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ORIGINAL ARTICLE

Changes in matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinases (TIMP) expression profile in Crohn's disease after immunosuppressive treatment correlate with histological score and calprotectin values

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Abstract
Background Matrix metalloproteinases (MMPs) constitute a family of enzymes capable of degrading various extracellular matrices (ECM) and basement membrane components playing a role in ECM turnover. They activate and degrade signaling molecules, such as cytokines and chemokines. MMPs are involved in inflammation and have been implicated in tissue degradation and repair occurring in inflammatory bowel disease. The aim of this study was to investigate the MMP profile of intestinal Crohn's disease (CD) patients before and after immunosuppressive treatment (anti-TNF-α agents or corticosteroids and conventional immunosuppressants azathioprine or methotrexate) to learn more about the therapeutic pathways for immunosuppressive agents.
Methods Expression of MMP-1, MMP-7, MMP-9, MMP-10, and MMP-26 and tissue inhibitors of metalloproteinases (TIMP)-1 and TIMP-3 was studied by immunohistochemistry in pretreatment and post-treatment tissue samples. Semiquantitative immunohistochemical scores were tested for correlations with fecal and serum inflammation markers as well as endoscopic and clinical disease activity scores.
Results Neutrophil MMP-9 (p=0.039) and MMP-26 (p=0.030) and stromal TIMP-1 (p=0.041) and TIMP-3 (p=0.029) decreased along with treatment. However, expression of TIMP-3 by enterocytes tended to increase. Total histological score demonstrated positive correlation with neutrophil MMP-9 (p=0.000), MMP-26 (p=0.014), and macrophage TIMP-1 (p=0.001). Calprotectin followed a similar pattern with stromal MMP-26 (p=0.011), TIMP-1 (p=0.000), and TIMP-3 (p=0.001). Crohn's disease endoscopic index of severity (CDEIS) value correlated positively with macrophage TIMP-1 (p=0.007) and stromal TIMP-3 (p=0.005). Epithelial TIMP-3 presented with negative correlations with CDEIS (p=0.006) and C-reactive protein values (p=0.004).
Conclusions Our results suggest that immunosuppressive drugs modulate disease activity in CD by downregulation of MMP-9 and MMP-26 positive neutrophils and stromal TIMP-1 and TIMP-3.

Keywords Calprotectin · CDAI · CDEIS · Infliximab · MMP-26

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Abbreviations

CD	Crohn's disease
MMP	matrix metalloproteinase
TIMP	tissue inhibitor of metalloproteinase
CDAI	Crohn's disease activity index
CDEIS	Crohn's disease endoscopic index of severity

Introduction

Crohn's disease (CD) is a chronic, relapsing inflammatory disorder that may affect any region of the gastrointestinal tract. It is characterized by transmural inflammation, narrowing of intestinal lumen, fistula formation, granulomas, and fibrosis [1]. Both genetic and environmental factors have been implicated in the pathogenesis of CD. A dysregulated response of the intestinal immune system to intraluminal antigens of bacterial origin predisposes to CD resulting in the activation and release of cytokines, eicosanoids, nitric oxide, and proteolytic enzymes, which initiate a cascade leading to intestinal injury [2, 3].

Matrix metalloproteinases (MMPs) constitute a family of 24 human enzymes collectively capable of degrading all extracellular matrices (ECM) and basement membrane components. However, they also regulate the activation of various growth factors and modify inflammatory reactions [4]. Several MMPs have been implicated in the pathobiology of human inflammatory bowel disease (IBD) being involved in tissue remodeling, angiogenesis, cell migration, and promotion of leukocyte extravasation in the actively inflamed area in the ulcer base in both CD and ulcerative colitis (UC) [5]. MMP-3 (stromelysin-1) is upregulated in CD [6–9] and both MMP-3 and MMP-9 (92 kDa gelatinase) have been associated with mucosal damage and fistulae in this disease [8, 10, 11]. MMP-9 is abundantly expressed in IBD and is pivotal for tissue damage [11]. The selective inhibition of both MMP-3 and MMP-9 has been suggested to be of therapeutic benefit in IBD [5]. MMP-1 (collagenase-1) is increased in the granulation tissue of CD [6, 12], may potentiate the inflammatory response, and take part in remodeling and monocyte migration [5]. MMP-1 is one of the highly upregulated genes in microarray studies on diseased areas of CD patients [13]. MMP-10 (stromelysin-2) is present in macrophages and T cells in CD stroma and in migrating enterocytes [14] and, together with MMP-7 (matrilysin-1), has been implicated to have a role in intestinal wound healing [15]. Instead, MMP-26 (matrilysin-2) is not significantly upregulated in the epithelium or stroma of CD patients [16]. Moreover, immune response can increase MMP-12 (matrix metalloelastase) in lamina propria (LP) macrophages [14, 17] and this proteinase may have a role in macrophage migration and tissue inflammation in CD [17].

A critical balance between MMPs and their tissue inhibitors (TIMPs) determines the outcome of tissue damage in CD. TIMP-1 and TIMP-3 are abundantly expressed in the granulation tissue of the stroma in active CD [6, 14]. TIMP-1 has been associated with fibrotic strictures of CD [8]. TIMP-3 is a potent inhibitor of TNF-α-converting enzyme. Single-nucleotide polymorphisms in genes coding for TIMP-1 and MMP-3 affect CD susceptibility and phenotype [18], further implicating the relevance of MMPs and TIMPs in the pathobiology of CD.

Corticosteroids represent standard treatment for CD but the use of *anti-TNF-α agents* is emerging in severe cases. The aim of this study was to assess the intestinal MMP profile of CD patients receiving corticosteroids and *conventional immunosuppressants* (*azathioprine* or *methotrexate*) or *anti-TNF-α agents* before initiation of treatment and after a few months to learn more about the therapeutic pathways for *immunosuppressive agents* and to identify possible therapeutic targets for selective MMP inhibitors.

Materials and methods

Patients and tissues

All patients gave their written informed consent for participation in this study, approved by the Ethics Committee of the Helsinki University Central Hospital, Finland. Seventeen adult patients with established CD diagnosis referred to ileocolonoscopy were recruited between January 2005 and June 2007 at Helsinki University Central Hospital, Helsinki, Finland. Of these patients, seven were female and ten were male, their age ranging between 19 and 47 years (mean 26.5 years). The disease duration, defined as months since diagnosis by endoscopy, ranged between 0 and 249 months (mean 74.76 months; Table 1). One patient was newly diagnosed. Indications for the baseline endoscopies were an acute flare (n=10), a chronic active disease (n=2), and a prolonged need of corticosteroid treatment (n=5). Endoscopy findings were scored according to Crohn's disease endoscopic index of severity (CDEIS) [19] and clinical activity according to Crohn's disease activity index (CDAI) [20]. In this study, CDAI <150 indicated clinically inactive disease and ≥150 active disease [21]. CDEIS 0 to 9 indicated inactive or mildly active CD and CDEIS >9 indicated moderately or severely active disease [22].

After endoscopy, 12 patients received *therapy with anti-TNF-α agent*, either infliximab infusion 5 mg/kg at week 0 and at week 8 (n=11) or subcutaneous adalimumab 80 mg followed by 40 mg every other week until week 8 (n=1; Table 1). Of the total 17 patients, five received steroid

Table 1 Clinical data for the patients

Patient	Sex	Age (years)	Indication for treatment	Disease location	Disease duration (years)	Treatment
1	M	22	Acute flare	Ileocolon	0.9	TNF-α-inhibitor, AZA
2	M	19	Chronically active disease	Ileocolon	0.4	TNF-α-inhibitor, AZA
3	F	23	Acute flare	Ileocolon	10.2	TNF-α-inhibitor, AZA
4	M	29	Acute flare	Ileocolon	8.1	TNF-α-inhibitor, AZA
5	M	26	Acute flare	Ileocolon	5.1	TNF-α-inhibitor, AZA
6	F	32	Chronically active disease	Ileocolon	15.5	TNF-α-inhibitor, AZA
7	M	25	Chronically active disease	Colon	13.3	TNF-α-inhibitor, AZA
8	F	22	Acute flare	Ileocolon	1.7	TNF-α-inhibitor, 6MP
9	M	20	Chronically active disease	Colon	2.1	TNF-α-inhibitor, AZA
10	M	22	Chronically active disease	Colon	2.8	TNF-α-inhibitor, AZA
11	F	23	Acute flare	Ileocolon	2.0	TNF-α-inhibitor, MTX
12	F	26	Chronically active disease	Ileocolon	6.2	TNF-α-inhibitor, MTX
13	F	47	Acute flare	Colon	2.5	Prednisone, MTX
14	M	34	Acute flare	Colon	9.3	Prednisone, AZA
15	F	23	Acute flare	Colon	0	Prednisone, AZA
16	M	31	Chronically active disease	Colon	5.2	Prednisone, AZA
17	M	26	Acute flare	Colon	1.6	Prednisone, AZA

AZA azathioprine, *6MP* 6-mercaptopurine, *MTX* methotrexate

induction with introduction of *conventional immunosuppressive treatment* (n=1 methotrexate, n=4 azathioprine). Two of the induction steroid treatments began 2–5 days prior to the first endoscopy. Endoscopic response was assessed in anti-TNF-treated patients around 3 months and in those treated with immunosuppressants 4–6 months after the beginning of the treatment. The ileum was reached in 31 out of 34 (91%) and the cecum in 33 out of 34 (97%) of the endoscopies. At the time of the endoscopies, patients provided blood samples for serum C-reactive protein (CRP, in milligrams per liter) and fecal samples for measurement of calprotectin. All but one patient responded to treatment, ten reaching remission of the disease. However, the patient with no improvement in CDEIS values demonstrated marked decrease in total histological score and CDAI and was thus included in the study.

Histology scoring

During all the endoscopies, four biopsies—targeted to most severely diseased areas—were taken from the ileum, right colon, transverse colon, and left colon, and rectum. Routine histology was performed on specimens stained with hematoxylin and eosin. For this study, the most severely affected colonic segment in the baseline endoscopy was selected for MMP stainings of pretreatment and post-treatment biopsy specimens (five right colon, six transverse colon, and six left colon). Histological findings of the corresponding segment were scored by an experienced gastrointestinal pathologist (P.K.) according to a scoring system for histological abnormalities in CD mucosal biopsy specimens [23]: epithelial damage was classified as normal (score=0), focal (score=1) or extensive (score=2) pathology; architectural changes as normal (score=0), moderately (<50%) disturbed (score=1), or severely (>50%) disturbed (score=2). The infiltration of mononuclear cells in the LP and polymorphonuclear cells in the LP was scored as normal (score=0), moderate (score=1), or severe (score=2). Polymorphonuclear cells in the epithelium were scored 1 if they were in the surface epithelium, 2 in the presence of cryptitis, and 3 in the presence of a crypt abscess. Presence of an erosion and/or an ulcer and presence of a granuloma were both scored "yes" (score=0) or "no" (score=1). In addition, the number of biopsy specimens affected were scored none (score=0), <33% (score=1), 33% to 66% (score=2), or >66% (score=3) [23]. The total histological score for the segment was a sum of these variables (minimum 0, maximum 16).

Fecal calprotectin

Fecal calprotectin was measured by a quantitative enzyme immunoassay (PhiCal Test, Calpro AS, Oslo, Norway; NovaTec Immunodiagnostica, Dietzenbach, Germany). The values quoted as normal in our laboratory were <100 μg/g [24, 25]. In this study, fecal calprotectin ≥200 μg/g indicated active CD [22].

Immunohistochemistry

Immunohistochemistry was performed using streptavidin–biotin–peroxidase complex technique (DakoCytomation, StreptABComplex/HRP Duet, Mouse/Rabbit, Glostrup, Denmark; Elite Goat IgG Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) and the antibody–polymer detection technique (PowerVision, Poly-HRP IHC Kit, ImmunoVision Technologies, Brisbane, CA, USA). Diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC) was used as chromogenic substrates and Mayer's hematoxylin as counterstain. Monoclonal antibodies were used to stain for MMP-1 (1:500, IM35L, Oncogene Research Products, La Jolla, CA, USA) [26], MMP-7 (1:130, IM40L, Calbiochem, La Jolla, CA, USA) [26–28], MMP-9 (1:100, MS-569-P1, Neomarkers, Fremont, CA, USA), MMP-10 (1:300, 5E4, Novocastra Laboratories, New Castle upon Tyne, UK) [28], TIMP-1 (1:100, IM63, Calbiochem) [26], and TIMP-3 (1:400, IM43L, Calbiochem) [26]. Polyclonal antibodies were used for MMP-26 (1:120, a gift from Prof. K. Isaka, Tokyo Medical University) [29]. MMP-1, MMP-9, and MMP-10 were pretreated with 1% trypsin solution for 30 min at +37°C. MMP-7, MMP-26, TIMP-1, and TIMP-3 were pretreated in a +95°C water bath for 20 min (Dako retrieval solution, pH 6; Dakocytomation). The incubation conditions for the antibodies were: +4°C overnight for MMP-1, MMP-7, MMP-9, TIMP-1, and TIMP-3; 2 h at room temperature for MMP-10; and 1 h at +37°C for MMP-26. For negative controls, parallel sections of the same samples were processed using preimmune sera or normal rabbit or mouse immunoglobulin. Immunohistochemical specimens were graded independently by two different investigators (L.M., U.S.-K.) in a semiquantitative fashion under a light-field microscope at ×100 magnification using a scale marking staining intensity as follows: 0=less than 20 positive cells; 1=20–50 positive cells; 2=50–200 positive cells; 3=over 200 positive cells.

Statistical analysis

Paired and independent samples *t* tests, nonparametric Wilcox and Mann–Whitney's and Spearman's correlation tests were performed to investigate the significance of results with the Statistical Package for Social Sciences 15.0 for Windows. A p value under 0.05 was considered as significant.

Results

When comparing the patient groups before and after treatment, both fecal and serological inflammation indicators decreased significantly (calprotectin means 2,614 vs. 249; p=0.000 and CRP 32 vs. 1; p=0.001), as did endoscopic findings according to CDEIS (14.9 vs. 3.5; p=0.000), total histological score of biopsies (7 vs. 4; p=0.004), and symptoms of the disease assessed by CDAI (214 vs. 61; p=0.000). When looking at pretreatment and post-treatment biopsies of *patients treated with anti-TNF-α agents or conventional immunosuppressants* separately, no significant differences in MMP or TIMP profiles could be observed. Both groups presented with the general patterns as discussed below.

MMP-1

MMP-1 was detected in the stroma and surface epithelium of all samples (Fig. 1a; Table 2). In the stroma, MMP-1 was expressed by macrophages (Fig. 1b). The expression pattern in both pretreatment and post-treatment biopsies was fairly similar (Table 2).

MMP-7

MMP-7 expression was detected in the epithelium in seven out of 17 of the initial samples and two out of 17 of the post-treatment specimens (Fig. 1c, d; Table 2). Stronger epithelial expression in initial samples was also confirmed in semiquantitative scoring of immunohistochemical specimens (means 0.47 vs. 0.12; p=0.055; Tables 2 and 3A). Expression of epithelial MMP-7 tended to be stronger in the group active for CDAI (≥150) compared to that of the inactive group (means 0.50 vs. 0.18; p=0.091). Epithelial MMP-7 expression tended to have a positive correlation with total histological score (correlation coefficient=0.334; p=0.053; Table 3B) and active disease as indicated by CRP (≥10 mg/l; mean 0.55 vs. 0.17; p=0.018).

MMP-9

MMP-9 protein was generally absent from the surface epithelium, but stromal expression by macrophages and neutrophils was initially strong (Fig. 1e–g; Table 2). In semiquantitative analysis of neutrophil MMP-9, higher stromal expression was observed in initial samples (means 2.12 vs. 1.29; p=0.039; Table 3A). Total histological score showed significant correlation with both macrophage (correlation coefficient=0.439; p=0.009) and neutrophil (correlation coefficient=0.577; p=0.000) MMP-9 (Table 3B). Stronger expression of MMP-9 in neutrophils was also observed in samples that were considered active for CDEIS (>9; mean 2.00 vs. 1.38; p=0.061) and CRP (≥10; mean 2.27 vs. 1.43; p=0.020).

MMP-10

Expression of MMP-10 in the surface epithelium was detected in 16 out of 17 initial samples and 14 out of 17

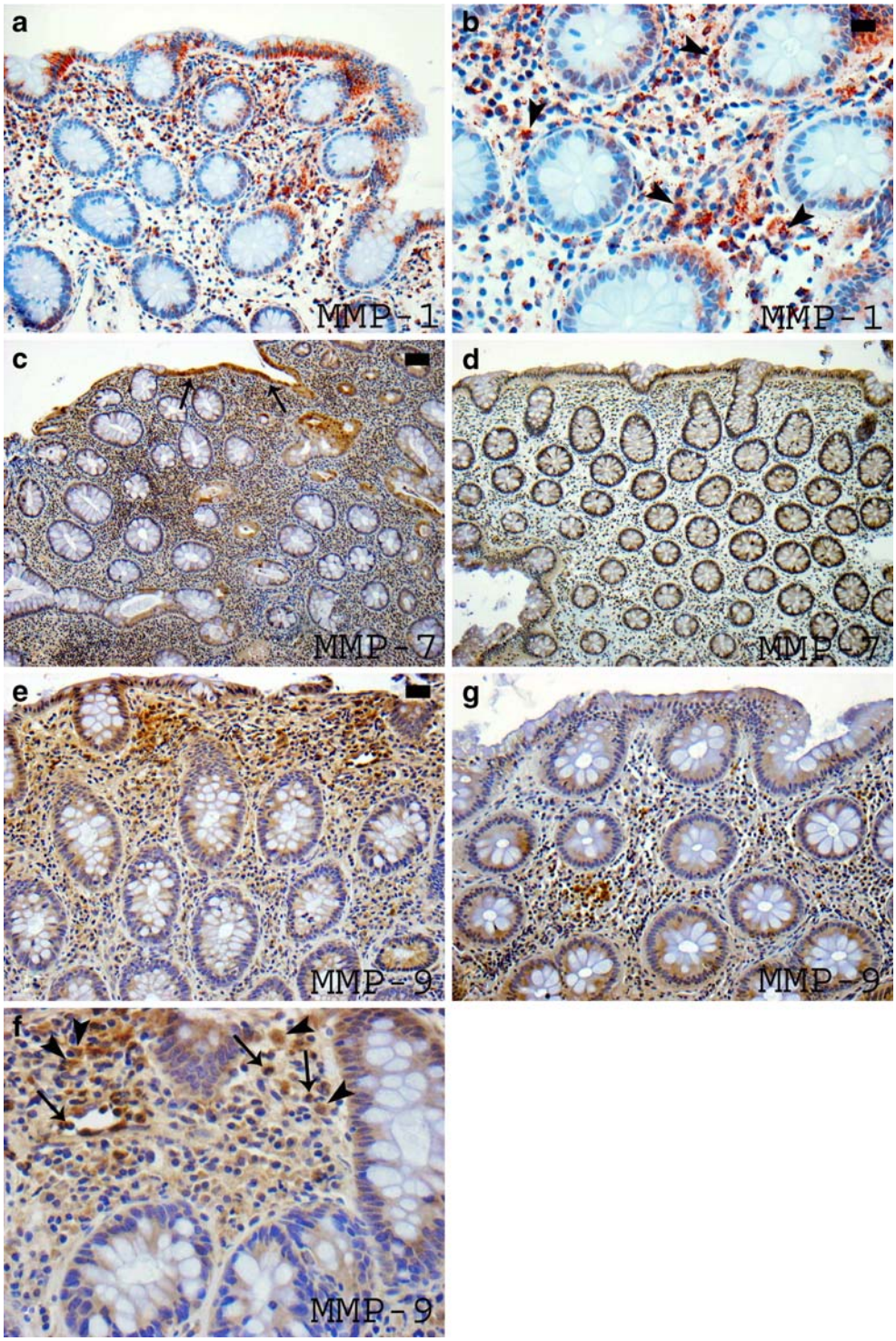

Fig. 1 Stromal and epithelial expression of MMP-1 (**a**) was detected in all samples and the expression pattern was similar in both sets of samples. **b** Higher magnification of **a** with MMP-1 positive macrophages (*arrowheads*). Epithelial expression of MMP-7 was stronger in initial samples (**c**) when compared to the ones taken after treatment (**d**). *Arrows* depict positive epithelial cells (**c**). MMP-9 was expressed in the stroma of most samples; however, expression was higher in initial samples (**e**) and decreased with treatment (**g**). Macrophages (*arrowhead*) and neutrophils (*arrow*) expressed MMP-9 (**f**). AEC was used as chromogenic substrate in **a** and **b**, DAB in **c**–**g**. Samples **a**–**c**, **e**, and **f** were taken before treatment. Samples **d** and **g** had been treated with *anti-TNF-α agents*. Scale bars=7.5 μm (**b**, **f**); 15 μm (**a**, **e**, **g**); 30 μm (**c**, **d**)

post-treatment samples (Fig. 2a; Table 2). All samples in both groups had positive stromal expression in macrophages and lymphocytes (Fig. 2a, b). Thus, expression in both sets of samples was very similar. Total histological score and fecal calprotectin tended to correlate positively with stromal MMP-10 (correlation coefficient=0.325; p=0.060 and correlation coefficient=0.353; p=0.040, respectively; Table 3B).

MMP-26

MMP-26 was not present in the surface epithelium of any sample. In the stroma, MMP-26 was positive in 15 of the pretreatment biopsies and in seven of the post-treatment samples in endothelial cells and neutrophils (Fig. 2c′; Table 2). MMP-26 expression in stromal neutrophils decreased with treatment (means 0.94 vs. 0.53; p=0.030;

Table 2 The inflammation scores and expression of MMPs and TIMPs in the colon of patients with CD

Sample	CDEIS	CDAI	Calpro	CRP	Total	MMP-1		MMP-7		MMP-9			MMP-10		MMP-26		TIMP-1			TIMP-3	
						e	s	e	s	e	s/mf	s/nf	e	s	e	s	e	s/mf	s/nf	e	s
1A	25.3	256	1,173	32	4	3	2	1	1	0	2	2	1	2	0	0	0	0	0	0	3
2A	14.6	183	15,326	0	8	2	2	0	0	0	3	0	1	2	0	1	0	2	0	2	3
3A	23.9	158	4,260	42	8	1	2	1	0	0	1	2	1	2	0	1	0	2	0	0	2
4A	16.5	317	2,434	0	8	2	2	0	0	0	1	1	2	3	0	1	1	3	0	2	3
5A	12.5	50	1,016	8	4	2	2	0	0	0	0	2	0	2	0	1	0	2	2	2	2
6A	9.8	61	813	23	8	1	2	1	0	0	3	2	1	2	0	1	1	1	0	0	2
7A	13	49	2,222	54	11	2	3	0	0	0	2	3	1	3	0	1	1	3	2	0	3
8A	14.4	605	1,019	7	10	3	3	0	0	0	3	3	3	3	0	1	1	3	2	1	2
9A	9.4	222	1,143	8	10	2	3	0	0	0	2	3	2	2	0	2	1	2	0	1	2
10A	10.1	174	1,891	21	8	3	3	0	0	0	3	2	2	3	0	1	1	2	0	2	3
11A	16	252	1,926	0	9	2	2	2	0	0	3	2	2	3	0	1	1	2	0	1	3
12A	17.4	133	2,775	13	7	3	3	0	0	0	3	2	2	3	0	1	1	2	2	1	3
13A[a]	13	229	2,588	211	12	2	2	1	0	0	2	3	1	3	0	1	0	2	0	2	3
14A[a]	13.2	325	802	20	10	2	2	1	0	0	1	2	2	2	0	1	0	0	0	1	2
15A[a]	15.8	164	1,282	65	3	1	2	0	0	0	0	2	1	3	0	1	0	0	0	1	3
16A[a]	11.9	45	1,484	18	5	3	3	1	0	0	3	3	2	3	0	1	0	2	0	3	3
17A[a]	16.8	419	2,277	29	0	3	3	0	0	0	0	2	3	2	0	0	0	2	0	1	3
1B	6.84	88	296	6	3	3	2	0	0	0	2	2	2	2	0	0	0	0	0	2	2
2B	5.2	77	1,419	0	5	3	3	0	0	0	2	3	2	3	0	2	0	2	0	1	3
3B	8.7	24	200	4	0	2	2	0	0	0	2	0	2	2	0	0	0	0	0	1	2
4B	1.6	54	13	0	2	2	2	0	0	0	0	0	0	2	0	1	1	1	0	3	3
5B	4.36	44	18	0	7	2	3	0	0	0	1	2	2	2	0	0	0	0	0	1	2
6B	1.06	47	42	0	5	2	3	0	0	0	3	2	1	2	0	0	0	1	0	2	2
7B	7.88	33	267	0	4	2	2	0	0	0	2	0	1	3	0	0	0	1	0	2	2
8B	0	101	27	0	4	2	2	0	0	0	0	1	0	2	0	0	0	0	0	1	2
9B	9.6	81	1,052	6	3	2	2	0	0	0	2	0	2	2	0	1	3	2	0	0	3
10B	1.76	30	25	0	9	2	3	1	0	0	2	3	2	3	0	1	1	3	0	2	3
11B	0.2	32	130	0	5	3	2	0	0	2	3	2	2	2	0	0	2	0	0	2	2
12B	5.38	80	271	0	3	3	3	0	0	0	0	1	2	2	0	2	2	2	0	2	3
13B[a]	2.7	93	21	0	3	2	2	0	0	0	0	2	2	2	0	0	0	0	0	2	2
14B[a]	1.72	28	136	0	0	2	3	1	0	0	2	0	2	3	0	0	0	0	0	2	2
15B[a]	1.32	68	69	0	3	2	2	0	0	0	0	2	2	2	0	1	0	0	0	2	2
16B[a]	0.2	50	166	0	4	3	3	0	0	0	3	1	3	3	0	1	0	1	0	2	2
17B[a]	0.2	115	77	0	5	2	2	0	0	0	1	1	0	2	0	0	0	0	0	2	2

A baseline endoscopy, *B* follow-up endoscopy, *CDEIS* Crohn's disease endoscopic index of severity, *CDAI* Crohn's disease activity index, *Calpro* fecal calprotectin, *Total* total histologic score, *s* stroma, *e* epithelium, *mf* macrophage, *nf* neutrophil.

[a] Patients who did not receive TNF-α inhibitor treatment

Fig. 2c, d; Table 3A). Fecal calprotectin and total histological score showed strong positive correlation with stromal MMP-26 (correlation coefficient=0.432; p=0.011 and correlation coefficient=0.418; p=0.014; Table 3B). Biopsies considered to represent active disease (calprotectin ≥200 or CDEIS >9) showed higher expression of stromal MMP-26 than biopsies from inactive disease (means for MMP-26 0.91 [calprotectin ≥200] vs. 0.36 [calprotectin <200]; p=0.013; and 0.94 [CDEIS >9] vs. 0.50 [CDEIS ≤9]; p=0.017).

TIMP-1

Epithelial expression of TIMP-1 did not statistically differ between pretreatment and post-treatment samples (Table 2). However, expression in stromal macrophages was higher in biopsies taken before treatment than after this (means 1.76 vs. 0.76; p=0.003; Fig. 2e–g; Table 3A). This pattern was also present in stromal neutrophils, as none of the post-treatment samples had positive neutrophils (means 0.47

Table 3 The means of semiquantitatively assessed scores for MMPs and TIMPs for baseline and post-treatment biopsies and association of MMP and TIMP expression with the total histological score, calprotectin, CDEIS, CDAI, and CRP values

	MMP-1		MMP-7		MMP-9			MMP-10		MMP-26		TIMP-1			TIMP-3	
	e	s	e	s	e	s/mf	s/nf	e	s	e	s	e	s/mf	s/nf	e	s
(A) Means of semiquantitatively assessed scores for MMPs and TIMPs																
Pretreatment (SEM)	2.18 (0.18)	2.41 (0.12)	0.47 (0.15)	0.06 (0.06)	0.00 (0.00)	1.88 (0.28)	2.12 (0.19)	1.59 (0.19)	2.53 (0.13)	0.00 (0.00)	0.94 (0.10)	0.47 (0.13)	1.76 (0.24)	0.47 (0.21)	1.18 (0.21)	2.65 (0.12)
Post-treatment(SEM)	2.29 (0.11)	2.41 (0.12)	0.12 (0.08)	0.00 (0.00)	0.12 (0.12)	1.47 (0.27)	1.29 (0.25)	1.59 (0.21)	2.29 (0.11)	0.00 (0.00)	0.53 (0.17)	0.53 (0.23)	0.76 (0.24)	0.00 (0.00)	1.71 (0.17)	2.29 (0.11)
p value	0.480	1.000	0.055	0.317	0.317	0.203	0.039**	0.917	0.157	1.000	0.030**	0.739	0.003***	0.041**	0.058	0.029**
(B) Association of MMP and TIMP expression																
Correlation coefficients																
Tot.hist.score	n.s.	n.s.	0.334*	n.s.	n.s.	0.439***	0.577***	n.s.	0.325*	n.s.	0.418**	n.s.	0.524***	n.s.	n.s.	n.s.
Calprotectin	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.353**	n.s.	0.432**	n.s.	0.586***	n.s.	−0.335*	0.541***
CDEIS	n.s.	n.s.	0.310*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.451***	0.312*	−0.462***	0.469***

Differences in MMP and TIMP profile were assessed using paired samples *t* test and nonparametric samples Wilcox test. Association was calculated using Spearman's rank correlation test. Comparisons were considered significant at a *p* value <0.05

e epithelium, *s* stroma, *mf* macrophage, *nf* neutrophil, *SEM* standard error of the mean, *Tot.hist.score* total histologic score, *CDEIS* Crohn's disease endoscopic index of severity, *n.s.* not significant

***$p<0.010$, **$p<0.050$, *$p<0.080$

vs. 0; $p=0.041$; Fig. 2e–g; Table 3A). For macrophage expression, there was significant positive correlation with indicators of inflammation, calprotectin (correlation coefficient=0.586; $p=0.000$), CDEIS (correlation coefficient=0.451; $p=0.007$), and total histological score (correlation coefficient=0.524; $p=0.001$; Table 3B). Biopsies considered active for CDEIS (>9) showed stronger stromal expression of TIMP-1 in macrophages (means 1.78 vs. 0.69; $p=0.002$) and neutrophils (means 0.44 vs. 0; $p=0.046$) than those considered inactive for CDEIS. Also, stronger expression by macrophages was present in biopsies grouped as active disease according to calprotectin (means 1.61 vs. 0.55; $p=0.005$).

TIMP-3

TIMP-3 was detected in the surface epithelium in both groups of biopsies (Table 2), showing a trend for weaker expression in pretreatment samples (means 1.18 vs. 1.71; $p=0.058$; Fig. 2h, i; Table 3A). Although all samples had positive stromal macrophages and plasma cells, TIMP-3 expression was significantly stronger in the pretreatment samples (means 2.65 vs. 2.29; $p=0.029$; Fig. 2h, i; Table 3A). On the contrary, epithelial TIMP-3 showed negative correlation with inflammation indicators: TIMP-3 had significant inverse associations with CRP and CDEIS (correlation coefficient=−0.486; $p=0.004$ and correlation coefficient=−0.462; $p=0.006$; Table 3B). Furthermore, inverse trends were present when comparing epithelial TIMP-3 with the fecal calprotectin (correlation coefficient=−0.335; $p=0.053$; Table 3B) and the group with low CRP (<10 mg/l; correlation coefficient=−0.404; $p=0.056$). Epithelial TIMP-3 expression was lower in biopsies considered active for CDEIS (means 1.11 vs. 1.81; $p=0.011$), calprotectin (means 1.22 vs. 1.99; $p=0.019$), and CRP (means 1.00 vs. 1.65; $p=0.028$). In contrast, stromal TIMP-3 showed positive correlation with calprotectin (correlation coefficient=0.541; $p=0.001$) and CDEIS values (correlation coefficient=0.469; $p=0.005$; Table 3B), as well as when separately looking at samples in the group active according to calprotectin level (correlation coefficient=0.578; $p=0.004$). Stromal expression was higher in the group considered active for CDEIS (means 2.67 vs. 2.25; $p=0.014$), calprotectin (means 2.61 vs. 2.18; $p=0.019$), and CRP (means 2.73 vs. 2.35; $p=0.039$).

Discussion

MMPs participate in T cell-mediated injury in the human intestine [30]. In fact, levels of specific MMPs in the urine may provide clinicians with useful diagnostic and prognostic information [31]. However, some MMPs may play a role

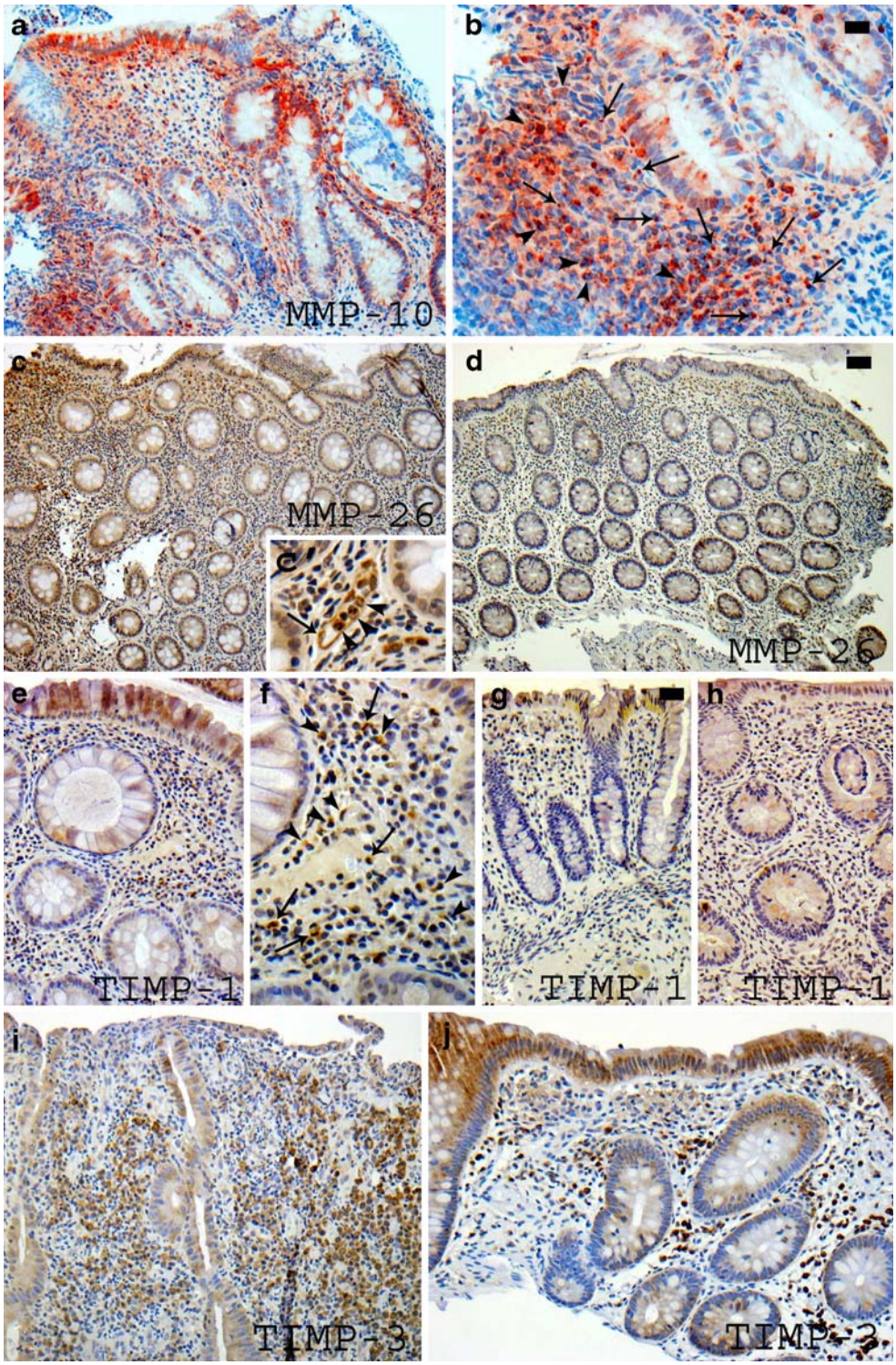

Fig. 2 MMP-10 was detected in the epithelium and stroma of most samples (**a**). MMP-10 was expressed by lymphocytes (*thin arrows*) and macrophages (*arrowheads*, **b**). MMP-26 showed stronger stromal expression in pretreatment samples (**c**) in neutrophils (*arrowhead*, **c′**) and endothelial cells (*arrow*, **c′**) when compared to post-treatment samples (**d**). TIMP-1 was expressed in stroma in macrophages (*arrowhead*, **f**) and neutrophils (*arrow*, **f**). When compared to pretreatment samples (**e**), the number of positive macrophages decreased with treatment and none of the post-treatment samples presented TIMP-1 positive neutrophils (**g**, **h**). Stromal expression of TIMP-3 was initially strong (**i**) but decreased with treatment (**j**). However, epithelial TIMP-3 increased with treatment. AEC was used as chromogenic substrate in **a** and **b**, DAB in **c**–**j**. Samples **a**–**c**, **e**, **f**, and **i** were taken before treatment, samples **d**, **h**, and **j** had undergone treatment with *anti-TNF-α agents* and sample **g** with *corticosteroids* and *a conventional immunosuppressant*. Scale bars=7.5 μm (**b**, **c′**, **f**); 15 μm (**e**, **g**–**j**); 30 μm (**a**, **c**, **d**)

in epithelial restitution and tissue remodeling. In this study, treatment with *anti-TNF-α agents* (*infliximab or adalimumab*) *or with corticosteroids and conventional immunosuppressants* (*azathioprine or methotrexate*) decreased the expression of epithelial MMP-7 and that of MMP-9, MMP-26, and their tissue inhibitors TIMP-1 and TIMP-3 in the stroma. While expression of MMP-1 and MMP-10 was generally not altered, epithelial TIMP-3 increased with treatment.

Treatment with *anti-TNF-α agents or conventional* immunosuppressants decreased the expression of MMP-9 by neutrophils that associated with a decrease in histological severity score. Similarly, downregulation of MMP-9 production of macrophages was observed to correlate with

decreases in histological score and CRP. MMP-9 is pivotal for tissue damage [11] and its selective inhibition has been suggested to be of therapeutic benefit in IBD [5]. In a previous study, infliximab treatment increased MMP-2 and decreased MMP-9 in serum of patients (the latter also in tissue) with CD [32]. The changes were not strictly associated with the response to treatment. Enhanced leukocyte MMP-9 expression in CD seemed to be regulated by TNF-α. Moreover, in intestinal mucosal explants from IBD, infliximab downregulated MMP-9 relative to TIMP-1 and TIMP-2 [33]. MMP-9 has been shown to respond to immunotherapeutic agents. There exist several studies on the effect of infliximab on tissue MMP profile particularly in arthritis and psoriasis. Infliximab decreased MMP-9 in psoriatic skin [34]. In rheumatoid arthritis, infliximab infusions downregulated serum MMP-1, MMP-3, MMP-9, TIMP-1, and TIMP-2 levels [35] and modulation of the MMP/TIMP system by infliximab can contribute to the anti-inflammatory and tissue-remodeling effects of TNF-α blockade in spondylarthropathies [36].

Expression of MMP-26 in neutrophils decreased with treatment, depicting a direct relationship with histological score and calprotectin. MMP-26 may thus be important in tissue destruction or migration of neutrophils as it is able to degrade various ECM and basement components, such as type I gelatin, vitronectin, fibronectin, and type IV collagen [37, 38]. MMP-26 has been previously detected in neutrophils [39] and endothelial cells [40], agreeing with our results. MMP-26 was not present in the epithelium of any sample. It has been previously shown to be present in the cytoplasm of migrating enterocytes in UC but not CD [16].

MMP-7 tended to be less expressed in the epithelium after treatment, correlating with a decrease in histological score in post-treatment samples. This agrees with our previous findings in necrotizing enterocolitis and further strengthens the putative role of this MMP in tissue destruction [40]. Expression of MMP-7 has also been discovered to correlate with the degree of inflammation in UC [41].

The lack of significant changes in MMP-1 is not in complete disagreement with previous studies. MMP-1 transcripts have been shown to be present in CD myofibroblasts, as well as MMP-1 protein, but the latter did not possess any bioactivity [42]. However, transcript levels of MMP-1 have been shown to correlate with CDAI in mucosal biopsy specimens of CD patients [43]. Furthermore, when mucosal biopsies of active CD were studied 8 weeks after initiation of infliximab therapy, MMP-1 levels post-treatment were able to predict patients achieving long-term remission [44]. In intestinal mucosal explants from IBD, infliximab downregulated MMP-1 relative to TIMP-1 and TIMP-2 and decreased MMP-1 and MMP-3 activities [33].

No statistically significant changes in semiquantitatively assessed epithelial or stromal MMP-10 were detected post-treatment. However, it tended to decrease when histological score and calprotectin became lower. In an experimental model of IBD using T cell activation of intestinal explants [30], MMP-10 was detected in areas with most severe injury. This discrepancy may be due to different experimental systems, i.e., in vivo biopsies vs. in vitro cultures.

Expression of TIMP-1 in macrophages and neutrophils was weaker in post-treatment samples. Also, histological score, calprotectin, and CDEIS values decreased together with stromal expression of TIMP-1. Our study thus portrayed a decrease in TIMP-1 after therapy. In rheumatoid arthritis, infliximab downregulated serum levels of TIMP-1 [35], but in colonic myofibroblasts isolated from patients with active CD, infliximab and adalimumab induced a dose-dependent increase TIMP-1 production [45].

Stromal TIMP-3 decreased with treatment, correlating with a decrease in calprotectin and CDEIS values. This agrees with our results in an experimental model in which both TIMPs were expressed abundantly at the beginning of culture but downregulated after T cell activation [30]. However, there was a trend for greater TIMP-3 expression in the epithelium in post-treatment samples, but lower values for calprotectin, CDEIS, and CRP. Thus, epithelial TIMP-3 may participate in epithelial regeneration.

Fecal calprotectin associates with the degree of histological inflammation in the colon [46] and showed a positive correlation with stromal MMP-10, MMP-26, TIMP-1, and TIMP-3. However, a negative correlation was seen with epithelial TIMP-3. When comparing the biopsies from inactive and active disease based on calprotectin values, an association with epithelial MMP-1 and stromal MMP-9 was observed and, for the latter, with stromal TIMP-1 and TIMP-3. Interestingly, calprotectin may affect various pathophysiological processes by competing with MMPs for zinc [47].

CDEIS, the activity index determined in endoscopy, depicted a direct relationship with epithelial and stromal MMP-7 and stromal TIMP-1. However, a negative association was present when comparing CDEIS with epithelial TIMP-3. Also, when looking at samples considered active for CDEIS, an inverse relationship was present between this and stromal MMP-26. When looking at all samples and CDAI, only a negative relationship with epithelial TIMP-3 could be seen, but looking separately at samples inactive and active for CDAI, the latter correlated positively with epithelial MMP-1 and MMP-10. CRP did not present as a good inflammation indicator, as correlation with MMPs and other inflammation indicators varied. Previous studies indicate that calprotectin is indeed a more precise marker for inflammation in CD and a useful surrogate marker for mucosal healing [46].

In conclusion, our results suggest that immunosuppressive drugs modulate disease activity in CD by down-regulation of MMP-9 and MMP-26 positive neutrophils and stromal TIMP-1 and TIMP-3. MMP-1 and MMP-10 do not seem to be important players in the mucosal wound-healing response elicited by TNF-α inhibition.

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References

1. Shanahan F (2002) Crohn's disease. Lancet 359:62–69
2. Podolsky DK (2002) Inflammatory bowel disease. N Engl J Med 347:417–429
3. Taylor CT, Keely SJ (2007) The autonomic nervous system and inflammatory bowel disease. Auton Neurosci 133:104–114
4. Parks WC, Wilson CL, López-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol 4:617–629
5. Ravi A, Garg P, Sitaraman SV (2007) Matrix metalloproteinases in inflammatory bowel disease: boon or a bane? Inflamm Bowel Dis 13:97–107
6. Saarialho-Kere UK, Vaalamo M, Puolakkainen P et al (1996) Enhanced expression of matrilysin, collagenase, and stromelysin-1 in gastrointestinal ulcers. Am J Pathol 148:519–526
7. Heuschkel RB, MacDonald TT, Monteleone G et al (2000) Imbalance of stromelysin-1 and TIMP-1 in the mucosal lesions of children with inflammatory bowel disease. Gut 47:57–62
8. Kirkegaard T, Hansen A, Bruun E et al (2004) Expression and localisation of matrix metalloproteinases and their natural inhibitors in fistulae of patients with Crohn's disease. Gut 53:701–709
9. Warnaar N, Hofker HS, Maathuis MH et al (2006) Matrix metalloproteinases as profibrotic factors in terminal ileum in Crohn's disease. Inflamm Bowel Dis 12:863–869
10. Bailey CJ, Hembry RM, Alexander A et al (1994) Distribution of the matrix metalloproteinases stromelysin, gelatinases A and B, and collagenase in Crohn's disease and normal intestine. J Clin Pathol 47:113–116
11. Baugh MD, Perry MJ, Hollander AP et al (1999) Matrix metalloproteinase levels are elevated in inflammatory bowel disease. Gastroenterology 117:814–822
12. Stumpf M, Cao W, Klinge U et al (2005) Reduced expression of collagen type I and increased expression of matrix metalloproteinases 1 in patients with Crohn's disease. J Invest Surg 18:33–38
13. Srivastava MD, Kulaylat MN (2004) Gene expression profiles of late colonic Crohn's disease. J Med 35:233–255
14. Vaalamo M, Karjalainen-Lindsberg ML, Puolakkainen P et al (1998) Distinct expression profiles of stromelysin-2 (MMP-10), collagenase-3 (MMP-13), macrophage metalloelastase (MMP-12), and tissue inhibitor of metalloproteinases-3 (TIMP-3) in intestinal ulcerations. Am J Pathol 152:1005–1014
15. Gill SE, Parks WC (2008) Metalloproteinases and their inhibitors: regulators of wound healing. Int J Biochem Cell Biol 40:1334–1347
16. Bister VO, Salmela MT, Karjalainen-Lindsberg ML et al (2004) Differential expression of three matrix metalloproteinases, MMP-19, MMP-26, and MMP-28, in normal and inflamed intestine and colon cancer. Dig Dis Sci 49:653–661
17. Pender SL, Li CK, Sabatino AD et al (2006) Role of macrophage metalloelastase in gut inflammation. Ann N Y Acad Sci 1072:386–388
18. Meijer MJ, Mieremet-Ooms MA, van Hogezand RA et al (2007) Role of matrix metalloproteinase, tissue inhibitor of metalloproteinase and tumor necrosis factor-alpha single nucleotide gene polymorphisms in inflammatory bowel disease. World J Gastroenterol 13:2960–2966
19. Mary JY, Modigliani R (1989) Development and validation of an endoscopic index of the severity for Crohn's disease: a prospective multicentre study. Groupe d'Etudes Therapeutiques des Affections Inflammatoires du Tube Digestif (GETAID). Gut 30:983–989
20. Best WR, Becktel JM, Singleton JW et al (1976) Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. Gastroenterology 70:439–444
21. Sostegni R, Daperno M, Scaglione N et al (2003) Review article: Crohn's disease: monitoring disease activity. Aliment Pharmacol Ther 17:11–17
22. Sipponen T, Savilahti E, Kolho KL et al (2008) Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. Inflamm Bowel Dis 14:40–46
23. D'Haens GR, Geboes K, Peeters M et al (1998) Early lesions of recurrent Crohn's disease caused by infusion of intestinal contents in excluded ileum. Gastroenterology 114:262–267
24. Kolho KL, Raivio T, Lindahl H et al (2006) Fecal calprotectin remains high during glucocorticoid therapy in children with inflammatory bowel disease. Scand J Gastroenterol 41:720–725
25. von Roon AC, Karamountzos L, Purkayastha S et al (2007) Diagnostic precision of fecal calprotectin for inflammatory bowel disease and colorectal malignancy. Am J Gastroenterol 102:803–813
26. Bister V, Mäkitalo L, Jeskanen L et al (2007) Expression of MMP-9, MMP-10 and TNF-alpha and lack of epithelial MMP-1 and MMP-26 characterize pyoderma gangrenosum. J Cutan Pathol 34:889–898
27. Impola U, Jeskanen L, Ravanti L et al (2005) Expression of matrix metalloproteinase (MMP)-7 and MMP-13 and loss of MMP-19 and p16 are associated with malignant progression in chronic wounds. Br J Dermatol 152:720–726
28. Kuivanen T, Jeskanen L, Kyllönen L et al (2006) Transformation-specific matrix metalloproteinases, MMP-7 and MMP-13 are present in epithelial cells of keratoacanthomas. Mod Pathol 19:1203–1212
29. Isaka K, Nishi H, Nakai H et al (2003) Matrix metalloproteinase-26 is expressed in human endometrium but not in endometrial carcinoma. Cancer 97:79–89
30. Salmela MT, MacDonald TT, Black D et al (2002) Upregulation of matrix metalloproteinases in a model of T cell mediated tissue injury in the gut: analysis by gene array and in situ hybridisation. Gut 51:540–547
31. Manfredi MA, Zurakowski D, Rufo PA et al (2008) Increased incidence of urinary matrix metalloproteinases as predictors of disease in pediatric patients with inflammatory bowel disease. Inflamm Bowel Dis 14:1091–1096
32. Gao Q, Meijer MJ, Schluter UG et al (2007) Infliximab treatment influences the serological expression of matrix metalloproteinase (MMP)-2 and -9 in Crohn's disease. Inflamm Bowel Dis 13:693–702
33. Meijer MJ, Mieremet-Ooms MA, van Duijn W et al (2007) Effect of the anti-tumor necrosis factor- alpha antibody infliximab on the ex vivo mucosal matrix metalloproteinase-proteolytic phenotype in inflammatory bowel disease. Inflamm Bowel Dis 13:200–210

34. Cordiali-Fei P, Trento E, D'Agosto G et al (2007) Effective therapy with anti-TNF-alpha in patients with psoriatic arthritis is associated with decreased levels of metalloproteinases and angiogenic cytokines in the sera and skin lesions. Ann N Y Acad Sci 1110:578–589
35. Klimiuk PA, Sierakowski S, Domyslawska I et al (2004) Effect of repeated infliximab therapy on serum matrix metalloproteinases and tissue inhibitors of metalloproteinases in patients with rheumatoid arthritis. J Rheumatol 31:238–242
36. Vandooren B, Kruithof E, Yu DT et al (2004) Involvement of matrix metalloproteinases and their inhibitors in peripheral synovitis and down-regulation by tumor necrosis factor alpha blockade in spondylarthropathy. Arthritis Rheum 50:2942–2953
37. Marchenko GN, Ratnikov BI, Rozanov DV et al (2001) Characterization of matrix metalloproteinase-26, a novel metalloproteinase widely expressed in cancer cells of epithelial origin. Biochem J 356:705–718
38. Uría JA, López-Otín C (2000) Matrilysin-2, a new matrix metalloproteinase expressed in human tumors and showing the minimal domain organization required for secretion, latency, and activity. Cancer Res 60:4745–4751
39. Li W, Savinov AY, Rozanov DV et al (2004) Matrix metalloproteinase-26 is associated with estrogen-dependent malignancies and targets alpha1-antitrypsin serpin. Cancer Res 64:8657–8665
40. Bister V, Salmela MT, Heikkilä P et al (2005) Matrilysins-1 and -2 (MMP-7 and -26) and metalloelastase (MMP-12), unlike MMP-19, are up-regulated in necrotizing enterocolitis. J Pediatr Gastroenterol Nutr 40:60–66
41. Matsuno K, Adachi Y, Yamamoto H et al (2003) The expression of matrix metalloproteinase matrilysin indicates the degree of inflammation in ulcerative colitis. J Gastroenterol 38:348–354
42. McKaig BC, McWilliams D, Watson SA et al (2003) Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. Am J Pathol 162:1355–1360
43. Stallmach A, Giese T, Schmidt C et al (2004) Cytokine/chemokine transcript profiles reflect mucosal inflammation in Crohn's disease. Int J Colorectal Dis 19:308–315
44. Schmidt C, Giese T, Hermann E et al (2007) Predictive value of mucosal TNF-alpha transcripts in steroid-refractory Crohn's disease patients receiving intensive immunosuppressive therapy. Inflamm Bowel Dis 13(1):65–70
45. Di Sabatino A, Pender SL, Jackson CL et al (2007) Functional modulation of Crohn's disease myofibroblasts by anti-tumor necrosis factor antibodies. Gastroenterology 133:137–149
46. Sipponen T, Savilahti E, Kärkkäinen P et al (2008) Fecal calprotectin, lactoferrin and endoscopic disease activity in monitoring anti-TNF-alpha therapy for Crohn's disease. Inflamm Bowel Dis 14:1392–1398
47. Isaksen B, Fagerhol MK (2001) Calprotectin inhibits matrix metalloproteinases by sequestration of zinc. Mol Pathol 54:289–292