

## CLINICAL STUDY

# Serum and synovial fluid concentrations of CCL2 (MCP-1) chemokine in patients suffering rheumatoid arthritis and osteoarthritis reflect disease activity

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**Abstract:** *Objective:* To determine serum and synovial fluid (SF) concentrations of monocyte chemoattractant protein-1 (MCP-1) or CCL2 chemokine, in patients suffering (RA) and osteoarthritis (OA) and to correlate the values to disease activity, and other patient- and disease-related parameters.

*Methods:* The CCL2/MCP-1 chemokine (CK) was measured in serum and SF of 30 RA and 15 OA patients using specific and very sensitive ELISA assay.

*Results:* The CCL2/MCP-1 CK was found in increased amounts in SF compared to serum ( $p < 0.001$ ) and in RA compared to OA patients ( $p < 0.001$ ). The values were significantly greater in RA patients with more active disease. Greater mean SF concentrations were observed in older RA patients, in patients with longer duration of RA disease and in those who had been treated with methotrexate. Also positive correlation was found between RA SF concentrations and SF leukocyte numbers ( $r = 0.497$ ,  $p < 0.05$ ).

*Conclusions:* The SF and serum CCL2/MCP-1 concentrations are significantly greater in RA than in OA and in hda-RA than in mda-RA; increased SF over serum concentrations suggest that CCL2/MCP-1 is mainly produced locally by activated cells where it may exacerbate and sustain inflammation by attracting pro-inflammatory leukocytes, predominantly monocytes (Tab. 1, Fig. 2, Ref. 50). Full Text (Free, PDF) [www.bmj.sk](http://www.bmj.sk). Key words: MCP-1, serum, synovial fluid, methotrexate, corticosteroids, rheumatoid arthritis, osteoarthritis, inflammation.

Chemokines (CKs) are large group of small (8-10 kDa), structurally related proteins that mediate and sustain inflammation by attracting and activating pro-inflammatory cells (1–11). The members of this family of proteins possess near their NH<sub>2</sub> terminus 4 conserved cysteine residues that form disulfide bonds which are critical for their biological activity (2, 4). With a few exceptions, CKs could be subdivided into two large subclasses based on the position of the first two cysteines: in CC CKs the first two cysteines are conserved, whereas in CXC CKs they are separated by another aminoacid. MCP-1 is a CC ligand-2 (CCL-2) CK which interacts with its specific receptor CCL2R.

Under normal physiological conditions many CKs are constitutively expressed and synthesized at low concentrations by resident cells in tissues. Released in the pericellular environment, they diffuse to the blood stream and bind to the specific receptors expressed on surface of endothelial and circulating mononuclear cells. Ligand-receptor coupling triggers a chain of complex and coordinated events that result in mononuclear cell roll-

ing, firm attachment to the vascular endothelium, trans-wall diapedesis and migration toward the site of CK production (12, 13). This chemoattractant activity is thought to play an important role in cell trafficking between hemopoietic tissues, blood stream and other tissues (6, 7, 12, 13). In inflammation much greater concentrations of inducible CKs contribute, together with other chemoattractant molecules such as complement-derived peptides, leukotriene B<sub>4</sub> and platelet-activating factor, to the accumulation of mononuclear cells in the synovial tissue and fluid (1–13).

*Rheumatoid arthritis* (RA) is a chronic painful inflammatory disease of unknown etiology, affecting primarily synovial tissue of multiple joints with symmetrical distribution (14, 15). RA synovial inflammation often results in the destruction of articular cartilage and adjacent bone causing deformities and severe impairment of joint function. Joint destruction is caused by hypertrophic inflamed synovial tissue (pannus) that has propensity to invade and destroy neighboring tissues such as ligaments, cartilage and bone (14). RA synovium contains many new capillaries (16, 17) and is heavily infiltrated by mononuclear cells, mainly activated T lymphocytes, plasmacells, monocytes and macrophages (14, 15, 18, 19), attracted by locally produced and released factors such as CKs. Hypertrophic synovial pannus and numerous SF cells overproduce and secrete excess amount of substances such as the metalloproteases, nitric oxide, free radicals, prostaglandins and others that sustain inflammation and promote

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tissue destruction (14, 15, 20). The proinflammatory cytokines and CKs are likely to be the key factors that drive cells to overproduce these deleterious agents thus accelerating tissue damage (1–15, 20).

In contrast to RA, *osteoarthritis* (OA) is a non-inflammatory, degenerative disease of one or several, most often weight-bearing joints and mobile segments of vertebral column. It is characterized by the focal destruction of articular cartilage resulting in secondary changes of the bone and synovial tissue. In most, if not all, OA cases, at least at the beginning of a disease, the synovial membrane is histologically normal or shows mild hypertrophy without perivascular cell infiltrates. The absence of important inflammation is also attested by minimal, if any, SF changes (22). However, in rare cases with severe joint destruction, the synovium could be more hypertrophic and inflammatory (23). This hypertrophy is mostly reactive to the presence of numerous osteo-cartilaginous fragments, engulfed by the synovium and in the process of resorption by macrophages (22). Such hypertrophic synovium is usually fibrotic and lacks the perivascular cell infiltrates.

The *CCL2/MCP-1* has been implicated in the pathophysiology of such inflammatory conditions like atheromatosis, multiple sclerosis and rheumatoid arthritis by attracting in the site of inflammation mainly monocytes and lymphocytes (10). It has been found in increased amounts in serum and SF of patients suffering RA (24–28), and in animals suffering experimentally-induced synovitis (29). Its plasmatic and SF concentrations could be decreased by anti-inflammatory drugs (25, 29). *CCL2/MCP-1* is produced locally by the cells from rheumatoid synovial tissue and fluid (30–36), and also to a certain extent by articular chondrocytes (37–40). In OA the serum and SF concentrations most often were found to be increased compared to control subjects (34–36), although much lower than in RA (28, 39–40).

The *CCL2/MCP-1* CK is believed to play a role as proinflammatory agent (10, 41). The single injection of this CK results in accumulation of monocyte/macrophages in the synovial tissue and fluid (24). The accumulation of lymphocytes and monocytes/macrophages is probably due to multiple cytokines (42). In “*in vivo*” role of *CCL2/MCP-1* and its capacity to attract leukocytes have been analyzed in several lines of transgenic mice (43–47). In these studies the capacity of *CCL2/MCP-1* to attract monocytes and macrophages was variably appreciated. In certain lines of transgenic mice where an over expression and production of *CCL2/MCP-1* was induced by gene manipulations an influx of lymphocytes and monocytes was observed (43), though in other lines it was not (44). In one line of mice, deficient in *CCL2/MCP-1*, intraperitoneal injection of thioglycollate does not cause the accumulation of monocytes and macrophages despite of normal numbers of leukocytes in blood (45). In addition the accumulation of monocytes in delayed-type hypersensitivity lesions was impaired. However, transgenic mice that over express and produce *CCL2/MCP-1* have increased susceptibility to infection and inflammation (43, 44). This abnormality was analyzed in one model of homozygotic (*-/-*) *CCL2/MCP-1* deficient mice (47). It was shown that such mice is unable to mount TH2 response

(humoral immunity) since lymph node cells from immunized *CCL2/MCP-1(-/-)* mice synthesize extremely low levels of IL-4, IL-5 and IL-10, but normal amounts of interferon gamma and IL-2 and were unable to produce specific antibodies.

In this study we have measured serum and SF concentrations of *CCL2/MCP-1* in 30 patients suffering RA and 15 patients suffering OA and correlated values to disease activity and other patient and disease relevant parameters.

## Material and methods

*Selection of patients.* All patients were evaluated by competent rheumatologists at the Institute of Niska Banja, Serbia and Montenegro. Thirty patients suffered definitive RA according to 1987 revised ARA criteria (48, 49) and 15 knee joint OA (minimum grade II, according to Kellgren and Lawrence (50)). All OA patients suffered pain on walking and had palpable knee joint effusion. In the RA group there were 26 females and 4 males (mean age  $53 \pm 13$  years, range 18–73 years); mean duration of the disease was  $8.3 \pm 8.1$  y (range 0–30 y). In OA group there were 13 females and 2 males (mean age  $64 \pm 7.7$  y, range 51–73 y); mean duration of the disease was  $6.5 \pm 4.9$  y (range 2–20 y). All RA patients were seropositive for rheumatoid factor and had active joint disease. They were subdivided in 2 subgroups: those with high disease activity (hda-RA) and those with moderate disease activity (mda-RA). Disease activity was evaluated based on American College of Rheumatology criteria (48, 49). Moderate disease activity was defined when the erythrocytes sedimentation rate was  $\leq 30$  mm/h, number of painful joints  $\leq 10$ , number of swollen joints 5 and morning stiffness  $\leq 1$  h. High disease activity group had at least three of the following criteria: erythrocytes' sedimentation rate  $> 30$  mm/h, number of painful joints  $> 10$ , number of swollen joints  $> 5$  and morning stiffness  $> 1$  h.

RA patients were further compared as a function of age, disease duration, erythrocytes sedimentation rate, leucocyte numbers in blood and SF and treatment regiment. Three age groups were compared  $< 40$ , 40–60, and  $> 60$  y; and three groups with disease duration of  $< 2$  y, 2–5 y and  $> 5$  y. As a function of treatment regiment, the patients were divided on those that are receiving or have received in the last three months one of the following drugs: chloroquine, gold or methotrexate. This group was further subdivided on those who have or have had chloroquine and those who have or have had methotrexate. The patients were also compared as a function of present or previous intra-articular (knee joint) injections of corticosteroids, and also as a function of daily dose of prednisone:  $< 10$  mg and  $> 10$  mg.

*Sample collection and preparation.* Blood was obtained by vein puncture and synovial fluid by puncture of the knee joints. SF was double diluted with phosphate buffered saline (PBS) and immediately centrifuged 5 min at 4 °C at a speed of 450 g. The supernatant was collected and stored at -20 °C until assayed. The SF sediment was suspended in a reconstituted volume of PBS and cells counted using Malassez hemocytometer. Blood cell count was done similarly in a routine way. Plasma was allowed to coagulate at room temperature and the serum collected and

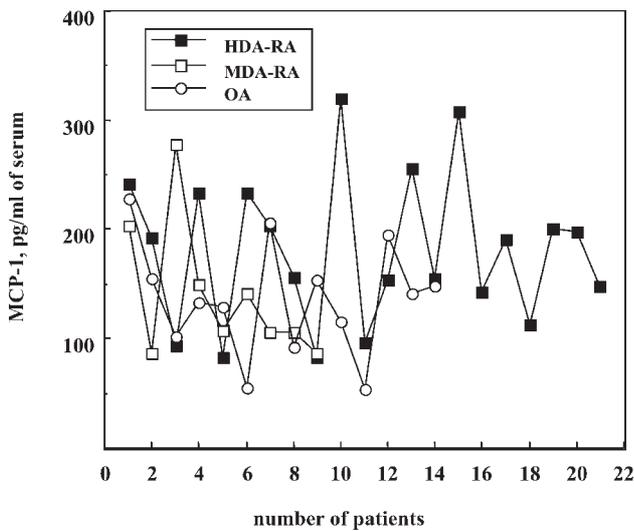


Fig. 1. MCP-1 serum concentrations in patients suffering RA and OA.

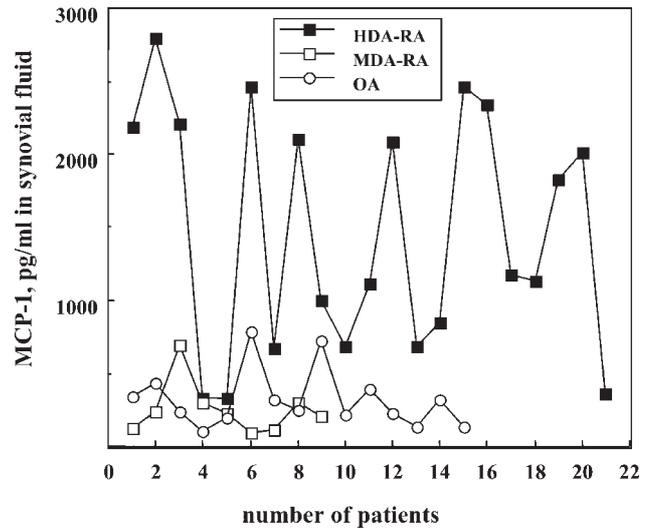


Fig. 2 MCP-1 synovial fluid concentrations in patients suffering RA and OA.

stored as above until assayed. All determinations were done on the same day. Prior analysis the samples were thawed at room temperature, centrifuged, and the supernatants used for the assays.

**CK determination.** CCL2/MCP-1 was measured in the serum and SF of all patients using specific ELISA-based Quantikine HS kits (R&D Systems Inc, Minneapolis, MN, USA). Lower detection limit in the assay was  $\pm 5$  pg/ml. Serum samples were assayed non diluted. SF samples were assayed diluted with PBS (v/v): 1/1, 1/2 and 1/3. The best fitting value with linear part of the concentration response curve given by the manufacturer was chosen to determine the concentration.

**Statistics.** The results were evaluated statistically using Windows statistical package for Social Sciences, version 10.0 which includes Student “t” test, Pearson X2 test and linear least square regression line. When “p” value was smaller than 0.05 for the samples number (n=1) the difference was considered significant. Highly significant difference had “p” value  $\leq 0.001$ .

**Results**

**Serum concentrations** (Fig. 1). The CCL-2/MCP-1 concentrations were:  $178 \pm 70$  pg/ml (range 82–320 pg/ml) for hda-RA,

Tab. 1. Mean MCP-1 synovial fluid concentration found in patients as a function of age, disease duration and treatment regiment.

Patient's age	hda-RA	mda-RA	RA (all)	OA
<40 years	1285±1305 (n=2)	250±65 (n=2)	767±962 (n=4)	–
40–60 years	1490±789 (n=14)	248±226 (n=6)	1216±916 (n=19)	266±124 (n=4)
>60 years	1615±922 (n=5)	206±132 (n=2)	948±815 (n=7)	340±223 (n=11)
disease duration				
<2 years	1580±1722 (n=2)	197±103 (n=3)	793±993 (n=5)	–
2–5 years	1451±981 (n=6)	177±90 (n=2)	1625±1121 (n=8)	312±227 (n=8)
>5 years	1457±680 (n=13)	336±249 (n=4)	1193±774 (n=17)	329±181 (n=8)
treatment with:				
metotrexate (n=6)			1588±600 (n=6)	
chloroquine (n=9)			1249±972 (n=9)*	
intra-articular CS:				
yes			1269±725 (n=14)	
no			1258±875 (n=16)	

\*p<0.05

140±63 pg/ml (range 86–276 pg/ml) for mda-RA, and 140±52 pg/ml (range 54–228 pg/ml) for OA.

SF concentrations (Fig. 2). The CCL-2/MCP-1 concentrations were 1,467±818 pg/ml (range 335–2,798 pg/ml) for hda-RA, 254±182 pg/ml (range 90–694 pg/ml) for mda-RA, and 320±200 pg/ml (range 105–726 pg/ml) for OA.

The differences were highly significant ( $p < 0.001$ ) between hda-RA group and other two groups for SF concentrations, only. Serum concentrations were significantly greater ( $p < 0.05$ ) in hda-RA compared to two other groups.

Greater concentrations also were found in the SF of RA patients in the age groups over 40 y, although the difference reached the statistical significance only in the age group 40–60 y (Tab. 1) ( $p < 0.05$ ). Also greater SF concentrations were found in the groups of patients that have suffered RA for 2 or more years or that have been treated with methotrexate compared to chloroquine (table). Statistically validated, positive correlation was observed when SF concentrations were compared to SF leukocyte numbers ( $r = 0.497$ ,  $p < 0.05$ ).

## Discussion

Our findings are in agreement with previously published results that have shown increased CCL2/MCP-1 concentrations in both plasma and synovial fluid of RA patients (24, 28) and much higher concentrations in patients suffering RA than OA (28, 40). Much more greater SF over plasma concentrations of CCL2/MCP-1 indicate that this CK is mainly produced by the cells of synovial tissue and fluid (30–36, 41).

In this study, we have confirmed previous reports claiming greater SF over plasma concentrations and greater SF concentration in patients suffering RA than in those suffering OA. In addition, we have shown greater serum and SF concentrations in RA patients suffering more active disease. The serum and SF concentrations found in patients suffering OA were significantly smaller than those of patients suffering RA but not very much different from those found in patients suffering less active RA. The differences were highly significant ( $p < 0.001$ ) when comparing hda-RA and each of the two other groups. These results suggest that CCL2/MCP-1 is an important, locally produced, mediator of rheumatoid inflammation. In support to this statement also are the findings of increased SF concentrations in patients suffering RA for longer than 2 years and positive correlation between SF CCL2/MCP-1 concentrations and respective SF leukocyte numbers.

As the patient's age and treatment regimens may interfere with the production and secretion of various agents by inflamed synovial tissue and fluid cells, we analyzed the results as a function of these parameters. Interestingly, increased SF values are found in patients older than 40 y and in patients who have been treated with methotrexate. Other treatment regimens, in particular, the intra-articular and systemic applications of corticosteroids do not seem to interfere with SF CCL2/MCP-1 concentrations.

Increased SF concentrations found in older patients may result from slower CCL2/MCP-1 clearance from SF, due to slower

SF drainage through thickened capillaries and lymphatics. The positive correlation found in this study between SF CCL2/MCP-1 concentrations and SF leukocyte numbers suggests that SF leukocytes are an important source of this CK. Similarly, greater SF CCL2/MCP-1 concentrations found in patients treated with methotrexate (which lowers leukocyte numbers) may be explained by slower clearance from the SF due to less CK binding to the specific cell surface based receptors and subsequent internalization and destruction in lysosomes.

Activated synovial fibroblasts, endothelial cells of small blood vessels, and proinflammatory resident cells, such as macrophages and dendritic cells (30–36, 41), and to a certain extent articular chondrocytes (37–40) are the main source of CKs production. Proinflammatory cytokines, interleukin-1 beta (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), and interferon gamma are known to activate these cells in RA inflammation (14–15). In “in vitro” they induce an over-expression of many specific genes and increase the synthesis of gene products, including CKs. Proinflammatory cytokines, TNF- $\alpha$ , IL-1 $\beta$  Interferon gamma and CXCL8/IL-8 seem to be potent inducers of CCL2/MCP1 synthesis (24, 30, 31, 36, 41).

Anti-inflammatory drugs are found to have variable effects on the production and secretion of CKs. In certain studies (25) serum and SF concentrations of CCL2/MCP1 were found decreased in RA patients treated with AINS and glucocorticosteroids. Others have reported an inhibitory effect on CCL2/MCP1 production by cultured RA synovioblasts obtained with dexamethasone or sodium thiomalate but not with AINS or methotrexate (36). Yet in another study, Tenidap, an AINS drug, has been reported to inhibit in “in vivo” and in “in vitro” the expression of specific mRNA and production of CCL2/MCP1 by synovial fibroblasts of pre-immunized rabbits challenged with the intra-articular injection of ovalbumin (29).

In our study serum concentrations were found to be slightly higher in RA patients than in OA patients but the difference was statistically significant ( $p < 0.005$ ) comparing the patients suffering hda-RA, only. The SF concentrations also were much higher in RA than in OA and in patient suffering more active RA. In spite of important patient to patient variations, these differences were statistically highly significant. The mean SF concentrations was about 8 times greater than mean serum concentrations in hda-RA group and about 2 to 3 times greater than serum concentrations in mda-RA and OA groups, respectively. Also it was approximately 5 times greater than mean SF concentrations in other two groups.

The analysis of these data suggests that CCL2/MCP1 is mainly produced locally and that the production is correlated to the degree of rheumatoid inflammation. In RA, only patients with more severe disease seem to have high SF concentrations.

In conclusion our results focus on CCL2/MCP1 as an important, locally produced and released, proinflammatory CK which, by attracting mononuclear leukocytes in great numbers in synovial tissue and fluid, significantly contributes to sustain inflammation and induce tissue damage in arthritis. Its SF concentration appears to reflect synovial inflammation and RA dis-

ease activity. It may help physician to correctly evaluate patient status and also incite researchers to develop specific chemokine/cytokine inhibitors that would presumably stop inflammation and tissue damage in arthritis.

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