Basic and clinical aspects of mucosal inflammation and healing in Crohn's disease

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Chapter 7

Infliximab influences the expression of matrix metalloproteinase (MMP)-2 and -9 in Crohn's disease

Short title: Infliximab and MMPs in CD

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Abstract

Background and aims: Infliximab, a chimeric IgG1 anti-tumor necrosis factor (TNF)-α monoclonal antibody, is an effective immunotherapeutic agent for severe Crohn's disease (CD). Matrix metalloproteinases (MMPs) are believed to be actively involved in the pathogenesis of CD. In the present study we assessed the effect of infliximab on the *in vitro* and *in vivo* expression of MMP-2 and MMP-9 in CD.
Patients and Methods: CD patients with fistulizing (n=10) or active disease (n=7) were administered infliximab and evaluated for serum levels of the MMPs and clinical response for 12 to 14 weeks, in an in-house study. Biopsies from some patients were evaluated immunohistochemically for the MMPs. Serum MMPs levels of fistulizing CD patients (n=42), with a follow-up of 18 weeks, and active CD patients (n=24) followed for 4 weeks, from two international placebo controlled infliximab studies, were also evaluated. In addition, in vitro whole blood cultures stimulated with LPS with/without infliximab were evaluated for MMP mRNA and protein levels by RT-PCR and ELISA, respectively.
Results: MMP-2 serum levels in CD patients with either fistulas or active disease increased during follow-up, both in the in-house and in the international study, with a decline at the end of the follow-up in the latter. However, a similar trend was observed in responders and non-responders to infliximab. Immunohistochemistry showed an immunoreaction to MMP-2 in the extracellular matrix (ECM) of the submucosa and the lamina propria and in endothelial cells without a clear change by the infliximab treatment. In contrast, serum MMP-9 levels showed a consistent pattern of decrease in most CD patients, particularly in the responding patients and less in non-responding patients, although at week 10 the MMP-9 level started to increase again. Immunohistocemically MMP-9 was predominantly present in polymorphonuclear leukocytes (PMNL) and showed a decrease by infliximab in 2 weeks.
In vitro short-term 1.5 hours LPS stimulation of whole blood increased the MMP-9 levels in plasma from both CD patients and healthy volunteers, but significantly higher in CD. However, the respective MMP-9 mRNA levels were downregulated to 50% from that of control samples. Infliximab was found not to affect the mRNA and protein levels of this short-term LPS stimulation. LPS stimulation for 24 h did not further increase the MMP-9 plasma levels of the CD patients, whereas in the healthy controls it raised further. The leucocyte MMP-9 mRNA levels simultaneously raised up to16-fold and infliximab did not affect protein but inhibited the MMP-9 mRNA induction by 80%.
Conclusion: MMP-2 and MMP-9 in serum of patients with CD exhibit an inverse changing pattern by treatment, i.e. an increase of MMP-2 and a decrease of MMP-9, the latter also in the intestine. However, these changes were not strictly associated with the response to treatment with infliximab, i.e., clinical improvement. *In vitro* studies indicate that the enhanced leucocyte MMP-9 expression in CD seems to be regulated by TNF-α.

Infliximab, a chimeric IgG1 anti-tumor necrosis factor (TNF)-α monoclonal antibody, is a successful immunotherapeutic agent for Crohn's disease (CD). The treatment with infliximab results in a high clinical efficacy, rapid onset of action and prolonged effect in patients with moderate to severe active CD, which have not responded to conventional therapy, and in fistulizing CD patients. Simultaneously the quality of life of these patients is essentially improved [1-3]. The proposed immunological mechanisms of infliximab include the suppression of TNF-α bioactivity and the lysis of TNF-α-producing cells, such as monocytes and lymphocytes, via complement fixation, antibody dependent cellular cytotoxicity (ADCC) and Fc portion binding of the IgG1 antibody. Furthermore, infliximab downregulates mucosal Th1 cytokines, reduces the expression of IFN-γ and other inflammatory molecules, such as intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule (VCAM)-1 [4-6].

Matrix metalloproteinases (MMPs) compose a family with over 20 members of Zn^{2+}-containing neutral proteinases [7]. Usually MMPs are synthesized as prepro-enzymes and are secreted in a proenzyme form that requires proteolytic cleavage for activation in most cases [8;9]. The activity of MMPs is precisely regulated within tissues by the balance between zymogen activation and enzyme inhibition. Factors which regulate activity of the MMPs include endogenous inhibitors, α-macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs) and the MMPs themselves [10;11]. MMPs are implicated in the inflammatory response, wound healing, tissue remodeling, cell growth, migration, apoptosis, cell-cell communication, tumor invasion and metastasis [12-16]. MMPs exert their activity by the degradation of a class of biological molecules which include not only the components of extracellular matrix (ECM) but also an increasing family of bioactive modulators, such as cytokines, growth factor receptors, other proteinases, coagulation factors, chemotactic molecules, and adhesion molecules [14]. In the pathogenesis of CD, MMPs are believed to be associated with the injury of gut tissue mediated by TNF-α and Th1 cytokines. One of mechanisms by which TNF-α causes intestinal tissue injury is believed to be the enhancement of the MMP production at local sites [17-20].

MMP-2 (72 kDa, gelatinase A) and MMP-9 (92 kDa, gelatinase B) are the two members of the gelatinase subgroup of MMPs. The substrates of MMP-2 and MMP-9 specifically include not only basement membrane (BM) type IV collagen and other components like gelatin, collagen type I, V, VII, X, lastin, laminin and fibronectin, but also numerous bioactive molecules, such as Fibroblast growth factor receptor (FGFR)-1, pro-interleukin (IL)-1 and ICAM-1 [21-24]. Previous studies already showed that MMP-2 and -9 are actively involved in the pathophysiological processes in the intestine of IBD patients [17;25-31]. After treatment with infliximab the elevated levels of MMP-1 and -3 in serum of patients with rheumatoid arthritis were reported to be reduced [32]. The role of MMPs in treatment of CD patients with infliximab, however, is still poorly understood. In the present study, we explored the relationship between the clinical efficacy of infliximab and the expression levels of MMP-2 and -9 in patients with CD, and described the *in vivo* and *in vitro* regulation of the expression of these two gelatinases by infliximab.

Materials and Methods

Clinical study

In the present study we included patients that participated in an expanded access program for infliximab treatment of CD in our institute, i.e. in-house study [3], and a subgroup of patients that participated in an international multicenter, placebo-controlled trial

of infliximab either for the treatment of fistulas in patients with CD or for the treatment of active CD [1;2]. The eligibility of patients in these studies is described previously [3;33]. Briefly, the age of confirmed patients with Crohn's disease had to be between 18 and 65 years. For inclusion in the fistula treatment groups, patients had to have single or multiple draining abdominal or perianal fistulas of at least three months' duration. Patients who had had Crohn's disease for at least six months, with CDAI scores equal or above 220 were eligible for the treatment with infliximab for active Crohn's disease. Analyses of efficacy evaluated the number of patients with a reduction of half or more in the number of draining fistulas from baseline as responders or those with complete healing (defined as the absence of any draining fistulas) at two consecutive visits. Changes in scores of the CDAI and the open fistulas scores were also evaluated. Failure of treatment was defined as changes in medication that were not permitted in the protocol, surgery related to Crohn's disease, or no return for follow-up visit.

Protocol. 1. **Fistulas**

Within two weeks of screening, eligible patients (n=10, one patient also with active disease) for the in-house study received infliximab 5 mg/kg (body weight), and patients from the international trial were randomly assigned to receive one of three treatments at weeks 0, 2 and 6: placebo (n=14) or 5 or 10 mg/kg of infliximab (total n=28). Study drug was administered intravenously over a 2-hour period. After the first infusion of study medication, patients returned for clinical and laboratory assessments at weeks 2, 6, 10, 14 and/or 18. Serum samples were collected, if possible, at each study visit through week 18.

Protocol 2. Active disease

Patients in the in-house study received a single dose of 5 mg of infliximab per kilogram of body weight (total n=7) in an intravenous infusion, administered over a two-hour period. Disease activity according to the CDAI and/or blood samples serum were assessed at day 0, day 3, week 2, 4, 8 and 12. In the international study patients were randomly assigned to receive a single dose of either placebo (n=7) or 5, 10 or 20 mg/kg of infliximab (total n=17). Disease activity was assessed and serum samples were collected at weeks 0 and 4.

***In vitro* study**

Patients, volunteers and blood samples

Patients with CD (n=7) were treated with infliximab for fistulizing and/or active disease in the in-house study. The healthy volunteers (n=5) were recruited from the laboratory.

Heparinized blood samples were obtained from patients before and two hours after a single infusion of infliximab of 5 mg/kg over 2 hours period. For the blood samples from the healthy volunteers a concentration of 75 μg infliximab per ml blood was added. At 37^{o}C and 5% CO_2, whole blood samples with/without infliximab were stimulated with/without Lipopolysaccharide (LPS) (Sigma, St. Louis, U.S.A) at 0.1 μg/ml blood for 1.5-24 hours for MMPs and TNF-α mRNA and protein determinations. The plasma was subsequently separated from the blood sample by centrifugation at 4^{o}C, and stored at -70^{o}C until further analysis.

Leucocytes isolation was performed by adding lysis buffer, containing 0.16M NH_4Cl, 10mM $KHCO_3$, and 0.01 mM K_2-EDTA (pH 7.4 at 0°C), to the samples. After erythrocytes were degraded the sample was centrifuged at 4°C and lysis was repeated to obtain pure leucocytes. The leucocytes were immediately used to isolate RNA.

Determination of MMPs by ELISA

MMP-2 and MMP-9 levels in the samples were measured by our highly specific enzyme-linked immunosorbent assays (ELISA), which measure the total of pro-enzyme, active- and inhibitor-complexed forms of the respective MMP, as described previously [34;35]. In brief, a polyclonal anti-MMP-2 antibody or monoclonal anti-MMP-9 antibody was used as catching antibody and appropriately diluted samples were incubated overnight at 4 °C. Immunodetection of MMP-2 was performed using polyclonal anti-MMP-2 followed by biotin-labelled goat anti-rabbit-IgG and of MMP-9 with biotin-labelled polyclonal anti-MMP-9 antibody. After incubation with avidin-peroxidase the chromogenic substrate 3,3', 5,5'-tetramethyl benzidine in the presence of hydrogen peroxide was added and the reaction was stopped with H_2SO_4 and the absorption was measured at 450 nm. The amount of MMP was calculated from the parallel standard curve and expressed in ng MMP per ml serum or plasma.

Immunohistochemical staining for MMPs

Standardized colonic biopsies of 6 patients from the in-house study (4 with fistulizing and 2 with active disease) were obtained at the start of the study, as well as at day 3 and week 2 of follow-up. To assess the localization of MMP-2 and MMP-9 within the intestinal tissues indirect immunohistochemical staining of the MMPs was performed as described previously [36]. In brief, paraffin tissue sections, treated with proteinase K for MMP-2 antigen retrieval, were incubated with rabbit polyclonal anti-human MMP antibodies, similar to those used in the ELISAs. Subsequently, the sections were incubated with biotinylated goat anti-rabbit Ig, peroxidase-labelled streptavidin, and stained with 3-amino-9-ethylcarbazole and hematoxylin.

The immunohistochemical staining was semiquantitatively assessed using the following scoring system: 0 = no staining, 1 = a few positive cells / areas of tissue or a low staining intensity in all cells, 2 = a minority of the cells / areas of tissue positive or a moderate staining intensity in all cells, 3 = a majority of the cells / areas of tissue positive and/or a moderate staining intensity in all cells, 4 = all cells or areas of tissue strongly positive.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Oligonucleotide primers (Table 1) for the RT-PCR were derived from the DNA sequence database of the NCBI (Bethesda, MD, USA). Primer sets were designed using the Primers3 Output computer program (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). The MMP-2 and MMP-9 PCR products span three introns to prevent interference of contamination by genomic DNA. Specificity of the primers was checked with the NCBI BLAST program. β2-microglobulin (β2-M) was used as a control to normalize PCR signals from the different samples.

Total RNA was isolated from blood samples by the method of Chomczynski and Sacchi [37]. The integrity and quality of the purified RNA were analyzed by 1.5% agarose gel/ethidium-bromide staining and the 260/280 nm absorbance ratio. Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase was used for cDNA synthesis from DNAse treated RNA. The PCR was started at 94°C for 3 min. followed by 30 cycles for MMP-2 and

MMP-9, and 28 cycles for β2-M. Each cycle consisted of a denaturation step (at 94°C for 30 sec.), an annealing step for 45 sec. (at 56°C for MMP-2 and β2-M, 59°C for MMP-9) and extension step (at 72°C for 1 min.), followed by a final elongation step (at 72°C for 7 min.). The reaction was performed in a Whatman T Gradient cycler (Biometra, Goettingen, Germany) and the amplified products were electrophorized on 1.5% agarose gels containing ethidium-bromide (0.5μg/ml) and visualized under ultraviolet light. A RT-PCR, which contained RNA but not M-MLV reverse transcriptase, was used to check contamination with genomic DNA. The Scion imaging program (Frederick, Maryland, USA. www.scioncorp.com) was used to semi-quantify the band density in the gels, expressed in arbitrary units (AU).

Table 1. Oligonucleotide primers for RT-PCR

mRNA	Gene	Sense primer	Antisense primer	Product size
NM-004530	MMP-2	AGGATCATTGGCTACACACC	AGCTGTCATAGGATGTGCCC	535
NM-004994	MMP-9	CGCAGACATCGTCATCCAGT	GGATTGGCCTTGGAAGATGA	406
NM-000594	TNF-α	CCCCAGGGACCTCTCTCTAA	GGAAGACCCCTCCCAGATAG	413
NM-004048	β2-M	CCAGCAGAGAATGGAAAGTC	GATGCTGCTTACATGTCTCG	269

Statistical analysis

The results of the MMP ELISAs are given as mean ± SEM., the clinical, immunohistochemical and mRNA data are presented as median with inter quartile range. The Wilcoxon signed-rank test or the paired Student t-test was used to evaluate difference between paired data and the Mann-Whitey *U*-test or the Student t-test for unpaired data, where applicable. Differences were considered significant when $P \leq 0.05$.

Results

Clinical studies

MMP-2

The serum MMP-2 level in patients with fistulas from the in-house study (n=6) responding to treatment with infliximab showed a steady increase from 605±78 (ng/ml) at day 0 up to 834±46 at week 14 (P=0.08). Correspondingly, the open/draining fistulas score in these patients was decreased at the end of follow-up from 3 (2-6) to 1 (0-1) (P=0.03), most of these responders already had signs of improvement at week 2. Except for the first three days, where the level of MMP-2 in the nonresponders of the patients with fistulas (n=4) decreased due to one outlier, also the non-responding patients showed a gradual increase up to 649±107 at the end of follow-up. The open fistulas scores in this group remained at 1 (Figure 1), and all these nonresponding patients had genitourinary fistulas.

Similarly, from day 0 to week 10 in the international study, the serum MMP-2 level in fistulizing CD patients increased in both placebo (n.s) and infliximab (P=0.003) treated patients, and in both responders/healers and nonresponders/nonhealers ($0.02<P<0.08$), with a general decrease after week 10. Baseline MMP-2 levels at inclusion were comparable between all sub-groups (Figure 2).

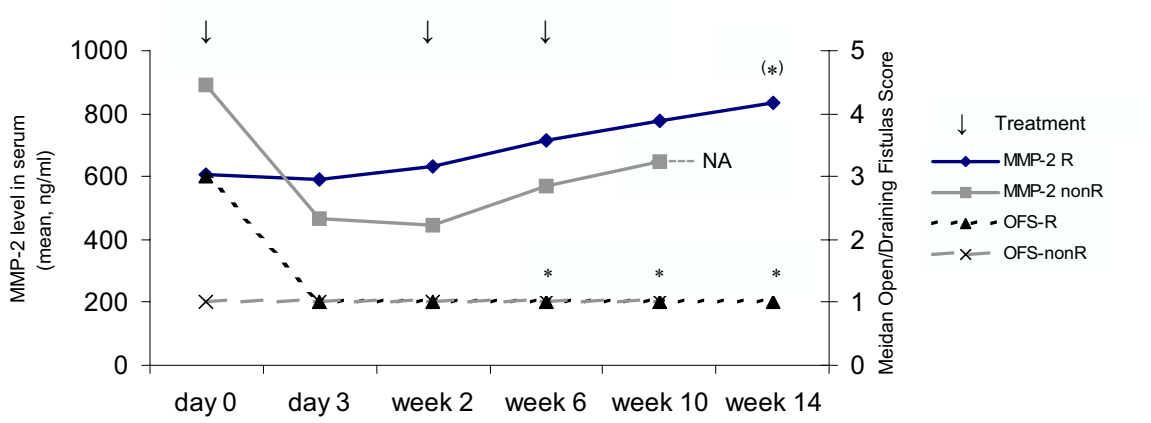

Figure 1. Serum MMP-2 levels in fistulizing patients from the in-house study showed a tendency to increase during follow-up in both responders (n=6) and non-responders (n=4).
NA: not available, (*): P = 0.08, *: P < 0.05.

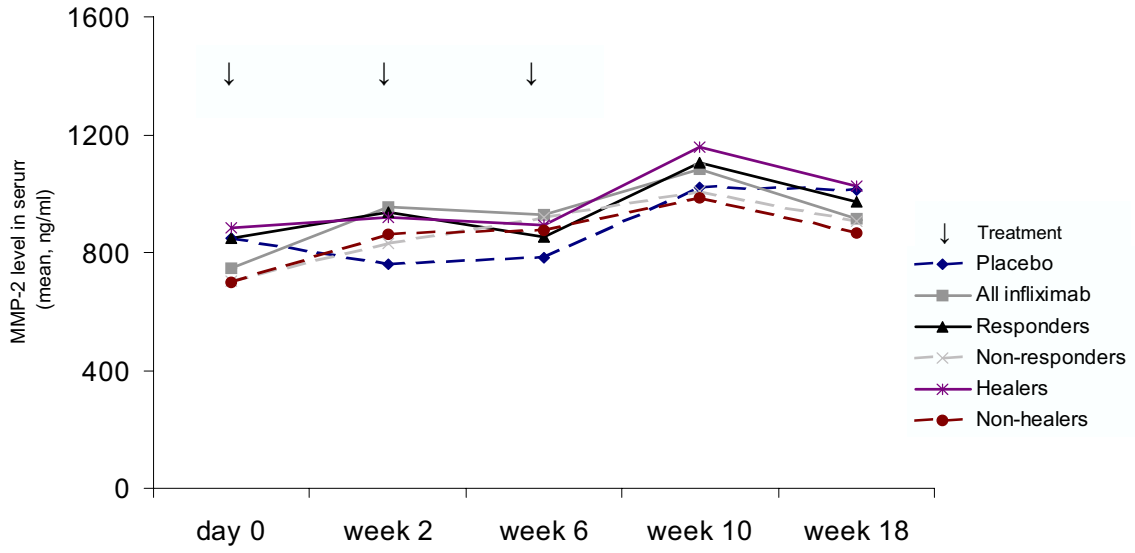

Figure 2. Serum MMP-2 levels in all sub-groups of patient with fistulas from the international study showed a similar pattern, i.e., slightly increasing during the follow-up, with a slight decrease at the end.

Also in CD patients with active disease (n=7) from the in-house study who responded to the treatment with infliximab, the improvement of disease activity was accompanied by a significant increase of the serum MMP-2 level from day 0, at week 4 to week 8 (P=0.03). The CDAI decreased significantly from 365 (264-461) down to 50 (10-189) at the corresponding time points (P=0.04). At the end of follow-up, i.e., week 12, the MMP-2 level started to decrease and the CDAI started to increase again. One patient showed no improvement by the infliximab treatment and the serum MMP-2 level in this patient remained stable over time (Figure 3).

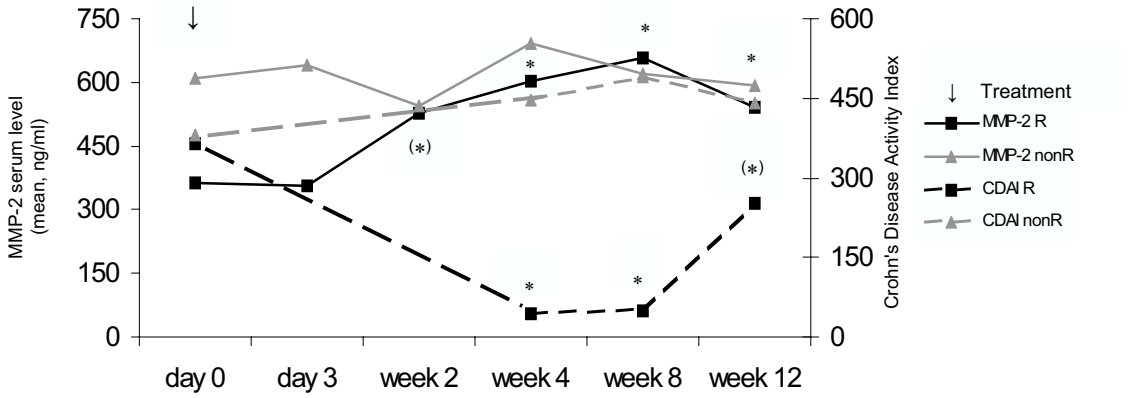

Figure 3. Active CD patients from the in-house study who responded to infliximab (n=7) showed a significant increase in serum MMP-2 levels from week 2 until the end of follow-up.
Non-responder: n=1, (*): 0.06 < P = 0.08, *: P < 0.05.

The serum MMP-2 level in patients with active disease from the international study did not show consistent and significant changes at the end of 4 weeks follow-up compared with day 0 in both placebo and infliximab treated groups (Table 2).

MMP-9

The MMP-9 serum level of the in-house patients with fistulizing disease in both responders and non-responders was hardly affected by the infliximab therapy (Figure 4). In the international study, there seemed to be a general trend to a decreased MMP-9 level where the infliximab treated patients and responders/healers seemed to have a slightly lower level at week 6 to 18 than the placebo treated and nonresponding/nonhealing patients, although no statistical significance was reached (Figure 5).

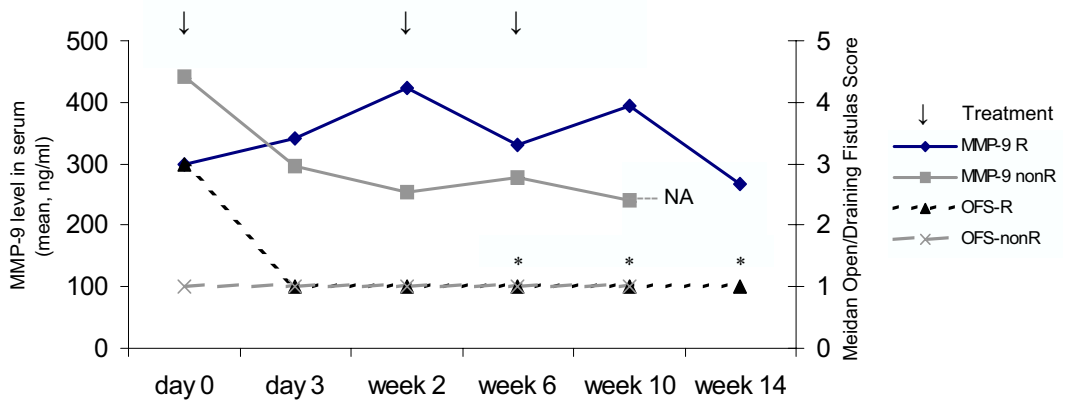

Figure 4. Serum MMP-9 levels in fistulizing patients from the in-house study remained stable or showed a tendency to decrease during follow-up in responders (n=6) and non-responders (n=4).
NA: not available, *: P < 0.05.

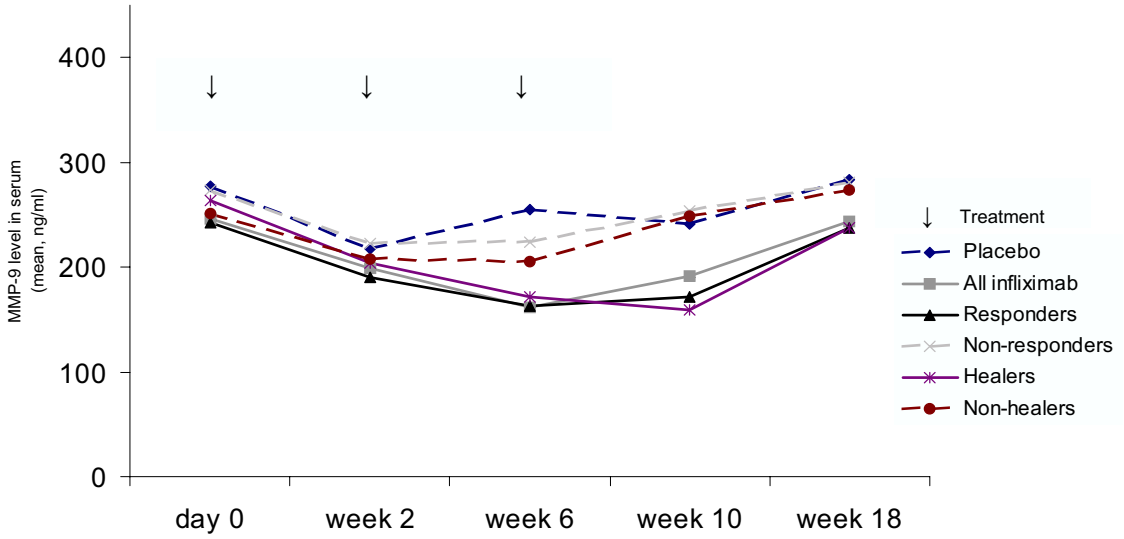

Figure 5. In contrast to the serum levels of MMP-2, MMP-9 levels in patients with fistulas from the international study showed a trend to decrease in all groups during the follow-up, with a slight increase at the end.
Placebo: n=14; All infliximab: n=28; Responders: n=22; Non-responders: n=19;
Healers: n=18; Non-healers: n=23.

Active disease patients from the in-house study who responded to infliximab had a decreased MMP-9 serum level from day 3 after treatment onwards. The MMP-9 serum level fell from 419±88 at the beginning of the study down to 236±26 at week 4 (P=0.05), and remained this at lower level to the end of the follow-up, 230±52 (P=0.05), accompanying the decrease in CDAI. The MMP-9 level in the one patient who did not respond to the treatment also showed a reduction of MMP-9 serum levels during the follow-up (Figure 6).

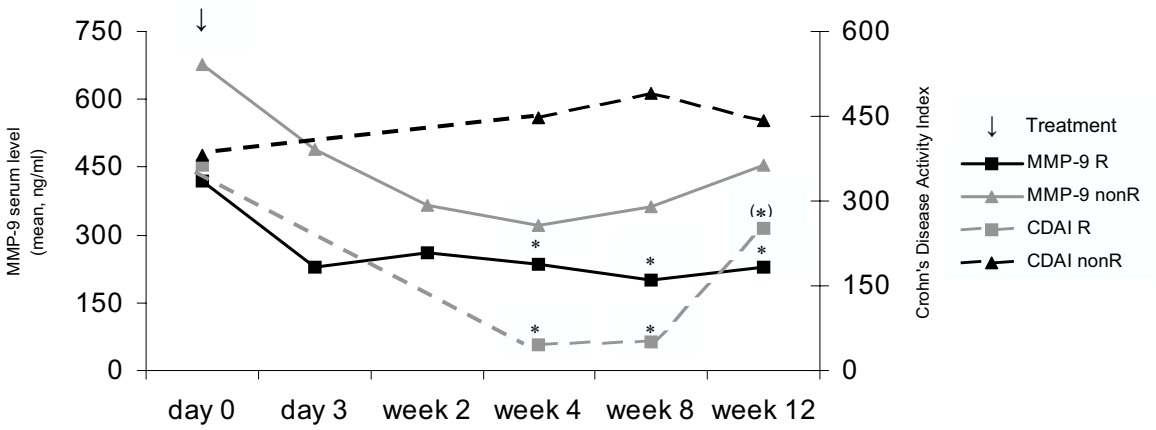

Figure 6. Active CD patients from the in-house study who responded to infliximab (n=7) showed a significant decrease in serum MMP-9 levels from week 4 until the end of follow-up.
Non-responder: n=1, (*): P = 0.08, *: P < 0.05.

In the international study the levels of MMP-9 in the active CD patients were also decreased in both the placebo and infliximab treated groups at the end of the follow-up, except for the 5 mg/kg infliximab group, but no statistical significance was reached (Table 2).

Table 2. Serum MMP-2 and –9 levels in patients with active disease included in the international study

		Placebo n=7	Infliximab 5 mg/kg n=4	Infliximab 10 mg/kg n=6	Infliximab 20 mg/kg n=7
MMP-2	day 0	780 ± 170	935 ± 411	658 ± 126	780 ± 80
	week 4	715 ± 225	879 ± 160	998 ± 118	898 ± 172
MMP-9	day 0	344 ± 79	180 ± 62	286 ± 111	255 ± 48
	week 4	207 ± 46	194 ± 65	232 ± 86	135 ± 29

MMPs presented in ng/ml (mean ± SEM).

Immunohistochemical results

A patchy and relatively strong positive immunoreaction to MMP-2 was present in the ECM of submucosa in non-inflamed tissues (Figure 7 A). In inflamed tissues a positive staining of MMP-2 was observed in endothelial cells and the ECM of the lamina propria (Figure 7 A and B). There were no major differences between patients with fistulas or with active disease. Overall the immunohistochemical expression pattern of MMP-2 did not seem to change by the treatment with infliximab therapy (data not shown).

The immunoreactivity for MMP-9 was predominantly present in the polymorphonuclear leukocytes (PMNL). A relatively high PMNL positive staining for MMP-9 was observed in the tissues before treatment [median score 2 (IQR 1-2.5)]. Follow-up biopsies after treatment with infliximab revealed a decreased intensity of MMP-9 staining already at day 3 [1 (1-1.8), n.s.] which was even lower at week 2 [0.5 (0-1.3), $P<0.05$] (Figure 8 A and B). Interestingly, in most of the tissue sections we found enteroendocrine cells to be positive for MMP-9, independent of treatment with infliximab (Figure 8 C).

In vitro study

Whole blood cultures revealed that the levels of MMP-2 in plasma of both CD patients and controls were not affected by stimulation with LPS or with LPS in the presence of infliximab for 1.5 or 24 hours. However, the levels of MMP-2 in the plasma of CD patients were in general lower than that in healthy controls (Table 3). With RT-PCR no detectable MMP-2 mRNA level was found in leucocytes of both CD patients and healthy volunteers.

	Patients (n=7)		Volunteers (n=5)	
	1.5 h	24 h	1.5 h	24 h
Blank	380 ± 108	477 ± 148	936 ± 200	771 ± 117
LPS	397 ± 140	639 ± 144	887 ± 120	833 ± 203
Infliximab+LPS	462 ± 152	421 ± 98	913 ± 346	718 ± 119

MMP-2 presented in ng/ml (mean ± SEM)

Table 3. Plasma MMP-2 levels from the in vitro whole blood cultures of CD patients and healthy volunteers

In contrast to the levels of MMP-2, MMP-9 levels in patients' plasma were higher, compared with those in healthy volunteers. After 1.5 hour LPS stimulation the levels of MMP-9 were significantly increased from 541±209 to 1132±242 (P<0.01) in CD patients, and from 126±18 to 364±75 (P<0.05) in controls, respectively, both more than 2-fold higher than the unstimulated cultures, and the increase in the CD patients was significantly higher than that in the healthy volunteers (P=0.05, Figure 9 and 10). However, the respective MMP-9 mRNA levels were downregulated to 50% from that of the blank samples, where TNF-α mRNA was increased 13-fold (Table 4). Infliximab was found not to affect MMP-9 protein levels of this short-term LPS stimulation (Figure 9 and 10).

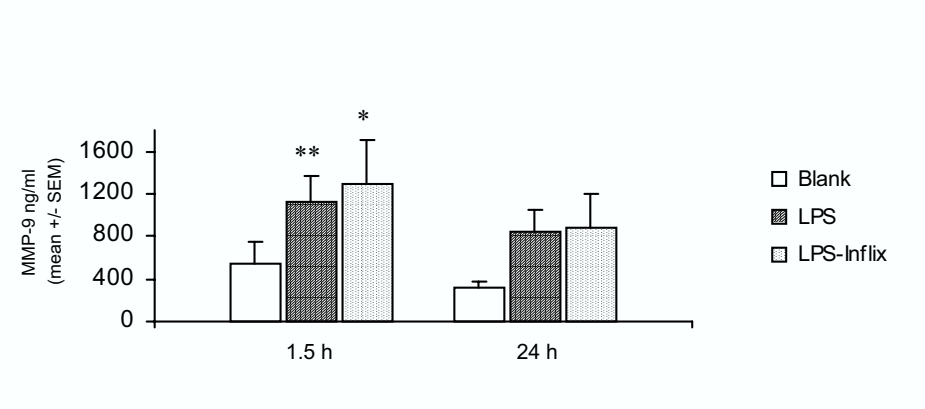

Figure 9. In CD patients (n=7) after 1.5 hour *in vitro* LPS stimulation of whole blood the plasma levels of MMP-9 were increased more than 2-fold compared to unstimulated blank samples, after 24 hours LPS did not further promote leucocytes to synthesize/release MMP-9. Infliximab did not affect the MMP-9 protein synthesis/release from leucocytes.
*: P < 0.05 vs blank; **: P < 0.01 vs blank.

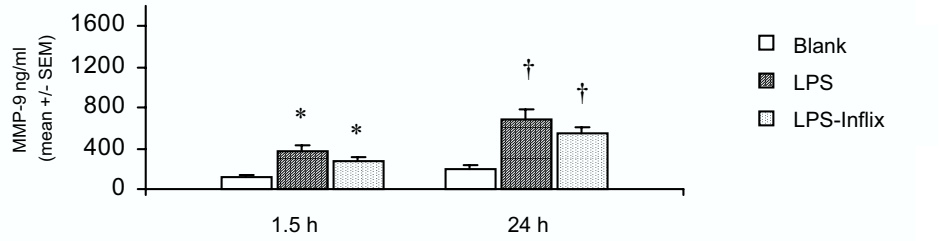

Figure 10. In healthy controls (n=5) 1.5 hour *in vitro* LPS stimulation of whole blood also increased the plasma levels of MMP-9 more than 2-fold compared to blank samples; further LPS stimulation significantly increased MMP-9 protein synthesis in leucocytes. Infliximab did not affect the MMP-9 protein synthesis at both 1.5 and 24 hour stimulation.
*: P < 0.05 vs blank; †: P < 0.05 vs 1.5 h.

After 24 hours, LPS stimulation did not further promote leucocytes to synthesize/secrete MMP-9 in CD patients, but in the healthy volunteers LPS stimulated the MMP-9 protein synthesis/secretion, increasing from 194±31 up to 679±106 (P=0.04). The LPS-stimulated level of MMP-9 protein after 24 hours was significantly higher in comparison with the level after 1.5 hours (P<0.05). Infliximab did not affect the MMP-9 protein synthesis after 24 hours in both patients and healthy volunteers (Figure 9 and 10).

More MMP-9 mRNA was transcribed in leucocytes after 24 hours stimulation with LPS, raising up to 16-fold. This transcription of MMP-9 mRNA was mediated by TNF-α as infliximab downregulated the mRNA level by almost 80%, (Table 4). At the same time, the TNF-α mRNA level in the 24 hour samples was enhanced by only 4-fold and even lower, i.e., 2-fold, in the presence of infliximab (Table 4).

Table 4. MMP-9 and TNF-α mRNA levels in cultured leucocytes from healthy volunteers

mRNA	1.5 h LPS	24 h LPS	24 h LPS + infliximab
MMP-9	0.5 (0.1-1.4)	16 (2-47)	3 (3-7)
TNF-α	13 (10-121)	4 (3-9)	2 (0.5-4)

The results represent 4 experiments, with mRNA levels expressed in median relative densitometry units (inter quartile range) in comparison with blank samples.

Discussion

Treatment with infliximab is very effective in patients with active or fistulizing Crohn's disease, although the mechanism(s) of action have not yet been fully elucidated [1;2;5]. In the present study, we found an increase of serum MMP-2 in both fistulizing and active CD patients by the treatment with infliximab, in comparison to baseline, with a decline at the end of follow-up after cessation of treatment. The cause of the increase in MMP-2 might be related to the turnover of the intestinal tissue in CD, especially the remodeling of the ECM components. CD is a chronic and recurrent inflammation of the alimentary tract, where remission and relapse of the disease alternate and almost inevitably occur. In the inflammatory process the destruction and healing of tissue seems to occur simultaneously. This could partially explain why the increase of serum MMP-2 is not strictly related to the criteria of clinical improvement. During these processes there is formation of granulation tissue, especially at ulcerative and fistulizing sites, where the remodeling of tissue actively takes place. This granulation tissue differs from the normal tissue in composition of cells and matrix components, containing many fibroblasts and endothelial cells. In addition, we previously showed that in the inflamed area MMP-2 is significantly increased [38]. The proliferation, differentiation and (neo)angiogenesis are highly promoted by stimulation through induced growth factors, such as transforming growth factor (TGF)-β, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). The increase of MMP-2 at local sites is probably to meet the demand of active ECM turnover. Ågren also believed that MMP-2 is important during the prolonged remodeling phase in wound healing [39]. Apparently, at moments of high tissue demand for MMP-2 the serum levels are low and at inclusion of controlled treatment protocols these serum levels start to increase, not strictly related to response. The sequestration of MMP-2 to ECM of intestinal IBD tissue may also partially contribute to the paradoxical phenomenon, i.e., the elevated expression of MMP-2 in inflamed tissue and low level in the circulation [20;25].

MMP-2 is constitutively expressed by many cells, especially mesenchymal cells, such as fibroblasts and endothelial cells, and has a ubiquitous tissue distribution [24]. TNF-α and other proinflammatory cytokines seem not to be major promoting factors for the expression of MMP-2 because the gene promoter lacks TPA-responsive element (TRE). TGF-β, however, is regarded to be a stimulator for the expression of MMP-2, which might be relevant to the remodeling processes [40]. Our *in vitro* results do confirm this since the levels of MMP-2 in plasma were not affected by the incubation of leucocytes with immunomodulators like LPS and/or infliximab.

Serum MMP-9 levels, in contrast to MMP-2, in the CD patients were found to reduced by the treatment, with an increase again at the end of the follow-up. Similar to the duration of the increase of the MMP-2 level, the decrease of MMP-9 also lasted for a time-period that coincides with the duration that infliximab is maintained at a detectable level in the circulation [5]. MMP-9 is thought to be an active participator the process of inflammation in CD, especially in the acute phase. Unlike other MMPs, MMP-9 is normally stored in secondary and tertiary granules of neutrophils poised for rapid release to participate in the reaction of the host to exogenous and endogenous stimulation. MMP-9 not only lyses components of the ECM, but influences also the generation or activation of c-x-c and other chemokines, which attract neutrophils to migrate across the BM of capillaries to inflammatory sites [41]. Kirkegaard et al. [31] recently found MMP-9 to be markedly upregulated in and contribute to intestinal fistula formation in CD. During the evolvement of tissue repair overexpression of MMP-9 has been speculated to prevent the healing process [42]. In contrast, Salo et al. [43] concluded that MMP-9 plays a prominent role because it participated in every step of the healing process, including detachment of epithelial cells from the basal membrane, rolling of cells to the wound matrix and remodeling of the granulation tissue.

The neutrophil is the most important source of MMP-9 in the acute phase of inflammation [44]. In the present study, immunohistochemical evaluation showed that MMP-9 predominately existed in the neutrophils and to a less extent in the ECM of severely inflammatory regions. The reduction of the MMP-9 expression in intestinal tissue from the infliximab treated CD patients is probably related to the decrease in number of the inflammatory cells, especially neutrophils and monocytes/macrophages [45;46]. MMP-9 was also found to be present in enteroendocrine cells. The significance of this observation needs to be elucidated further. Perhaps there is a similarity with the presence of MMP-7 in intestinal Paneth cells, which is believed to related to the activation of α-defensin [47;48].

Proinflammatory cytokines, such as TNF-α, IL-1β, are able to activate the MMP-9 gene through nuclear factor (NF)-κB to enhance the MMP-9 production [43;49]. LPS stimulates monocytes to express MMP-9, which is partly depending on TNF-α because neutralization of TNF-α significantly downregulated the production of MMP-9 [50]. Our *in vitro* study also showed that MMP-9 is released from leucocytes of both CD patients and healthy volunteers after short term 1.5 hours LPS incubation. The release of MMP-9 from leucocytes of the CD patients was significantly higher than from healthy volunteers. Most likely, the abundance of MMP-9 in the CD neutrophils has occurred during the process of their maturation activation in response to different stimulators, such as TNF-α, and bacterial products [51;52]. TNF-α seemed not to be involved in the process of MMP-9 secretion by neutrophils *in vitro,* as infliximab did not affect the level of MMP-9 in plasma. The transcription of MMP-9 mRNA was found to be inhibited by short term LPS stimulation, probably because of the increased production of other immediate response mRNAs like that of TNF-α.

Further incubation with LPS for 24 hours increased the level of MMP-9 significantly in healthy volunteers rather than in CD patients. The MMP-9 mRNA was also strongly upregulated. Infliximab did suppress this increase of MMP-9 mRNA, therefore we conclude that TNF-α is involved in the promotion of the transcription of MMP-9 mRNA. In the CD patients the MMP-9 producing cells probably lost their potential ability to further induce MMP-9 synthesis. Contrary to the situation in patients, leucocytes from healthy volunteers still had the capability to increase MMP-9 synthesis at both the mRNA and protein level, rather than only secrete the ready-on MMP-9 from leucocytes in response to LPS and TNF-α. We speculate that monocytes are the main MMP-9 producing cell type responding to the long term LPS incubation in this study. Pugin et al. [50] previously reported that the maximal level of monocytic MMP-9 synthesis was between 24 to 48 hours after LPS stimulation. Other studies also showed that LPS could activate monocytes to express the MMP-9 gene and protein, although they did not implicate TNF-α in their studies [53;54].

In conclusion, the serum MMP-2 and MMP-9 level in CD patients display an inverse changing pattern, i.e. an increase of MMP-2 and a decrease of MMP-9 during the treatment with infliximab, although not strictly related to the clinical effect of infliximab. The enhanced leucocyte MMP-9 expression in CD seems to be regulated by and responsive to TNF-α mediation.

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