

ORIGINAL ARTICLE

Plasma levels of matrix metalloproteinase-9 in chronic urticaria patients correlate with disease severity and C-reactive protein but not with circulating histamine-releasing factors

A. Tedeschi¹, R. Asero², M. Lorini^{1,3}, A. V. Marzano⁴ and M. Cugno^{3,5}

¹Unità Operativa di Allergologia e Immunologia Clinica, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Milano, Italy, ²Ambulatorio di Allergologia, Clinica San Carlo, Paderno Dugnano, Milano, Italy, ³Dipartimento di Medicina Interna, Università di Milano, Milano, Italy, ⁴Istituto di Scienze Dermatologiche, Università di Milano, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS, Milano, Italy and ⁵Unità Operativa di Medicina Interna 2, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione IRCCS, Milano, Italy

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Summary

Background Matrix metalloproteinase-9 (MMP-9) is an endopeptidase produced by many inflammatory cells that has been found in increased amounts in plasma from patients with chronic urticaria (CU).

Objective To evaluate plasma levels of MMP-9 and its tissue inhibitor of metalloproteinase-1 (TIMP-1) in CU patients in relation with disease severity, C-reactive protein (CRP) and circulating histamine-releasing factors.

Methods Fifty-two consecutive CU patients were included in the study and disease activity was graded from 0 to 3. Plasma MMP-9, TIMP-1 and CRP levels were measured by enzyme immunoassays. Circulating histamine-releasing factors were assessed using *in vivo* (autologous serum skin test) and *in vitro* (basophil histamine release) tests. Seven CU patients were studied both during active disease and during remission. Thirty healthy subjects were used as normal controls.

Results Plasma levels of MMP-9, TIMP-1 and CRP were significantly higher in CU patients than in healthy controls ($P = 0.0001$, 0.003 and 0.005 , respectively) and a trend towards a higher MMP-9/TIMP-1 molar ratio was found ($P = 0.051$). A significant correlation was found between plasma MMP-9 levels and urticaria severity score ($r = 0.48$, $P < 0.0001$). CRP levels correlated with MMP-9 levels ($r = 0.37$, $P = 0.008$) and CU severity score ($r = 0.52$, $P = 0.0001$), but not with TIMP-1 ($r = 0.13$) concentrations. MMP-9, TIMP-1 and CRP plasma levels and MMP-9/TIMP-1 molar ratio did not differ in patients either with or without an evidence of circulating histamine-releasing factors. Seven patients evaluated during remission showed a significant reduction of MMP-9 and CRP plasma levels.

Conclusion Plasma levels of MMP-9 and its inhibitor TIMP-1 are increased in CU patients. MMP-9 levels are associated with disease severity and CRP levels, but not with skin reactivity to autologous serum and with circulating histamine-releasing factors. These findings suggest that in CU there is an ongoing inflammatory process independent of the presence of circulating histamine-releasing factors.

Keywords autologous serum skin test, chronic urticaria, C-reactive protein, histamine-releasing factors, matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1

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Correspondence:

Dr Alberto Tedeschi, Unità Operativa di Allergologia e Immunologia Clinica, Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Via Pace, 9, 20122 Milano, Italy. E-mail: albitedi@alice.it

Introduction

Matrix metalloproteinase-9 (MMP-9, gelatinase B) is a zinc-dependent endopeptidase, that is responsible for the tissue remodelling and degradation of the extracellular

matrix [1, 2]. It is secreted as a 92 kDa zymogen (pro-MMP), which is activated by a variety of proteinases and inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) and by $\alpha 2$ -macroglobulin [3, 4]. Increased plasma levels of MMP-9 have been found in

patients with chronic urticaria (CU) and have been shown to be associated with the disease severity [5]. The main cellular source of MMP-9 in CU has not been defined, but it has been suggested that it could be derived at least in part by degranulating mast cells. In fact, according to Kessel et al. [5], the correlation between urticaria severity and the circulating MMP-9 suggests a relationship between MMP-9 levels and cutaneous mast cell degranulation. *In vitro* experiments have shown that human mast cells derived from peripheral blood can release MMP-9 on contact with activated T cells and a similar mechanism might be operating in CU [6]. The sites of wealing in CU patients are characterized by a perivascular infiltrate of lymphocytes (mainly of the CD4⁺ type), monocytes, neutrophils and eosinophils recalling the late-phase antigen-induced reaction [7–10]. It has been hypothesized that activated CD4⁺ T cells might reside in close proximity to mast cells and induce mast cell degranulation by cell-cell contact thus contributing to the appearance of urticaria symptoms. In the last two decades, it has been demonstrated that CU is often an autoimmune disorder due to histamine-releasing autoantibodies, namely autoantibodies directed against the α -subunit of the high-affinity IgE receptor or against IgE [11, 12]. However, histamine-releasing autoantibodies can be detected in about 30–50% of CU patients, and the mechanism operating in the other cases still has to be defined [13]. Direct stimulation by activated T cells might be an alternative mechanism of mast cell degranulation independent of the presence of histamine-releasing autoantibodies [6, 14].

The aim of this study was to evaluate MMP-9 and TIMP-1 levels in a population of CU patients in relation to the presence of circulating histamine-releasing factors, as assessed *in vivo* by autologous serum skin test (ASST) and *in vitro* by basophil histamine release assay (BHR), and to C-reactive protein (CRP) plasma concentrations.

Subjects and methods

Subjects

Fifty-two consecutive patients with chronic ordinary urticaria (CU) (14 males and 38 females, mean age 42.7 years) were evaluated at the allergy centres of Ospedale Maggiore Policlinico of Milano and Clinica S. Carlo, Paderno Dugnano, Italy, and selected for the study. CU was diagnosed on the basis of the recurrence of spontaneous weals with or without angioedema for >6 weeks. Patients with physical urticaria were excluded. All patients had active urticaria at the time of the study. Disease activity was estimated according to the number of weals present at the time when blood samples from these 52 patients were collected, as described previously by Sabroe et al. [15]: 1–10 small (<3 cm in diameter) weals = grade 1 (mild); 10–50 small weals or 1–10 large

weals = grade 2 (moderate); and >50 small weals or >10 large weals = grade 3 (severe). An intradermal test with 0.05 mL of fresh autologous serum (ASST) was carried out at least 5 days after stopping the antihistamine therapy (cetirizine, levocetirizine, loratadine, desloratadine or fexofenadine in all cases). ASST was performed following the method by Sabroe et al. [16], reading the weal and flare reaction at 30 min. Intradermal injection of saline solution (0.9% weight/volume NaCl) was performed as a negative control and skin prick test with 10 mg/mL histamine as positive control. Patients showing a red weal with a diameter at least 1.5 mm greater than the control saline solution were considered positive.

At the time of blood sampling, none of the patients had associated chronic inflammatory disorders, hepatitis C or an history of recent acute infectious disease. In seven patients, blood samples were taken during active disease and during remission. All subjects participating in the study gave their informed consent to ASST and peripheral blood collection for BHR, MMP-9, TIMP1 and CRP determinations. Thirty healthy subjects (12 males and 18 females, mean age 42.3 years) served as a control group for serum and plasma measurements. The study was approved by the local Institutional Review Board.

Matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 determinations

Blood samples for MMP-9 and TIMP-1 determinations were collected using heparin as an anticoagulant and were centrifuged (15 min at 1200 g) within 30 min after removal. The samples were then stored at –20 °C until assayed. MMP-9 concentration was measured using a sandwich enzyme immunoassay manufactured by R&D Systems (Minneapolis, MN, USA) using two monoclonal and polyclonal antibodies specific for MMP-9. The sensitivity of the assay is 0.156 ng/mL. Plasma TIMP-1 concentration was measured using a sandwich enzyme immunoassay manufactured by R&D Systems. The assay used a solid-phase bound monoclonal antibody specific for TIMP-1 and an enzyme-linked polyclonal antibody specific for TIMP-1; the sensitivity is 0.156 ng/mL. The intra- and inter-assay variability was lower than 10% for both MMP-9 and TIMP-1 methods. The molar ratio between MMP-9 and TIMP-1 levels was calculated for each patient assuming a molecular weight of 92 kDa for MMP-9 and 28 kDa for TIMP-1.

C-reactive protein determination

CRP plasma concentration was measured using a sandwich enzyme immunoassay (Zymutest CRP, Hyphen BioMed, Neuville-sur-Oise, France). Intra- and inter-assay variability was lower than 11%.

Basophil histamine release assay

Leucocyte suspensions from normal blood donors were prepared by dextran sedimentation of peripheral venous blood, anticoagulated with 0.01 M EDTA and mixed with 6% dextran in saline solution (Plander 70, Fresenius Kabi Italia, Isola della Scala, Verona, Italy) and 30 mM dextrose (Sigma Chemicals, St Louis, MO, USA). The cells were allowed to settle for 60–90 min at room temperature, according to Lichtenstein and Osler [17]. The leucocyte-rich plasma was aspirated and centrifuged at 300 g for 15 min at 4 °C, and the cell button was washed twice in Tyrode's buffer, pH 7.4, containing (mM) 140 NaCl, 5.5 dextrose, 2.7 KCl, 0.36 NaH₂PO₄ and 12 NaHCO₃. Leucocytes (with about 7×10^4 basophils) were then resuspended in 100 µL volume of Tyrode's buffer with 1.8 mM CaCl₂ and 0.5 mM MgCl₂ and incubated with 100 µL of the serum under examination, making a final volume of 200 µL. After incubation for 40 min at 37 °C, the reaction was stopped by the addition of 800 µL of ice-cold buffer solution and centrifugation at 1000 g for 10 min at 4 °C. After centrifugation, the supernatants were aspirated, mixed with an equal volume of 6% HClO₄ and centrifuged at 1000 g for 10 min at 4 °C. Histamine concentration in the supernatants was measured by an automated fluorometric method, according to Ruff et al. [18]. Spontaneous histamine release was evaluated by measuring the histamine concentration in the supernatant of unstimulated cells, incubated for 40 min at 37 °C. Total histamine content was obtained by adding 100 µL of 6% HClO₄ to 100 µL of cell suspension. Net histamine release was calculated as a percentage of total histamine content, after the subtraction of spontaneous release. A 5% release cut-off value was used, as described previously [19]. Sera were tested with leucocyte suspensions from three normal donors whose basophils were shown previously to release 30% of the total histamine content on challenge with an optimal dose of goat polyclonal anti-human IgE (10 µg/mL, Sigma Chemicals). In order to detect the presence of functionally active anti-FcεRI autoantibodies, basophil histamine release was also evaluated after the stripping of membrane-bound IgE with 10 mmol/L lactic acid (pH 3.9, incubation for 3.5 min at room temperature), a procedure that allows dissociation of FcεRI-bound IgE without damaging basophil functional activity [20]. One assay per serum was performed to detect the histamine-releasing activity. Histamine-release assay was carried out using the same sera used for ASST.

Statistical analysis

Results were expressed as mean ± SEM and were log-transformed before analysis because they were positively skewed. Differences between means were assessed by Student's *t*-test for unpaired values, and correlation was

assessed by the Pearson's test. The correlation with urticaria clinical score was assessed by the Spearman's rank test. *P*-values lower than 0.05 were considered significant.

Results

Plasma levels of MMP-9 and TIMP-1 were higher in CU patients than in healthy controls (mean ± SEM: 51.9 ± 7.5 vs. 23.3 ± 1.9 ng/mL, $P = 0.0001$ and 63.9 ± 2.7 vs. 52.8 ± 1.9 ng/mL, $P = 0.003$, respectively) (Figs 1 and 2). MMP-9/TIMP-1 molar ratio was higher in patients than in controls, although the difference did not reach statistical significance (0.24 ± 0.03 vs. 0.16 ± 0.03 , $P = 0.051$) (Fig. 3). MMP-9 levels paralleled the severity of CU, and a significant correlation was found between plasma concentrations and the clinical score ($r = 0.48$, $P < 0.0001$) (Fig. 4). Also, MMP-9/TIMP-1 molar ratio was associated with urticaria severity ($r = 0.34$, $P = 0.011$). TIMP-1 levels were not associated with disease severity ($r = 0.18$, P , NS), and no significant differences were found between patients with different degrees of urticaria severity. Because of the limited sample supply, CRP plasma levels were determined in 50 of 52 CU patients and were significantly higher than those found in healthy controls (2.98 ± 0.68 µg/mL, range 0.21–18.66 vs. 0.71 ± 0.8 µg/mL, range 0.10–1.60 µg/mL, $P = 0.005$). A significant correlation was found between MMP-9 and CRP plasma levels ($r = 0.37$, $P = 0.008$), but not between TIMP-1 and CRP plasma levels ($r = 0.13$, P , NS). CRP levels were also associated with CU severity ($r = 0.52$, $P < 0.0001$).

In the seven patients observed during CU exacerbation and during remission, MMP-9 and CRP plasma levels significantly decreased during remission (MMP-9 from

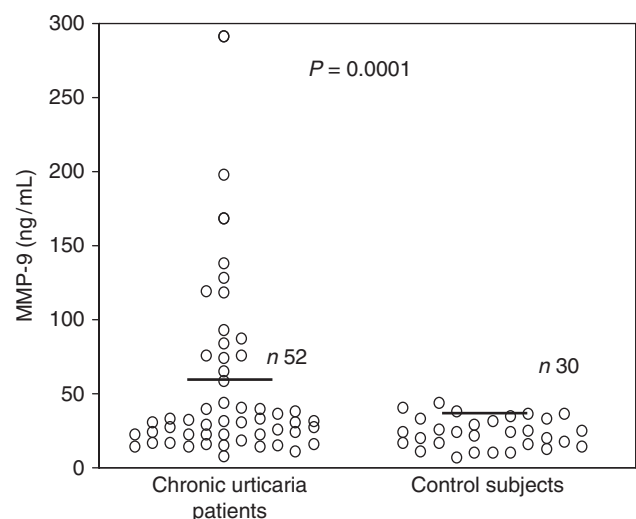


Fig. 1. Plasma levels of matrix metalloproteinase-9 (MMP-9) in patients with chronic urticaria (CU) and in normal controls. Results are expressed as ng/mL, and horizontal lines represent the mean values. MMP-9 plasma levels were significantly higher in patients with CU than in controls.

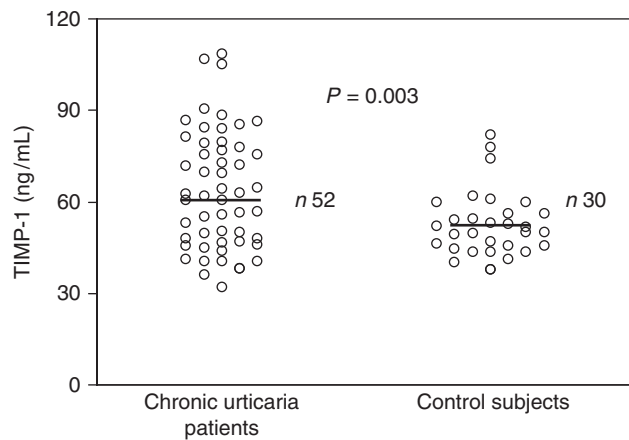


Fig. 2. Plasma levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) in patients with chronic urticaria (CU) and in normal controls. Results are expressed as ng/mL, and horizontal lines represent the mean values. TIMP-1 plasma levels were significantly higher in patients with CU than in controls.

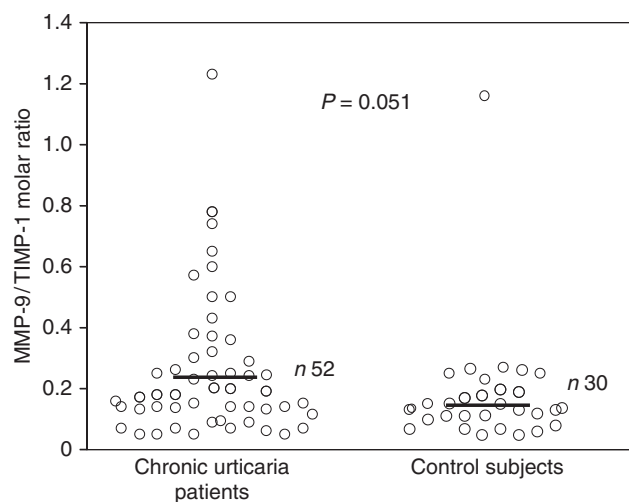


Fig. 3. Molar ratio between plasma levels of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in patients with chronic urticaria and in normal controls. Horizontal lines represent the mean values. MMP-9/TIMP-1 molar ratio tended to be higher in patients than in controls, although the difference did not reach statistical significance.

167.22 ± 37.0 to 23.62 ± 3.76 ng/mL, $P = 0.0007$, and CRP from 5.64 ± 2.19 to 1.6 ± 0.66 μ g/mL, $P = 0.008$). Also, TIMP-1 plasma levels decreased during remission but to a lesser extent without reaching statistical significance (from 55.78 ± 2.7 to 41.24 ± 5.51 ng/mL) (Fig. 5).

ASST, performed in 43 patients who accepted to undergo the test, was positive in 24 patients (55.8%) and negative in 19 (44.2%). No significant difference was found between ASST-positive and ASST-negative patients regarding MMP-9, TIMP-1 and CRP levels, and MMP-9/TIMP-1 molar ratio (Table 1). BHR was positive in 18 patients out of 52 patients (34.6%), with a mean release

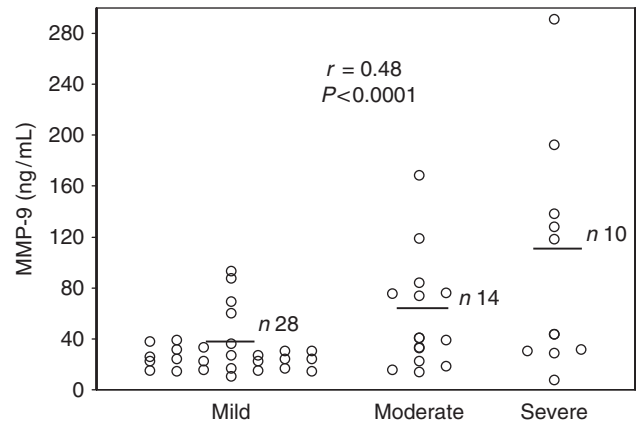


Fig. 4. Plasma levels of matrix metalloproteinase-9 (MMP-9) in patients with chronic urticaria divided into three different subsets according to the severity of the disease (mild, moderate and severe). Results are expressed as ng/mL, and horizontal lines represent the mean values. A significant correlation was found between MMP-9 plasma levels and urticaria severity score, as assessed by the Spearman's rank test.

of $19.80 \pm 2.92\%$. None of the BHR-negative sera converted to BHR positive after lactic acid stripping of basophil-bound IgE; however, an increase in histamine release after stripping of membrane-bound IgE was found in three patients who already tested weakly positive (from 5.86 ± 0.46 to $12.56 \pm 2.79\%$). None of the sera from the 30 healthy subjects caused histamine release above 5% of total histamine content (maximum release 3.1%). MMP-9, TIMP-1 and CRP plasma levels as well as MMP-9/TIMP-1 molar ratio were not significantly different in BHR-positive and BHR-negative patients (Table 2). In BHR-positive patients, no significant correlation was found between percentage of histamine release and urticaria severity score, MMP-9 levels or TIMP-1 levels.

Discussion

The present study shows that plasma levels of MMP-9 and TIMP-1 are significantly higher in CU patients than in normal subjects. MMP-9 levels as well as MMP-9/TIMP1 molar ratio paralleled the severity of CU, and a significant correlation was found between plasma concentrations and clinical score. In contrast, TIMP-1 levels were not associated with disease severity and no significant differences were found between patients with different degrees of urticaria severity. Although plasma levels of both MMP-9 and TIMP-1 are elevated in CU patients, it appears that the former is increased to a greater extent than the latter, suggesting that the increased release of MMP-9 in CU patients is not balanced by an adequate release of the natural inhibitor TIMP-1. Accordingly, the MMP-9/TIMP-1 molar ratio was higher in CU patients than in normal subjects, but the difference was not statistically significant. These findings are in agreement with the observations of Kessel et al. [5], who found increased plasma

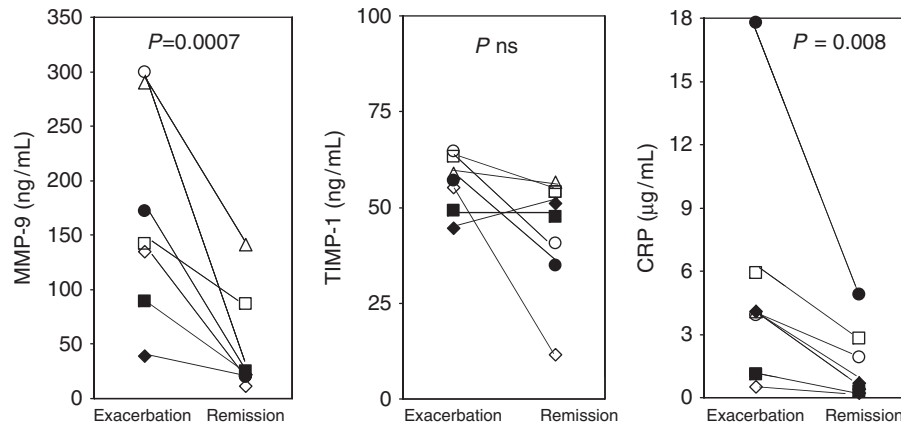


Fig. 5. Matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1) and C-reactive protein (CRP) levels in plasma samples taken during active disease and during remission in seven chronic urticaria patients.

Table 1. Matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1) and C-reactive protein (CRP) plasma levels, and MMP-9/TIMP-1 molar ratio in CU patients divided according to the results of autologous serum skin test (ASST)

Patient category	n	MMP-9 (ng/mL)	TIMP-1 (ng/mL)	CRP (μg/mL)	MMP-9/TIMP-1 molar ratio
ASST positive	24	51.93±10.68	68.69±4.18	2.89±0.82	0.23±0.04
ASST negative	19	50.17±14.92	61.19±4.70	3.13±1.30	0.24±0.06

Results are expressed as mean±SEM and are not significantly different in the patient groups.

Table 2. Matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1) and C-reactive protein (CRP) plasma levels, and MMP-9/TIMP-1 molar ratio in chronic urticaria (CU) patients divided according to the results of basophil histamine release assay (BHR)

Patient category	n	MMP-9 (ng/mL)	TIMP-1 (ng/mL)	CRP (μg/mL)	MMP-9/TIMP-1 molar ratio
BHR positive	18	60.31±12.98	60.32±4.84	2.33±0.82	0.28±0.05
BHR negative	34	71.42±17.33	65.94±3.18	3.36±0.97*	0.22±0.04

Results are expressed as mean±SEM and are not significantly different in the patient groups.

*CRP levels were measured in 32 BHR-negative patients out of 34 because of the limited sample supply.

levels of MMP-9 in CU patients and observed a good correlation with the clinical severity score. Antiga et al. [21] found increased MMP-9 plasma levels in patients with chronic autoimmune urticaria (i.e. with positive ASST), but not in patients with chronic idiopathic urticaria (i.e. with negative ASST). While confirming the higher plasma levels of MMP-9 in CU patients when compared with healthy controls, Altrichter et al. [22] failed to find a positive correlation with disease activity, although a slight and not significant tendency to higher MMP-9 levels in patients with more severe disease was observed. Interestingly, these authors found higher plasma MMP-9 levels in CU patients with elevated CRP serum levels than in CU patients with normal CRP serum concentrations. Furthermore, Altrichter et al. [22] observed no significant difference between ASST-positive and ASST-negative patients regarding plasma MMP-9 levels, in agreement with our findings. The results of our study also indicate that CU patients have raised CRP plasma levels that correlate with disease severity, but not with the presence of circulating histamine-releasing factors. In fact, CRP plasma levels were not significantly different in subsets of CU patients

testing positive or negative on ASST and BHR. Indeed, CU is associated with an increase in an inflammatory biomarker as CRP independently from the presence of detectable circulating histamine-releasing factors. Also, MMP-9 plasma levels were found to correlate with the severity score and CRP plasma levels, but not with the presence of histamine-releasing factors, as assessed by ASST and BHR. Interestingly, in the seven CU patients evaluated during activity and remission, both MMP-9 and CRP plasma levels significantly decreased during remission. It is conceivable that the elevated MMP-9 plasma levels reflect the inflammatory process occurring in CU patients independently from the presence of circulating histamine-releasing factors. This view is in line with the presence of a cutaneous cellular infiltrate consisting mainly of neutrophils, eosinophils and T lymphocytes in CU patients either with or without evidence of circulating histamine-releasing autoantibodies [9]. So far, the main cellular source of MMP-9 in CU has not been defined, although it has been demonstrated that several cell types detected in CU skin infiltrate are able to produce and release it. MMP-9 can be secreted as a 92 kDa zymogen (pro-MMP) by monocytes,

macrophages, neutrophils, eosinophils, mast cells and T lymphocytes [6, 23–25]. TIMP-1, an inducible protein that forms non-covalent complexes with MMPs, blocking the access of substrates to the MMP catalytic site, is also synthesized by many cells and tissues [4, 26]. Many physiological functions of TIMP-1 are linked to the functions of MMPs and an altered balance of MMP and TIMP production has been described in pathological conditions such as arthritis, tumour growth and metastasis [26]. The balance between MMP-9 and TIMP-1 is also altered in CU patients because MMP-9 is increased to a greater extent than TIMP-1, thereby contributing to the creation of a pro-inflammatory environment.

Whatever the cellular source of MMP-9 and TIMP-1, the data of the present study support the concept that CU is an inflammatory disorder that may, or may not, be associated with circulating histamine-releasing autoantibodies. In some CU patients, namely in those with ASST and BHR positivity, mast cell activation can be primarily ascribed to histamine-releasing autoantibodies; in the other patients with negative ASST and BHR, skin inflammation might be driven by cell-mediated mechanisms. In this respect, a role for T cells has been hypothesized following the *in vitro* observation that they have the capacity to activate mast cells directly by cell-to-cell contact [5, 14]. Although direct activation by T cells has been demonstrated for human mast cells derived from peripheral blood [6], but not yet for skin mast cells, Kessel et al. [5] have suggested that activated CD4⁺ T cells that reside in close apposition to cutaneous mast cell might cause mast cell degranulation and secretion of MMP-9. Further support for a possible role of T cells in CU pathophysiology stems from the observation of a positive correlation between a marker of T-cell activation (the soluble IL-2 receptor) and the mast cell-derived tryptase in CU patients [27].

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