studies a fixed catheter technique for perfusion of the rectum<sup>86</sup> or simultaneous rectal and sigmoid perfusion<sup>85</sup> were applied. Since the distribution of the collagenous layer and the inflammatory cell infiltrates in CC is non-homogenous<sup>21, 65, 81, 89</sup>, it is of interest to examine different parts of the large intestine. Therefore, we adapted the perfusion segment to a colonoscope and made it possible to investigate an optional segment of the colon up to splenic flexure. Few attempts were done to pass the splenic flexure and we found that further refinement of the technique, using a stabilized perfusion segment, is required to examine the proximal colon in a safe manner.

Introduction of the instrument and perfusion segment into the rectum was easy and were successful in 33 cases out of 37, i.e. the rate of success was 89%. The technical problems were minor in most cases, except for 2 attempts where the perfusion procedures were discontinued because of pain. Otherwise, the discomfort experienced by these patients during perfusion was minimal. No mucosal damage could be seen in the rectum after the perfusions, although the endoscope had passed this area when it was introduced to, and withdrawn from, the descending colon. Regarding the perfusion of the descending colon, all subjects experienced some degree of pain, and it was technically more challenging to enter the descending colon with the perfusion segment attached to the endoscope compared with routine colonoscopy. The frequency of success in descending was 80%, i.e. 20 out of 25 attempts. The fact that all patients but one had a normal endoscope appearance in the descending and sigmoid colon after completion of the second perfusion, still indicates that the technique apparently was non-traumatic.

The recovered perfusate volumes varied over time in the colonic segments but not in rectal perfusions. This was also the case in previous studies, using the fixed catheter system<sup>85</sup>. This is probably due to normal contractions/dilations of the perfused intestinal segment which are more marked in the colonic segment than in the rectal segment. The significant difference found in the volumes collected in our first study (paper I), is probably due to the small number of colonic perfusions. Most likely the difference will disappear when more investigations are done.

The small or negligible concentrations of phenol red detected in the perfusates, indicate minor or no contamination by intestinal fluids proximal to the closed segment. The minor discomfort (once the position was reached) experienced by the subjects, further supports that segmental perfusion can be performed in an optional part of the colon-rectum.

### The role of ECP, bFGF and VEGF in CC tissue remodelling

The two- to five-fold increase of ECP concentration in perfusates from CC patients compared to controls in paper I, and the decrease of ECP under oral steroid treatment in paper IV, confirms the finding in other studies<sup>24, 90, 91</sup> that local activation of eosinophils exists in CC. Furthermore, it can be concluded that this local eosinophil activation is down-regulated by corticosteroids.

The eosinophilic granulocytes are regarded as potent proinflammatory and profibrotic cells with the capacity to release several highly cytotoxic proteins, such as eosinophil cationic protein (ECP), transforming growth factor-beta (TGF- $\beta$ 1) and major basic protein (MBP)<sup>92</sup>. During injury, these proteins cause damage to airway epithelium and perpetuate chronic inflammation leading to fibrosis in diseases such as asthma bronchiole, idiopathic hypereosinophilic syndrome, lung fibrosis, IBD, etc<sup>24, 91, 93, 94</sup>. Perfusion



studies on UC patients have shown that ECP granule protein concentrations are increased in perfusates compared to controls <sup>95</sup>, and their concentrations in perfusates were reduced during corticosteroid treatment <sup>96</sup>. These findings and similar observations in Crohn's disease, imply that there is activation of eosinophils in IBD <sup>97</sup>, including CC, even if the degree of activation is much higher in both Crohn's disease and UC <sup>95-97</sup>, compared to CC. Therefore we suggest that eosinophils, through their proinflammatory and fibrotic cytokines, promote remodelling of ECM in, CC resulting in subepithelial collagen and Tenascin deposition.

Beside ECP, another inflammatory mediator in the present study is bFGF, which is a potent multifunctional growth factor that stimulates proliferation and differentiation of fibroblasts, and participates in wound healing and angiogenesis <sup>98</sup>. An interesting finding was a marked increase of bFGF concentration observed in CC patients as compared to controls (paper II). The elevated levels of bFGF concentrations were completely reversed following administration of corticosteroids. Thus, it is reasonable to assume that bFGF, among others, contributes to the mucosal changes in these patients and are, probably, even responsible to at least some of the clinical symptoms recorded in these CC patients.

Higher perfusate bFGF concentrations in CC patients compared to controls (paper II), significant downfall of bFGF concentrations and the disappearance of the correlations between bFGF and ECP or VEGF during steroids treatment (paper IV), implies that bFGF have a role to play in the pathogenesis of CC. This is in line with findings in IBD, where in vitro experiments showed that bFGF-stimulated fibroblasts/myofibroblasts from IBD patients, produce more collagen compared to corresponding cells from controls <sup>67</sup>. A segmental and rectal perfusion study conducted on UC patients showed that the median concentration of bFGF was much higher, almost 10-fold compared to healthy controls <sup>99</sup>. But the corresponding values in CC patients were only 4-fold compared to controls (paper II), probably reflecting the fact that mucosal inflammation is milder in CC than in UC. These findings in IBD and CC enable us to suggest that the proinflammatory and profibrotic nature of bFGF are parts of the pathophysiology in these diseases. In CC, increased numbers of pericryptal myofibroblasts with sign of synthetic activity are entrapped within the collagen layer <sup>23</sup>. The numbers of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), expressing myofibroblasts, were also higher in CC compared to in normal colonic mucosa <sup>47</sup>, suggesting once again that the myofibroblasts in CC are synthetically active. Thus, these observations and our findings put forward the hypothesis that pericryptal myofibroblasts are synthetically active in CC <sup>41, 47, 60</sup>, leading to pathological subepithelial collagen accumulation. In addition, the decrease of bFGF concentrations during steroid treatment suggests that the activation of myofibroblasts, and their secretion and release of bFGF, are down-regulated by corticosteroids in CC. This hypothesis is further supported by the down-regulating effect of corti-

costeroids on the proliferative action of bFGF on fibroblasts in asthma and nasal polypoid lesions<sup>100,101</sup>. This may also be the explanation for the diminished collagen layer and other inflammatory signs observed in corticosteroid-treated CC<sup>102-104</sup> and asthma<sup>105</sup> patients. Our finding of a correlation between bFGF and ECP perfusate concentrations suggests that bFGF, separately or in relation with other proteins, perpetuates chronic inflammation and trigger tissue remodelling in CC.

The pathophysiological complexity of CC is further illustrated by our detection of significantly higher VEGF concentrations in perfusion fluids of descending colon and rectum in CC patients compared to controls, and the significant decrease of VEGF concentrations during steroid treatment. VEGF mediates numerous biological effects<sup>68-70</sup> that are both physiological and pathophysiological. VEGF is a potent angiogenic, mitogenic, inflammation, permeability and fibrosis enhancing peptide<sup>69,71</sup>, and it is secreted by different types of cells, including eosinophils and mesangial cells<sup>93,106</sup>.

VEGF in active IBD enhances fibrosis, vascular permeability, angiogenesis, mitogenic stimulation of endothelial cells and inflammation leading to alterations of the ECM of the colonic mucosa<sup>71,72</sup>. It has been demonstrated that VEGF levels are significantly increased in intestinal mucosa of active UC and Crohn's disease, but not in their non-active states<sup>73,107</sup>. These findings and our observations that VEGF can be detected at significantly higher concentrations in perfusion fluids of descending colon and rectum in CC patients compared to controls, together with decreased VEGF during concentrations under corticosteroids treatment, support that VEGF participates in the pathogenesis of IBD including CC. It is possible that VEGF, through its induction of Connective Tissue Growth Factor (CTGF)<sup>70</sup> and as one of the eosinophil lysates, enhances proliferation of fibroblasts<sup>108,109</sup>, leading to tissue remodelling in CC. This possibility is further strengthened by the correlations found between VEGF, ECP and bFGF before corticosteroid treatment, and their absence after four weeks of treatment.

The tissue remodelling which occurs in CC is most probably a result of both increased synthesis<sup>40,41,60,65,66,110</sup> and decreased degradation<sup>43,45</sup> of ECM including collagen. It is discussed above that eosinophilic granule protein ECP, bFGF and VEGF participate in the tissue remodelling process by enhancing collagen and other components of ECM synthesis. In contrast, there are other data showing that the pathological accumulation of collagen in CC is the product of decreased collagen degradation, and not synthesis. The absence of increased collagen mRNA levels, the imbalance of MMP/TIMP<sup>43</sup> in favour of TIMP, as well as increased TGF- $\beta$ <sup>24</sup> are the main reasons for impaired degradation of ECM in CC. It is assumed that high matrix turnover is associated with excessive accumulation of the non-collagenous matrix component Tenascin<sup>43,44,46</sup>. Tenascin is the dominating part in immature ECM of tumours and inflammatory states, and its staining is more sensitive than collagen staining in the diagnosis of CC. VEGF and Tenascin

interaction, result in cell proliferation and altered ECM. Furthermore, in immature ECM, Tenascin is degraded at higher speeds by MMP-1 because VEGF stimulates MMP-1 and inhibits TIMP<sup>111,112</sup>. The findings in our studies concerning VEGF, underline the suggestion of other authors that VEGF alters the balance between MMP/TIMP<sup>48</sup>, leading to tissue remodelling through the deposition of immature ECM in CC.

An additional role for eosinophils in repairing injured epithelium in UC, has been put forward because of high eosinophil activation during inactive UC<sup>113</sup>. Tissue repair is associated with the ability of eosinophils to produce growth factors like TGF- $\beta$ <sup>114</sup> and VEGF<sup>48</sup>. In CC, VEGF immunostaining was significantly increased within the epithelium and the inflammatory cells of lamina propria. This expression remained significant within the epithelium even if clinical remission was accompanied by normalization of CC histopathological changes after budesonide treatment, suggesting that VEGF has a role to play in tissue repair.

The entrapment of superficial capillaries within the thickened subepithelial collagen band is common in CC, and this may be associated with the angiogenic property of VEGF. Based on VEGF findings in our study and the observation that VEGF expression is abundant within the epithelium and in the inflammatory cells of the lamina propria<sup>48</sup>, we suggest that VEGF has a role to play in the growth of these blood vessels, as in the case of metastasis of tumour, tissue repair and angiodysplasia of the colon<sup>115</sup>.

### ECP and VEGF and their role in mucosal permeability

VEGF is a potent vascular permeability enhancing cytokine, and it is over-expressed in inflammatory disorders, tumour growth and other conditions<sup>116,117</sup>. The central role that VEGF plays in promoting permeability in these disorders is not controversial. However, the relevance of this property in CC is not fully established. The positive correlation we found between elevated concentrations of ECP (paper I) and VEGF (paper III), with albumin in colonic perfusates of CC patients, suggests that ECP and VEGF, separately or together with other mediators, enhance permeability. In paper IV, the decreases of both VEGF and albumin concentrations run parallel to each other during corticosteroid treatment. But the timely reduction of albumin was not significant, and therefore the correlations we found before corticosteroid treatment should be interpreted with caution. Possibly, the reduction was not significant because the number of patients included in the study was too small, and perfusion was carried out in rectum, where the inflammatory process in CC is less expressed. Moreover, it is possible that VEGF increases mucosal vascular permeability in CC by modulating the effects of calcium-sensitive enzymes, thereby inducing increased production of nitric oxide (NO), which is associated with diarrhoea. The reduction of VEGF secretion and its availability in the colonic mucosa of CC patients caused by

corticosteroids in our study (paper IV), suggest that corticosteroids inhibit the enhanced NO production resulting in the diminished diarrhoea frequency (paper IV).

### Immunohistochemistry

Immunohistochemical staining of bFGF and VEGF is an expression mainly on the surface of colon epithelial and inflammatory cells, fibroblasts and ECM. Unexpectedly, there were no significant differences in intensity of the bFGF and VEGF expressions between CC and control patients, with the exception of VEGF expression in rectal and colonic lamina propria of control patients, which was more intensely stained compared to CC patients. One possible explanation for this result is that immunohistochemical detection and grading is less sensitive, compared to quantitative analysis based on perfusates from a larger part of the colonic mucosa. The patchy distribution of inflammation in the colonic mucosa of CC patients could have contributed to the result, in addition to technical failure. A recent study showed that VEGF immunohistochemical staining was significantly expressed in CC patients compared to controls <sup>48</sup>. The immunohistochemical staining technique used in that study was different from ours, regarding the origin of antibody and the method of staining. Such a kind of study concerning bFGF is not yet available in CC. Finally, more sensitive methods exist, such as in situ hybridization, to locate synthesis of bFGF and VEGF in colonic mucosa. These methods should be used in future studies concerning the role of bFGF and VEGF in CC pathophysiology.

## Conclusions

- A colonoscope-based, segmental perfusion technique was developed which enabled safe perfusion in the rectum and descending colon.
- Patients with collagenous colitis had elevated perfusate concentration of ECP, suggesting that eosinophilic activation may participate in the inflammatory reaction taking place in the colonic mucosa of CC patients.
- VEGF and bFGF concentrations were increased in the intestinal perfusates of CC patients, which supports the hypothesis that these inflammatory mediators enhance fibrosis, including collagen synthesis, in CC.
- The high perfusate albumin concentrations and the correlations between albumin and ECP or VEGF observed in these studies, imply that permeability is increased in CC and is probably triggered by ECP and VEGF.
- Steroid treatment improved the clinical features in our CC patients, and a concomitant marked reduction of the concentrations of ECP, bFGF and VEGF in colonic perfusates was observed. Our findings support a role of these inflammatory proteins in the pathogenesis of CC.
- The presented perfusion technique is beneficial when studying processes restricted to the colonic mucosa, which do not induce changes in the steady state conditions and serum concentrations of the studied inflammatory mediators.

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