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Elevated serum matrix metalloproteinase-2 and -9 and their correlations with severity of disease in patients with community-acquired pneumonia

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Background/aim: To determine the role of matrix metalloproteinases (MMPs) and their relationship with the clinical course of community-acquired pneumonia (CAP).

Materials and methods: Sixty-two consecutively hospitalized patients with CAP were enrolled and their pneumonia severity index (PSI), time to clinical stability (TCS), treatment response, and complications were recorded. The pre- and posttreatment serum concentrations of MMPs and their inhibitors were analyzed by ELISA. The activities of MMPs were evaluated by gelatin zymography.

Results: MMP-2 and -9 serum levels and their activities were higher in CAP patients than controls ($P < 0.001$ and $P < 0.001$, respectively). Low-risk patients had lower levels of MMP-2 and TIMP-1 than high-risk patients ($P = 0.044$, $P = 0.001$, respectively). Pretreatment serum TIMP-1 level was higher in patients with TCS of >3 days ($P = 0.004$) and was correlated with oxygenation and PSI scores. Posttreatment serum levels of MMP-9 and TIMP-1 were decreased after antibiotics ($P = 0.0001$ and $P = 0.017$, respectively).

Conclusion: Although MMP-2, MMP-9, and TIMP-1 correlate with many poor prognostic factors, more studies are required to prove their possible role in predicting the severity of CAP.

Key words: Matrix metalloproteinases, matrix metalloproteinase inhibitors, pneumonia, severity of illness index

1. Introduction

Community-acquired pneumonia (CAP) is the sixth most common cause of death in the United States and the leading cause of death from infectious diseases (1). It is related to morbidity and mortality, which place a major economic burden on the healthcare system (1). CAP has an incidence of 3–5 cases per 1000 persons and a mortality of 5%–15% in hospitalized patients, with higher mortality if the initial treatment is inappropriate (2,3). Despite modern antibiotics, mortality has still not declined owing to difficulties in choosing an appropriate antibiotic regime since CAP can be caused by multiple organisms (1–3).

In processing bacterial CAP, blood leukocytes respond to bacteria or bacterial products by secreting various substances, such as proinflammatory cytokines, chemokines, enzymes, and oxygen and nitrogen radicals (4–6). Matrix metalloproteinases (MMPs) are a family of zinc-neutral endopeptidases that are critical for disintegrating and remodeling the extracellular matrix during infection, inflammation, and wound healing (7). These enzymes are secreted as inactive proenzymes (or zymogens) and are either autoactivated or activated by other on-site proteolytic enzymes (7). It has been shown that inflammatory cells like neutrophils, lymphocytes, macrophages, endothelial cells and epithelial cells, and fibroblasts can produce a variety of proteases within the lungs (7–9). Although MMP-2 and MMP-9 (also known as gelatinases A and B) share some substrate specificities, MMP-2 is synthesized by structural cells (fibroblasts and endothelial/epithelial cells), whereas MMP-9 is mainly produced by inflammatory cells (10,11).

The major physiological inhibitors of MMPs in vivo are α-2 macroglobulin and TIMPs, the family of specific tissue inhibitors of MMPs. The TIMP family comprises four structurally related members, TIMP-1, TIMP-2, TIMP-3, and TIMP-4, with relative molecular masses ranging from 22 to 30 kDa, which bind with high affinity in a 1:1 molar ratio to the catalytic sites of active MMPs, resulting in loss of proteolytic activity (12,13).

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MMPs play an important role in the pathogenesis of several pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) (14,15), cystic fibrosis (16,17), bronchiectasis (18,19), asthma (11,15), pulmonary fibrosis (20), adult respiratory distress syndrome (21), and pneumonia (6,22–28). Higher levels and activities of MMP-8 and -9 have been detected in patients with hospital-acquired and ventilator-associated pneumonia (22–24). Recently, increased MMP-9 activity and MMP-9 levels in the plasma of CAP patients were observed in three studies from the same institution (6,26,27). In contrast to our study, the relationships of MMPs and their inhibitors with some clinical variables of CAP, such as radiologic involvement, time to clinical stability, or etiological microorganism, were not reported in those studies. Furthermore, the exact roles of MMPs need further examination to verify whether MMPs could serve as useful markers for predicting disease severity. The present study aims to determine the relationships of serum MMP-2 and -9, the MMP-9/TIMP-1 molar ratio, and MMP inhibitors to both clinical outcomes and disease severity in hospitalized CAP patients.

2. Materials and methods

Sixty-two consecutively hospitalized CAP patients were included in the study regardless of disease severity. CAP was defined according to the American Thoracic Society Community-Acquired Pneumonia Guideline (3). Initial treatment included commonly used antibiotics, such as cephalosporin, aminopenicillin, and macrolide, following the Turkish Thoracic Society Community-Acquired Pneumonia Guideline (29). Exclusion criteria were as follows: 1) immunosuppression or immunosuppressive therapy (including a daily dose of ≥20 mg prednisolone equivalent for >2 weeks); 2) prior antimicrobial treatment before hospital admission; 3) diagnosis of cancer, leukemia, or active tuberculosis; 4) discharge from hospital in the 10 days before latest admission; 5) pregnancy (30).

We calculated the pneumonia severity index (PSI) for each patient within 24 h of admission as proposed by Fine et al. (31). We also evaluated time to clinical stability (TCS) and response to empirical treatment (defined as normalization of temperature within 72 h after admission) by daily visits (32). At the end of the third day, patients were grouped as early or late stabilized cases. Patients were also placed into two groups as early (≤10 days) or late (>10 days) radiological resolution of pneumonia according to complete resolution time of pneumonic changes on the chest X-ray. That is why the patients underwent radiological evaluations every 5 days or less as needed. Detection of systemic inflammatory response was taken to indicate the presence of sepsis (33).

All routine biochemical and hematological studies and arterial blood gas analyses were done at admission before beginning antibiotic treatment. We used Gram staining and sputum, blood, and thoracentesis cultures (if pleural effusion was present) to obtain any potentially pathogenic microorganisms (PPMs); we did not use invasive diagnostic procedures, such as fiberoptic bronchoscopy or transthoracic needle aspiration. Age- and sex-matched healthy subjects (n = 30) were also enrolled in the study as controls.

Enzyme-linked immunosorbent assay (ELISA) for detection of IgM antibodies against *Mycoplasma pneumoniae* (Demeditec Diagnostics GmbH, Germany), *Chlamydia pneumoniae* (Medac Diagnostica, Germany), and *Coxiella burnetii* (Panbio Inc., USA) and for detection of IgG and IgM antibodies for *Legionella pneumophila* serogroups 1–7 (Virion/Serion GmbH, Germany) was performed on the baseline serum samples, which were immediately stored at −80 °C until analyzed. Detection of IgM antibodies against atypical agents was accepted as indicating active infection.

Pretreatment and posttreatment (n = 20, randomly selected) levels of MMP-2, MMP-9, and their inhibitors, TIMP-1 and α-2 macroglobulin, in the serum were determined with commercially available ELISA kits (R&D Systems, UK for MMP-2; Raybiotech, USA for MMP-9; BioSource International, USA for TIMP-1; Immundiagnostik AG, Germany for α-2 macroglobulin) following the manufacturer's instructions.

MMP-2 and -9 serum activities were measured by zymogram protease as previously described (34). We performed 7.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) for each serum sample. Enzyme activity was quantified by densitometry analysis using a Kodak Image 200MM (Kodak, USA). Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. The pro- and active forms of MMP-2 and MMP-9 were identified as bands at 72 and 67 kDa and at 92 and 87 kDa, respectively, by the relation of log*M*r to the relative mobility of Sigma SDS-PAGE low molecular weight marker protein.

Statistical analysis was performed using SPSS 15.0 for Windows (SPSS Inc., USA). All data are presented as means ± SEM. Correlation analysis was performed using Spearman's rank and Pearson rank correlations for nonparametric and parametric values, respectively. For two-group comparisons, the Mann–Whitney U-test was used, and the Kruskal–Wallis one-way analysis of variance was used to compare more than two groups. Pre- and posttreatment test values were analyzed with the Wilcoxon test because posttreatment serum samples were available in only 20 patients. A P-value of less than 0.05 was considered significant. The study protocol was reviewed

and approved by the local ethics committee. Diagnostic work-up was performed according to the Declaration of Helsinki's ethical principles for medical research involving human subjects, and informed consent was obtained from all study subjects prior to their inclusion in the study.

3. Results

Clinical characteristics of the 62 CAP patients are summarized in Table 1. Twenty-five patients (40.3%) had never smoked, while 17 patients (27.4%) were current smokers with a mean of 27.9 ± 5.6 packs per year. Comorbidities were detected in 42% patients: 16.1% had COPD, 6.5% had diabetes mellitus, 6.5% had chronic heart failure, 4.8% had chronic renal failure, 4.8% had suffered a cerebrovascular accident, 4.8% had nursing care at home, and 11.3% had other comorbidities.

Serum levels and activities of MMP-2 and MMP-9 were significantly higher in CAP patients than control subjects ($P < 0.001$ and $P < 0.001$, respectively) (Figures 1 and 2). In this study, neither MMP-2 nor MMP-9 serum levels showed any significant correlation with leukocyte or neutrophil counts in CAP patients. However, we found that TIMP-1 level was significantly correlated with oxygenation, specifically PaO_2 ($r = -0.266$, $P = 0.044$), PaO_2/FiO_2 ($r = -0.367$, $P = 0.005$), PSI scores ($r = 0.447$, $P = 0.0001$), CRP level ($r = 0.292$, $P = 0.026$), and clinical

Table 1. Demographic data (mean ± SEM) of all CAP patients.

	All CAP patients	PSI class I–III	PSI class IV–V	P
Number, n	62	44	18	-
Sex (male/female)	51/11	38/6	13/5	-
Age, years	43.2 ± 3.1	32.4 ± 2.8	69.4 ± 3.3	0.0001
Leukocytes, n × 10^9/L	18.47 ± 1.31	17.18 ± 1.38	21.62 ± 2.93	ns
Neutrophils, n × 10^9/L	15.20 ± 1.27	13.67 ± 1.36	18.76 ± 2.66	ns
Oxygenation index, PaO_2/FiO_2	285 ± 10.9	304.1 ± 13.4	236.0 ± 10.6	0.003
CRP, mg/L	158.4 ± 7.0	151.9 ± 9	173.2 ± 9.8	ns
Time to clinical stability, days	4.1 ± 0.3	4.2 ± 0.4	3.75 ± 0.4	ns
Sepsis	52 (83.9%)	36 (81.8%)	16 (88.9%)	ns
Bilateral infiltration	10 (16.1%)	5 (11.4%)	5 (27.8%)	ns
TCS >3 days	25 / 60	18 (40.9%)	7 (43.8%)	ns
Radiologic resolution >10 days	36/59	24 (55.8%)	12 (75%)	ns
PSI	65.5 ± 5.7	41.8 ± 3.5	123.2 ± 7.4	0.0001

CRP: C-reactive protein; FiO_2: inspired oxygen fraction; PSI: pneumonia severity index; PaO_2: arterial oxygen pressure; ns: not significant; TCS: time to clinical stability.

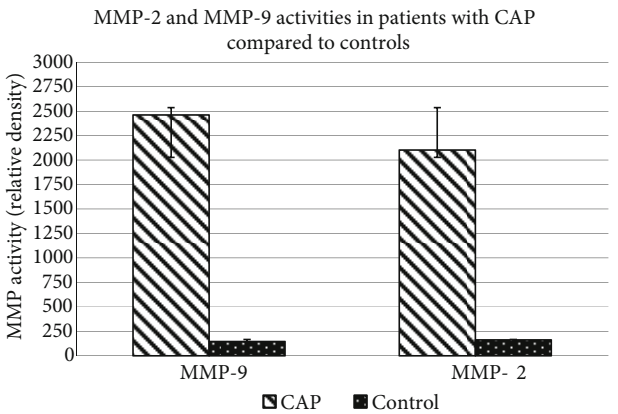

Figure 1. Serum MMP-2 and MMP-9 activities in patients with CAP compared to control subjects.

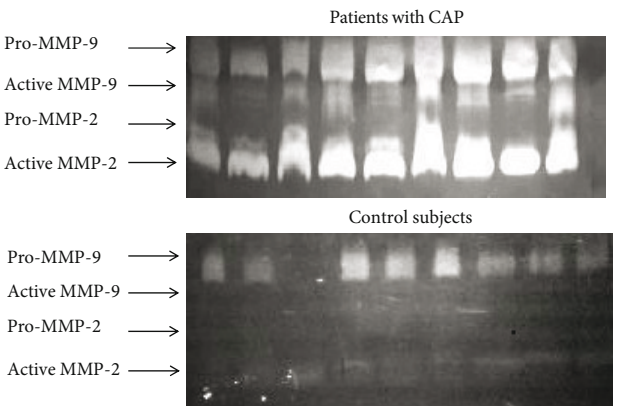

Figure 2. Representative serum MMP-2 and MMP-9 zymographs of CAP patients and control subjects.

variables such as hypotension (r = 0.365, P = 0.004) and tachypnea (r = 0.370, P = 0.003).

Table 2 and Figure 3 show MMP-2, MMP-9, TIMP-1, and α-2 macroglobulin serum levels in relation to various clinical variables. Systemic hypotension, tachypnea, and systemic inflammatory response syndrome was detected in 10 (16.1%), 8 (12.9%), and 52 (83.9%) patients, respectively. Higher TIMP-1 levels were detected in the serum of patients with hypotension or tachypnea than in those without (P = 0.004 and P = 0.004, respectively).

We found that serum levels of MMP-2, MMP-9, and TIMP-1 were similar in CAP patients with or without comorbidity, but serum α-2 macroglobulin level was significantly lower in patients with comorbidity (2.60 ± 0.23 mg/L) than those without comorbidity (3.10 ± 0.24 mg/L) (P = 0.037). We also could not find any relationship between smoking status and the serum levels of MMP-2, MMP-9, TIMP-1, or α-2 macroglobulin. Furthermore, no significant differences were detected in the serum levels of MMP-2, MMP-9, TIMP-1, and α-2 macroglobulin in patients with or without COPD.

Etiological microorganisms were identified in 19 of the 62 patients (30.6%), specifically *Mycoplasma pneumoniae* (n = 9), *Streptococcus pneumoniae* (n = 8), *Coxiella burnetii* (n = 4), *Legionella pneumophila* (n = 3), *Chlamydia pneumoniae* (n = 1), and polymicrobial etiology (n = 5). Serum α-2 macroglobulin levels were lower while MMPs and TIMP-1 levels were higher in patients infected with *S. pneumoniae*, although this was not statistically significant.

Compared to pretreatment levels, posttreatment serum levels of MMP-9 (95% CI: 785.6–2100.6) (P = 0.0001) and TIMP-1 (95% CI: 24.7–184.6) (P = 0.017) were lower but serum level of MMP-2 was higher 95% CI: –63.24 to 1.01) (P = 0.044) (Figure 4). We did not observe any significant difference between α-2 macroglobulin pre- and posttreatment serum levels.

4. Discussion

Extracellular matrix degradation is a well-known feature of MMPs, some of which, especially MMP-2 and MMP-9, play important roles in the pathogenesis of certain pulmonary diseases (6,11,14–24,26–28). However, only a few studies in the literature have investigated the role of MMPs in pneumonia pathogenesis (6,22–24,26–28). These have shown that patients with hospital-acquired pneumonia (HAP) have excessive MMP-8 and -9 concentrations in plasma and mini-BAL fluids compared to healthy controls (22–24). Excessive serum MMP-9 levels and MMP-9 activities have also been reported in the few studies of patients with CAP (6,26–28). We performed a zymographic analysis to determine active and inactive MMPs in the serums of CAP patients and controls. Active forms of MMP-2 and MMP-9 were 13 and 17 times higher, respectively, in CAP patients than controls, making our findings consistent with the results of previous studies (6,23,24,26–28).

There is only one study in the English-language literature showing that plasma TIMP-1 concentration

Table 2. Serum levels of MMP-2, MMP-9, TIMP-1, and α-2 macroglobulin, and the ratio of MMP- 9/TIMP-1 according to the some clinical criteria.

Variables	n (%)	MMP-2, ng/mL	MMP-9, ng/mL	TIMP-1, ng/mL	MMP-9/ TIMP-1	α-2 macroglobulin, mg/L
PSI						
Low risk (I–III)	44 (71)	191.9 ± 9.5	8078.8 ± 58.7	524.7 ± 22.2	23.7 ± 6.4	3.13 ± 0.2
High risk (IV–V)	18 (29)	222.4 ± 11.0	8092.7 ± 115.1	647.2 ± 86.6	12.8 ± 0.6	2.31 ± 0.2
P-value		0.044	0.368	0.001	0.002	0.010
Radiologic involvement						
Unilateral	52 (83.9)	200.5 ± 8.9	8077.9 ± 58.3	551.6 ± 20.5	21.8 ± 5.4	2.51 ± 0.18
Bilateral	10 (16.1)	202.9 ± 11.3	8108.8 ± 131.3	607.9 ± 34.6	13.9 ± 1.1	2.96 ± 0.2
P-value		0.907	0.433	0.311	0.315	0.473
Fever response						
Present	54 (86.2)	204.7 ± 8.9	8132.5 ± 48.5	536.6 ± 22.1	22.4 ± 5.5	2.99 ± 0.2
Absent	8 (13.8)	150.9 ± 15.4	7917.1 ± 190.8	636.2 ± 30.8	12.7 ± 0.7	2.47 ± 0.4
P-value		0.005	0.479	0.098	0.041	0.127
TCS						
≤72 h	36 (58.3)	198.1 ± 11.8	8144.0 ± 55.8	501.9 ± 27.5	25.8 ± 8.0	3.2 ± 0.2
>72 h	26 (41.7)	197.0 ± 11.6	8050.8 ± 87.7	614.1 ± 23.9	13.9 ± 0.9	2.6 ± 0.2
P-value		0.827	0.942	0.004	0.002	0.025

PSI: Pneumonia severity index; TCS: time to clinical stability.

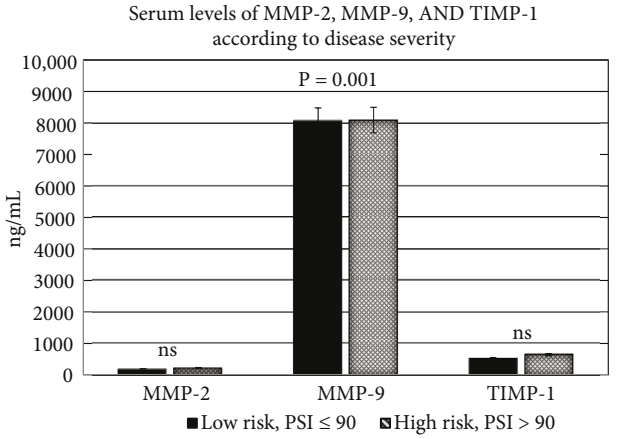

Figure 3. Serum levels of MMP-2, MMP-9, and TIMP-1 determined by ELISA analysis in CAP patients according to disease severity.

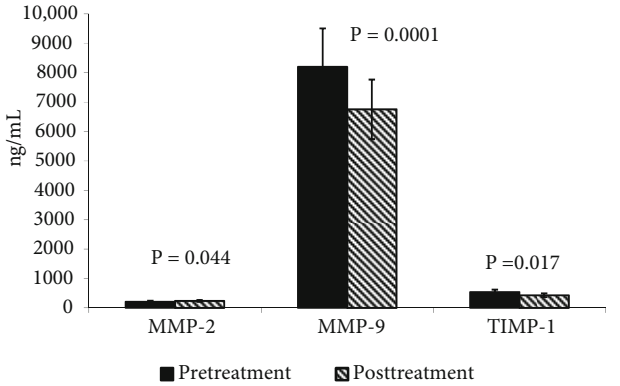

Figure 4. Serum levels of MMP-2, MMP-9, and TIMP-1 determined by ELISA analysis in 20 patients with CAP before and after the antibiotic treatment.

positively correlates with pneumonia severity indexes (PSI, CURB, and APACHE II) and length of hospital stay (27). Our results support this finding that patients with severe CAP have higher serum TIMP-1 levels and that protease/antiprotease imbalance is more prominent in severe CAP cases. This finding leads us to suggest that the MMP-9/TIMP-1 molar ratio may be used as a useful marker for determining disease severity in CAP patients. Furthermore, patients with severe pneumonia have higher MMP-2 levels and lower α-2 macroglobulin levels than nonsevere CAP patients, but the MMP-9 serum levels are the same. On the other hand, Hartog et al. (24) reported higher MMP-8 and MMP-9 levels both in serum and mini-BAL fluid in HAP patients with a clinical pulmonary infection score (CPIS) of ≥7 than in patients with CPIS of <7, but no difference in TIMP-1 levels. The CPIS was originally proposed by Pugin et al. (35,36) based on six variables (fever, leukocytosis, oxygenation, radiographic infiltrates, and tracheal aspirates and semiquantitative cultures of tracheal aspirates with Gram stain). It has a sensitivity of 93% and specificity of 100% for diagnosis of ventilator-associated pneumonia (35,36). Thus, it should be used as a prognostic index for pneumonia.

We identified pathogenic microorganisms in 19 of the 62 patients (30.6%), with *M. pneumoniae* and *S. pneumoniae* being the most frequently isolated microorganisms. Contrary to our expectations, we could not find any statistically significant differences in mean serum levels of MMP-2, MMP-9, TIMP-1, and α-2 macroglobulin between the patient groups whose pneumonia was caused by typical or atypical pathogen microorganisms or whose pneumonia was caused by an unidentified agent. Some bacteria, such as *S. aureus, P. aeruginosa*, and *E. coli*, may be associated with severe pulmonary damage and severe pneumonia (necrotizing pneumonia). However, only one study in the literature investigated MMP levels in relation to etiological microorganisms in CAP patients, showing that *M. pneumoniae* induces activity of MMP-9 in the patients' peripheral circulation (28). In patients with HAP, high-risk bacteria, such as *S. aureus* and *P. aeruginosa*, are related to increased concentrations and activities of pulmonary MMP (19). A similar finding was also reported in another study (20) showing increased levels of MMP-8 and MMP-9 in mini-BAL fluids of patients with HAP infected with PPMs compared with the non-PPM group.

Previous studies found that leukocytes/neutrophils can release a variety of extracellular matrix-degrading proteases, with a significant positive correlation between MMP-9 concentration and activity in mini-BAL fluid or serum (6,24). In contrast to these findings, however, we found no significant correlation between MMPs and leukocytes in the serum, although we did obtain similar results to those of Chiang et al. (27), namely that MMP-9 and TIMP-1 serum levels and CRP, leukocyte, and neutrophil counts decreased following successful antibiotic treatment. In our study, we observed an increase in serum levels of MMP-2 in patients with CAP after successful antibiotic treatment. This result may be related to the fact that MMP-2 is produced by mainly epithelial or endothelial cells. We had very few patients who had both pre- and posttreatment serum samples (only 20 patients). This may have been why we failed to identify any difference between pre- and posttreatment serum levels of α-2 macroglobulin and increase in posttreatment serum levels of MMP-2.

It is well known that morbidity and mortality are more common in CAP patients that have comorbidities, or who are immune-compromised or elderly. Many studies show that some comorbidities (COPD, bronchiectasis, or

diabetes mellitus) and even smoking may increase MMP activity compared to healthy controls (7,9,11,14,15,21–24). However, all patients in our study had pneumonia, which may be responsible for the increased levels of MMPs, rather than comorbid conditions or smoking status. We did not observe further increases in serum MMP-2, MMP-9, or TIMP-1 levels in those who had comorbid conditions compared to those who did not. To the best of our knowledge, serum MMP levels have not been compared in CAP patients with or without comorbidities in any study. In our study, on the other hand, the presence of high MMP-2, MMP-9, and TIMP-1 levels in patients with bilateral pneumonia; high TIMP-1 level in patients with systemic hypotension or tachypnea; and correlations between MMP-2 and TIMP-1 with hypoxia suggest that MMPs and TIMP-1 are related to several factors indicating a worse clinical prognosis. Thus, they can be used as prognostic markers for CAP patients.

In conclusion, MMP-2 and -9 activities were higher in CAP patients than controls and were correlated with several poor prognostic factors for pneumonia, including patient oxygenation index and TCS. MMPs and their specific inhibitors may therefore be used to determine disease severity in CAP patients. However, further studies with larger patient groups are necessary to confirm our results and to assess the prognostic impact of MMPs in CAP patients.

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