Published in final edited form as:

J Infect. 2009 July ; 59(1): 49–55. doi:10.1016/j.jinf.2009.05.004.

MATRIX METALLOPROTEASES IN BRONCHOALVEOLAR LAVAGE FLUID OF PATIENTS WITH TYPE III PSEUDOMONAS AERUGINOSA PNEUMONIA

Ali A. El-Solh, MD, MPH^{1,2}, Daniel Amsterdam, PhD³, Ahmad Alhajhusain, MD^{1,2}, Morohonfolu E. Akinnusi, MD¹, Ranime G. Saliba, MD¹, Susan V. Lynch, PhD⁴, and Jeanine P. Wiener-Kronish, MD⁵

¹ The Veterans Affairs Western New York Healthcare System; Buffalo, NY

²Western New York Respiratory Research Center, Department of Medicine, School of Medicine and Biomedical Sciences, Buffalo, NY

³Department of Laboratory Medicine, Erie County Medical Center, Buffalo, NY

⁴ Cardiovascular Research Institute University of California San Francisco, San Francisco, CA

⁵Department of Anesthesia and Critical Care, Massachusetts General Hospital, Boston, MA

SUMMARY

Objectives—In patients with ventilator associated pneumonia (VAP), *Pseudomonas aeruginosa* type III (TTSS) secreting isolates have been linked to poor clinical outcomes. Differential expression of matrix metalloproteinases (MMPs) induced by type III effector proteins may herald an irreversible lung injury.

Methods—Serial bronchoalveolar lavage fluids collected from 41 patients with *P. aeruginosa* at onset of VAP, day 4, and day 8 after antibiotic therapy were assayed for MMP-8, MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), and α -2 macroglobulin levels.

Results—At the onset of VAP, isolates secreting ExoU had the highest MMP-9 levels. The response to antimicrobial therapy showed a differential drop in MMPs with significant decrease in MMP-8 and MMP-9 levels on days 4 and 8 in patients with TTSS[−] compared to TTSS⁺ phenotype. The ratio of MMP-9/TIMP-1 was significantly associated with α -2 macroglobulin at end of therapy ($r=0.4$, $p=0.02$). Patients who survived had a lower MMP-9/TIMP-1 ratio than those who died ($p=0.003$).

Conclusions—VAP linked to *P. aeruginosa* Type III phenotype portrays a divergent antibiotic treatment response in regards to the concentrations of metalloproteinases in the alveolar space. The imbalance between MMP-9 and TIMP-1 may determine the intensity of alveolocapillary damage and ultimate outcome of *Pseudomonas aeruginosa* VAP.

Keywords

Pseudomonas aeruginosa; exotoxin; metalloproteinases; ventilator associated pneumonia

Please address any correspondence and reprint request to: Ali El Solh, MD, MPH Veterans Affairs Western New York Healthcare System Medical Research Building 20 3495 Bailey Avenue, Buffalo, NY 14215–1199 Tel (716) 862–7366 Fax (716) 862–8632 Email: solh@buffalo.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Pseudomonas aeruginosa remains a significant cause of nosocomial infection and septic mortality in patients admitted to the intensive care units (1). Since the early 1980s, the incidence of *P. aeruginosa* pneumonia has been steadily rising. Based on isolation of the organism from sputum culture, *P. aeruginosa* currently ranks as the leading cause of nosocomial pneumonia in the United States (2). The bacterium is uniquely problematic because of a combination of inherent resistance to many drug classes and its ability to acquire resistance to all relevant treatments. With 5570 open reading frames, *P. aeruginosa* genome is among the largest genomes of the prokaryotic world and encodes a vast array of proteins involved in regulation, transport and virulence factors, which may explain the high versatility and adaptive capacity of the species (3). Among the virulence factors described is the type III protein secretory system (TTSS). This syringe-like apparatus allows bacteria to directly translocate a set of toxins, termed effector proteins, into the eukaryotic host cell, where they modify signal transduction pathways to subvert the host immune response (4). Previous studies have reported that 77% to 90% of *P. aeruginosa* isolates from patients with acute respiratory infections secreted type III proteins and that the presence of type III secreting isolates is associated with worse outcomes in this setting (5,6). Recently, we have shown that type III phenotype is linked to the persistence of *P. aeruginosa* in the alveolar space of patients with ventilator-associated pneumonia (VAP) despite appropriate antimicrobial therapy (7). One of the mechanisms proposed for the failure to eradicate these strains is induction of neutrophil apoptosis.

Neutrophils could also release abundant matrix metalloproteinases (MMPs) upon activation (8,9). MMPs belong to a family of zinc- and calcium-dependent endopeptidases, which are capable of degrading connective tissue. MMP-8 (collagenase-2) and MMP-9 (gelatinase B) are produced by neutrophils (8,9), although MMP-8 can be also produced by non-neutrophil-lineage cells such as chondrocytes (10), synovial fibroblasts, and airway epithelial cells (11). MMP-8 and MMP-9 are normally stored in the intracellular secondary and tertiary granules respectively, prior to their release by neutrophils. Once released, the activity of MMPs is controlled by regulation of expression and secretion, by proteolytic activation of pro-enzymes and by the tissue inhibitors of metalloproteinases (TIMPs) (12,13). TIMPs form 1:1, non-covalent complexes with MMPs, blocking access of substrates to the MMP catalytic site.

Levels of MMP-8 and MMP-9 are elevated in the bronchoalveolar lavage fluid (BALF) of patients with hospital acquired pneumonia (14), but little is known about their expression in VAP attributed to *P. aeruginosa* with type III secretory phenotype and their response to therapy. Hence, we hypothesized that VAP patients with TTSS⁺ *P. aeruginosa* phenotype would have higher levels of MMP-8, MMP-9, and TIMP-1 in BAL than those infected with TTSS⁻ *P. aeruginosa* phenotype.

MATERIALS AND METHODS

Study population

The study protocol was approved by the Institutional Review Board of the State University of New York at Buffalo. Written informed consent was obtained from all subjects or their legal representatives. Forty one patients with documented *Pseudomonas aeruginosa* VAP were recruited. Some of the subjects included in this investigation were part of previous investigations (7,15). Only patients with first episode of *Pseudomonas aeruginosa* VAP defined as 1) fever with body temperature $\geq 38^{\circ}\text{C}$, or hypothermia defined as body temperature $\leq 35^{\circ}\text{C}$; 2) leukocytosis with white blood cell count $\geq 10,000/\text{mm}^3$ or leukopenia with total white blood cell count $< 4500/\text{mm}^3$ or $> 15\%$ immature neutrophils (bands) regardless of total peripheral white blood cell count; 3) new or progressive pulmonary infiltrate; and 4) bacterial

growth of 10^4 colony forming unit (CFU)/ml or more from BALF were enrolled. Eight control patients without cardiac or pulmonary disease who were intubated for airway protection were also enrolled. In all patients, tidal volumes were maintained as 6–8 mL·kg⁻¹ using a pressure-controlled or pressure-support mode. Positive end-expiratory pressure (PEEP) levels were set according to a strict protocol, in which optimal PEEP was defined as the lowest level of PEEP with maximum P_{aO_2} . Levels of PEEP were 9.0 (8.0–11.5) cmH₂O in patients with VAP, and 8.0 (7.3–13.0) cmH₂O in mechanically ventilated controls ($p=0.9$). All patients received a total of 7 days of antimicrobial therapy in accordance with the American Thoracic Society guidelines for the management of ventilator-associated pneumonia (16). Exclusion criteria included acute respiratory distress syndrome, extrapulmonary or polymicrobial infection, bacteremia, discordant antimicrobial therapy, immunosuppression, organ transplantation, and the presence of hematologic or solid organ malignancies.

Data Collection

Demographic and clinical data were recorded on study enrollment and included age, gender, duration of mechanical ventilation before study onset, prior antibiotic therapy, temperature, leukocyte count, ratio of PaO_2/FiO_2 , Acute Physiology and Chronic Health Evaluation (APACHE) II score (17), and the Clinical Pulmonary Infection Score (CPIS) (18).

Collection and processing of respiratory specimens

Mini-BAL fluid was obtained in a standardized fashion prior to initiation of antimicrobial therapy (15). BALF was filtered through one layer of sterile gauze and centrifuged at 500g for 10 minutes to separate the supernatants from the cell pellet. BALF was stored as aliquots at -70°C for further analysis. The cell suspension of the pooled BALF samples was washed three times in Eagle's minimal essential medium (MEM) containing 0.2% bovine serum albumin and 0.1% EDTA or in Hank's solution and resuspended in MEM. The total cell count was performed on a haemocytometer (Coulter Electronics Ltd) and cell viability was assessed by trypan blue exclusion. Differential cell counts were obtained from smears stained with May-Gruenwald-Giemsa. The viability of neutrophils was more than 96% in each group. A repeat non-bronchoscopic-directed BAL was performed on day 4 (± 1 day) and on day 8 (± 1 day) of VAP onset.

Immunoblot analysis of PcrV, ExoS and ExoU

To determine whether strains were capable (TTSS⁺) or incapable (TTSS⁻) of secreting the two most potent type III cytotoxins (ExoU and ExoS), immunoblot analysis was performed. Isolates were cultured under TTSS-inducing conditions in MIN-S medium (19). Cultures were incubated with shaking overnight at 37 °C prior to dilution to OD₆₀₀ of 0.1 in fresh MIN-S medium and cultured for further 5 h. Bacterial cells were harvested by centrifugation and the supernatant removed. Cell-free supernatant from each sample was concentrated using Centricon tubes (MWCO 10 KDa; Millipore, MA). Concentration of protein in all preparations was determined by the Biorad Dc protein quantification kit (Biorad, CA). Standardized protein concentrations (20 µg) were loaded onto 12.5% Tris polyacrylamide gels (Biorad, CA) and run under denaturing conditions. Polyacrylamide gels were transferred to PVDF membrane and immunoblotted with anti-PcrV, anti-ExoS or anti-ExoU as previously described (5,7).

Measurement of MMP-8, MMP-9, and TIMP-1

ELISA was performed to detect MMP-8, MMP-9, TIMP-1 in BAL samples and cell supernatants using Quantikine kits (R&D Systems, Minneapolis, MN, USA). The MMP-8 assay measures total MMP-8 (pro- and active MMP-8). The mean minimum detectable dose is 0.02 ng/ml. The MMP-9 assay recognizes also the pro- and active forms of MMP-9. The sensitivity of the assay is 0.156 ng/ml. Cross reactivity with MMP-9 with other MMPs is

<0.1%. The TIMP-1 assay recognizes complex and free TIMP-1. The cross reactivity with other MMPs or TIMPs is <0.1%. Sensitivity of the assay is <0.08 ng/ml.

Determination of α -2 macroglobulin

The levels of α 2-macroglobulin were measured using an ELISA kit (GenWay Biotech, San Diego, CA). The minimal detectable concentration is 7.8 ng/ml. The intraassay and interassay coefficients of variation were between $3.8 \pm 6.0\%$ and $3.1 \pm 7.2\%$, respectively.

Statistical analysis

The data were analyzed using non-parametric tests. The Mann-Whitney U test was used for unpaired samples, the Wilcoxon signed rank test was used for paired samples, and correlations were analyzed with Spearman's rank correlation. Cook's distance was used to assess for potential outliers. Analysis of variance for repeated measures was done for sequential measurements and *post hoc* tests were used for comparison of all pairs of columns. Parametric data are presented as mean (SD) and non-parametric data as median with 95% confidence interval or range. A p value of <0.05 was determined as significant. Calculations were carried out using statistical software NCSS 2000 (Salt Lake City, UT).

RESULTS

Twenty nine VAP patients with TTSS⁺*P. aeruginosa* and 12 VAP patients with TTSS⁻*P. aeruginosa* were studied. Of the 29 strains analyzed, 14 secreted ExoS⁺ PcrV, 13 secreted ExoU⁺ PcrV and two secreted PcrV only. There were no significant differences in age, gender, underlying comorbidities, CPIS scores, or severity of illness between the two groups (table 1). Only the duration of mechanical ventilation prior to VAP was significantly longer for those patients with *P. aeruginosa* TTSS⁺ than those with *P. aeruginosa* TTSS⁻ (p=0.01). The overall mortality was also higher for the TTSS⁺ phenotype (66%; 95% confidence interval [CI] 0.46–0.82) compared to the TTSS⁻ phenotype (33%; 95% CI 0.10–0.65) but the difference did not attain statistical significance (p=0.12).

Cellular composition of the BALF of patients with VAP is provided in table 2. The total cell number in BALF and the percentage of neutrophils were not significantly different between the two *P. aeruginosa* phenotypes at the onset of VAP. Following initiation of antibiotic therapy, there was a decline in the cellular composition of the BALF in both VAP groups although patients with *P. aeruginosa* TTSS⁺ had higher neutrophil composition in BAL at end of therapy than those with *P. aeruginosa* TTSS⁻ (p<0.001).

The concentrations of MMP-8 and MMP-9 levels in BALF were significantly increased in patients with VAP compared with controls at the onset of VAP (p<0.001 for both TTSS⁺ and TTSS⁻ phenotype) (figure 1). However, the initial levels of MMP-8 and MMP-9 were not significantly different between cases infected with TTSS⁺ and TTSS⁻ *Pseudomonas* strains (p=0.3 and 0.12, respectively). Similarly, the TIMP-1 levels were comparable between the control group and the VAP patients of either phenotype (p=0.14 for TTSS⁺ and p= 0.21 for TTSS⁻). Of interest, the highest MMP-9 levels were documented in patients with isolates that secreted ExoU (figure 2).

When analysis was performed in all patients with VAP, a significant positive correlation between MMP-8 and MMP-9 and the percentage of neutrophils was observed (r= 0.41, p=0.008 and r= 0.47, p=0.001; respectively). The correlation was more prominent in patients with ExoU secreting *Pseudomonas* (r=0.62 for MMP-9, p=0.02). Such association was not detected between the percentage of neutrophils and TIMP-1 levels.

Compared to VAP onset, levels of MMP-8 and MMP-9 showed a progressive decline on day 4 and day 8 following antibiotic administration ($p<0.001$ by ANOVA for ranks). However, the rate of decline varied according to the *P. aeruginosa* TTSS phenotype (figure 3). On day 4 of therapy, the levels of MMP-8 and MMP-9 in BALF of patients with TTSS⁺ dropped by 28% and 22% compared to 66% and 61% for patients with TTSS⁻ phenotype, respectively. At end of therapy, both MMP-8 and MMP-9 concentrations in those infected with TTSS⁻ phenotype were comparable to the control group levels ($p=0.15$ and $p=0.11$, respectively). In contrast, the levels of MMP-8 and MMP-9 in patients with TTSS⁺ phenotype remain significantly higher compared to controls ($p<0.001$ and $p<0.001$, respectively). Antibiotic therapy resulted also in significant reduction in the TIMP-1 levels during the course of therapy ($p=0.004$ for TTSS⁺ and $p=0.01$ for TTSS⁻ by ANOVA for ranks). Pairwise comparison showed that the decrease in TIMP-1 concentrations were significant only between the levels at VAP onset and at end of treatment.

To estimate the alveolocapillary leakage, α -2 macroglobulin was measured in BALF. The median level of α -2 macroglobulin at the onset of VAP was 237 ng/ml (range 56–584) for TTSS⁺ phenotype and 211 ng/ml (range 31–489) for TTSS⁻ phenotype ($p=0.3$). Although there was no correlation between the drop of MMPs and α -2 macroglobulin during the course of antibiotic administration, the ratio of MMP-9/TIMP-1 was significantly associated with α -2 macroglobulin at end of therapy ($r=0.4$, $p=0.02$) (figure 4). Moreover, the ratio of MMP-9/TIMP-1 was significantly higher for patients who died compared to those who survived ($p=0.003$) (figure 5).

DISCUSSION

In this study we have demonstrated that 1) levels of MMP-8 and MMP-9 are increased in patients with VAP compared to controls without pneumonia; 2) the metalloproteinases response to antimicrobial therapy varies according to the *P. aeruginosa* phenotype; and 3) the imbalance between MMP-9 and TIMP-1 may determine the intensity of alveolocapillary damage.

Previous studies have shown that the concentrations of MMP-8 and MMP-9 in mini-BALF of 30 patients with hospital acquired pneumonia were increased 10 fold whereas the levels of TIMP-1 did not concomitantly match the rise of metalloproteinases (14). Our findings extend these observations by providing a temporal evolution of the expression of MMP-8, MMP-9, and TIMP-1 in patients with VAP vis-à-vis the phenotypic characteristics of *P. aeruginosa* isolates. When compared to patients with *P. aeruginosa* TTSS⁻ phenotype, enzyme-linked immunosorbant assays demonstrated that MMP-8 and MMP-9 levels remained elevated in patients with *P. aeruginosa* TTSS⁺ phenotype despite adequate antimicrobial therapy. These observations suggest that proteases are overexpressed in *P. aeruginosa* TTSS⁺ phenotype, leading to free activity in the alveolar compartment. A number of studies have shown that local production of extracellular proteases plays a key role in immune lung disorders (20). The increased expression of extracellular proteases can affect the host's immune response against opportunistic infectious agents. In cystic fibrosis, for instance, opsonization of *Pseudomonas aeruginosa* is ineffective because neutrophil-derived elastase released in the extracellular space cleaves immunoglobulins and digests the C3b receptor on neutrophils, thereby limiting phagocytosis of pathogens (20). The same principle could explain our prior observations of persistent *P. aeruginosa* type III TTSS phenotype in the alveoli of patients with VAP after 7 days of adequate antibiotic therapy (7).

The current study confirmed prior findings linking the increased number of PMN in BALF to levels of MMP-8 and MMP-9. Betsuyaku and colleagues (21) found similar results in patients with emphysema as did Suga and coworkers (22) in idiopathic interstitial pneumonia. More

importantly, our results suggest that the association is even more pronounced in the presence of isolates with TTSS⁺ phenotype. *P. aeruginosa* strains producing ExoU are capable not only of destroying cellular monolayers during short infection periods (23) but also of inducing PMN cell death (24,25). The release of neutrophil elastase activates pro-MMP-9 to an active protease which contributes among others to the degradation of TIMP-1 (26). Hence, the excess MMP-8 and MMP-9 levels observed in patients with ExoU producing strains of *P. aeruginosa* might represent the cumulative effect of active release of proteases from newly recruited neutrophils and passive spill out of proteolytic enzymes from necrotic PMN into the alveolar space.

The exuberant metalloproteinase response in pneumonia might initiate a vicious cycle leading to a perpetuation of the inflammatory response in the lung. Indeed, high levels of MMP-9 in the lungs have been shown to promote the infiltration of inflammatory cells and to exacerbate the symptoms associated with bronchial asthma (27). The local production of matrix metalloproteinases can alter also the local tissue architecture directly by degrading the proteins of the extracellular matrix (28). Yet, by studying the response to antimicrobial therapy, the initial imbalance in MMP-8 and MMP-9 to TIMP-1 levels revealed that the perturbation in the extracellular proteases in patients with VAP *P. aeruginosa* might be a short lived event that is highly dependent on the phenotype of the invading pathogen. A normalization of the ratio MMP-9 to TIMP-1 could forecast a favorable prognosis while a persistently mismatched ratio as in the case in TTSS⁺ phenotype could entail alveolocapillary leakage with increased risk of acute lung injury progressing to acute respiratory distress syndrome. In such cases, inhibition of MMP activity may attenuate several crucial pathogenetic steps in the inflammatory cascade. For example, there is a converging body of evidence that synthetic MMP inhibitors that directly inactivate proteolytic activity may be able to tackle released enzyme, and hence contain neuronal damage in infectious meningitis effectively (29,30) or prevent acute lung injury from septic shock (31).

Alternatively, it is plausible that the increased activity of metalloproteinases in patients with *Pseudomonas* TTSS⁺ phenotype might be a marker of disease severity that is intended to benefit the host (32); for example, metalloproteinases promote the secretion of mucus, and assist in bacterial clearance and removal of microorganisms from the lung (33). Moreover, studies with mice lacking MMP-9 have shown that this enzyme is essential for an adequate inflammatory response and tissue repair, as these animals have an increased lung damage after the induction of peritonitis (34) and a poor outcome after an experimental brain hemorrhage (35). Similarly, lack of MMP-8 is on one side protective of acute liver injury (36) but can delay the resolution of inflammation in skin and lungs (37,38). These observations suggest that MMPs might have a dual effect with the ultimate outcome being a function of peak concentration, duration of exposure, site of release, and the characteristics of the offending pathogen.

The present study has the following limitations that have to be considered for the interpretation of the results. First, the sample size was relatively small and the bacteria selected originated from a single environmental ecosystem. Second, although we have included only patients who had received adequate therapy, we cannot dismiss the potential impact of different antibiotic classes on MMPs or TIMP-1 expression. Third, we do not have any clear explanation for the higher prevalence of TTSS⁺ strains in those with longer period of mechanical ventilation. Prior investigations (5,6) that have studied TTSS⁺ *P. aeruginosa* did not report on the duration of ventilation prior to VAP. Accordingly, it is unclear whether our observation represents a true difference (acquired by horizontal transmission or de novo mutation) or a coincidental finding (type II error). A surveillance study with daily culture would be required to answer this question. Fourth, we were unable to perform a serial lavage on the control group because 9 out of the 12 patients were extubated within 48 hours. At that point, we refrained, per IRB recommendation, from subsequent lavage as the risks of the procedure would outweigh the benefits. Finally, it is theoretically plausible that the increased levels of MMPs in those patients

with TTSS⁺ *Pseudomonas aeruginosa* strains might have been a byproduct of prolonged ventilation, however there is no evidence to our knowledge that lung injury is amplified or perpetuated while using lung-protective ventilation.

In conclusion, ventilator associated pneumonia linked to *P. aeruginosa* portrays a divergent antibiotic treatment response in regards to the concentrations of metalloproteinases in the alveolar space. The time evolution of MMP-8 and MMP-9 seems to be highly influenced by the type III cytotoxin secretion phenotype of *P. aeruginosa*. The imbalance between MMP-9 and TIMP-1 at end of therapy may determine the intensity of lung injury and ultimate outcome of VAP.

Acknowledgements

Grant support: Research for Health in Erie County [AES], American Lung Association [SVL], NIH AI075410 [SVL and JPWK], SCCOR HL 74005 [JPWK], HL69809 [JPWK]

REFERENCES

1. Rossolini GM, Mantengoli E. Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. Clin Microbiol Infect 2005;11:17–32. [PubMed: 15953020]
2. Rello J. Bench-to-bedside review: Therapeutic options and issues in the management of ventilator-associated bacterial pneumonia. Crit Care 2005;9:259–265. [PubMed: 15987380]
3. LaBaer J, Qiu Q, Anumanthan A, Mar W, Zuo D, Murthy TV, et al. The *Pseudomonas aeruginosa* PA01 Gene Collection. Genome Res 2004;14:2190–2200. [PubMed: 15489342]
4. Mota L, Sorg I, Cornelis G. Type III secretion: the bacteria-eukaryotic cell express. FEMS Microbiol Lett 2005;252:1–10. [PubMed: 16216444]
5. Roy-Burman A, Savel RH, Racine S, Swanson BL, Revadigar NS, Fujimoto J, et al. Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. J Infect Dis 2001;183:1767–1774. [PubMed: 11372029]
6. Hauser RA, Cobb E, Bodi M, Mariscal D, Vallés J, Engel JN, Rello J. Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. Crit Care Med 2002;30:521–528. [PubMed: 11990909]
7. El Solh AA, Akinnusi ME, Wiener-Kronish JP, Lynch SV, Pineda LA, Szarpa K. Persistent infection with *Pseudomonas aeruginosa* in ventilator associated pneumonia. Am J Respir Crit Care Med 2008;178:513–519. [PubMed: 18467510]
8. O'Connor CM, FitzGerald MX. Matrix metalloproteinases and lung disease. Thorax 1994;49:602–609. [PubMed: 8016800]
9. Corbel M, Lagente V, Boichot E. Pulmonary inflammation and tissue remodeling: role of metalloproteinases. Eur Respir Rev 2000;10:260–263.
10. Chubinskaya S, Huch K, Mikecz K, Cs-Szabo G, Hasty KA, Kuettner KE, et al. Chondrocyte matrix metalloproteinase-8. Upregulation of neutrophil collagenase by interleukin-1 beta in human cartilage from knee and ankle joints. Lab Invest 1996;74:232–240. [PubMed: 8569187]
11. Prikk K, Maisi P, Pirila E, Sepper R, Salo T, Wahlgren J, et al. In vivo collagenase-2 (MMP-8) expression by human bronchial epithelial cells and monocytes/macrophages in bronchiectasis. J Pathology 2001;194:232–238.
12. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. Eur J Cell Biol 1997;74:111–122. [PubMed: 9352216]
13. Bode W, Fernandez-Catalan C, Nagase H, Maskos K. Endoproteinase-protein inhibitor interactions. APMIS 1999;107:3–10. [PubMed: 10190274]
14. Hartog C, Wermelt J, Sommerfeld C, Eichler W, Dalhoff K, Braun J. Pulmonary matrix metalloproteinase excess in hospital-acquired pneumonia. Am J Respir Crit Care Med 2003;167:593–598. [PubMed: 12588713]

15. El Solh AA, Choi G, Schultz MJ, Pineda LA, Mankowski C. Clinical and hemostatic responses to treatment in ventilator-associated pneumonia: role of bacterial pathogens. *Crit Care Med* 2007;35:490–496. [PubMed: 17205031]
16. Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia: Official statement of ATS and IDSA. *Am J Respir Crit Care Med* 2005;171:388–416. [PubMed: 15699079]
17. Knaus WA, Draper EA, Wagner DP, Zimmerman J. APACHE II: A severity of disease classification system. *Crit Care Med* 1985;13:818–829. [PubMed: 3928249]
18. Pugin J, Auckenthaler R, Mili N, Janssens JP, Lew PD, Suter PM. Diagnosis of ventilator-associated pneumonia by bacteriologic analysis of bronchoscopic and non-bronchoscopic blind bronchoalveolar lavage fluid. *Am Rev Respir Dis* 1991;143:1121–1129. [PubMed: 2024824]
19. Nicas TI, Iglewski BH. Isolation and characterization of transposon-induced mutants of *Pseudomonas aeruginosa* deficient in production of exoenzyme S. *Infect Immun* 1984;45:470–474. [PubMed: 6086529]
20. Greenberger PA. Immunologic aspects of lung diseases and cystic fibrosis. *JAMA* 1997;278:1924–1930. [PubMed: 9396654]
21. Betsuyaku T, Nishimura M, Takeyabu K, Tanino M, Venge P, Xu S, et al. Neutrophil granule proteins in bronchoalveolar lavage fluid from subjects with subclinical emphysema. *Am J Respir Crit Care Med* 1999;159:1985–1991. [PubMed: 10351949]
22. Suga M, Iyonaga K, Okamoto T, Gushima Y, Miyakawa H, Akaike T, et al. Characteristic elevation of matrix metalloproteinase activity in idiopathic interstitial pneumonias. *Am J Respir Crit Care Med* 2000;162:1949–1956. [PubMed: 11069839]
23. Finck-Barbançon V, Goranson J, Zhu L, Sawa T, Wiener-Kronish JP, Fleiszig SM, et al. ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Mol Microbiol* 1997;25:547–557. [PubMed: 9302017]
24. Dacheux D, Attree I, Schneider C, Toussaint B. Cell Death of Human Polymorphonuclear neutrophils induced by a *Pseudomonas aeruginosa* cystic fibrosis isolate requires a functional Type III secretion system. *Infect Immun* 1999;67:6164–6167. [PubMed: 10531282]
25. Diaz MH, Shaver CM, King JD, Musunuri S, Kazzaz JA, Hauser AR. *Pseudomonas aeruginosa* induces localized immunosuppression during pneumonia. *Infect Immun* 2008;76:4414–4421. [PubMed: 18663007]
26. Gaggari A, Li Y, Weathington N, Winkler M, Kong M, Jackson P, et al. Matrix metalloproteinase-9 dysregulation in lower airway secretions of cystic fibrosis patients. *Am J Physiol Lung Cell Mol Physiol* 2007;293:L96–L104. [PubMed: 17384080]
27. Kumagai K, Ohno I, Okada S, Ohkawara Y, Suzuki K, Shinya T, et al. Inhibition of matrix metalloproteinases prevents allergen-induced airway inflammation in a murine model of asthma. *J Immunol* 1999;162:4212–4219. [PubMed: 10201949]
28. Shapiro SD. Elastolytic metalloproteinases produced by human mononuclear phagocytes. Potential roles in destructive lung disease. *Am J Respir Crit Care Med* 1994;150:S160–S164. [PubMed: 7952653]
29. Paul R, Lorenzl S, Koedel U, Sporer B, Vogel U, Frosch M, et al. Matrix metalloproteinases contribute to the blood-brain barrier disruption during bacterial meningitis. *Ann Neurol* 1998;44:592–600. [PubMed: 9778257]
30. Lindberg RLP, Sorsa T, Tervahartiala T, Hoffmann F, Mellanen L, Kappos L, et al. Gelatinase B [matrix metalloproteinase (MMP)-9] and collagenases (MMP-8/-13) are upregulated in cerebrospinal fluid during aseptic and bacterial meningitis in children. *Neuropathol Appl Neurobiol* 2006;32:304–317. [PubMed: 16640649]
31. Steinberg J, Halter J, Schiller H, Gatto L, Carney D, Lee HM, et al. Chemically modified tetracycline prevents the development of septic shock and acute respiratory distress syndrome in a clinically applicable porcine model. *Shock* 2005;24:348–356. [PubMed: 16205320]
32. Bottcher T, Spreer A, Azeih I, Gerber J. Matrix metalloproteinase-9 deficiency impairs host defense mechanisms against streptococcus pneumoniae in a mouse model of bacterial meningitis. *Neurosci Lett* 2003;338:201–204. [PubMed: 12581831]

33. Stockley RA. Neutrophils and protease/antiprotease imbalance. *Am J Respir Crit Care Med* 1999;160:S49–S52. [PubMed: 10556170]
34. Renckens R, Roelofs J, Florquin S, de Vos AF, Lijnen HR, van't Veer C, et al. Matrix metalloproteinase-9 deficiency impairs host defense against abdominal sepsis. *J Immunol* 2006;176:3735–3741. [PubMed: 16517742]
35. Tang J, Liu J, Zhou C, Alexander JS, Nanda A, Granger DN, et al. MMP-9 deficiency enhances collagenase-induced intracerebral hemorrhage and brain injury in mutant mice. *J Cereb Blood Flow Metab* 2004;24:1133–1145. [PubMed: 15529013]
36. Van Lint P, Wielockx B, Puimege L, Noël A, López-Otin C, Libert C. Resistance of collagenase-2 (matrix metalloproteinase-8)-deficient mice to TNF-induced lethal hepatitis. *J Immunol* 2005;175:7642–7649. [PubMed: 16301674]
37. Balbin M, Fueyo A, Tester AM, Pendás AM, Pitiot AS, Astudillo A, et al. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. *Nat Genet* 2003;35:252–257. [PubMed: 14517555]
38. Gutierrez-Fernandez A, Inada M, Balbin M, Fueyo A, Pitiot AS, Astudillo A, et al. Increased inflammation delays wound healing in mice deficient in collagenase-2 (MMP-8). *FASEB J* 2007;21:2580–2591. [PubMed: 17392479]

Figure 1.

Levels of MMP-8, MMP-9, and TIMP-1 in bronchoalveolar lavage fluids of two groups of patients with ventilator-associated pneumonia attributed to *Pseudomonas aeruginosa* with and without type III secretion system at the onset of pneumonia. A cohort group of patients ventilated for airway protection was included as controls. * indicates $p < 0.05$

Figure 2. Comparison of MMP-8, MMP-9, and TIMP-1 levels in bronchoalveolar lavage fluids of patients with ventilator-associated pneumonia attributed to ExoU and ExoS effector proteins of *Pseudomonas aeruginosa* with type III secretion system. * indicates $p < 0.05$

Figure 3.

Time course of metalloproteinase s MMP-8, MMP-9, and TIMP-1 levels in the bronchoalveolar lavage fluids of patients with *Pseudomonas aeruginosa* ventilator-associated pneumonia. * $p < 0.05$ for comparison between TTSS⁺ and TTSS⁻ phenotype.

Figure 4. Correlation between the ratio of MMP-9/TIMP and α -2 macroglobulin in the bronchoalveolar patients with *Pseudomonas aeruginosa* ventilator-associated pneumonia. Patients infected with type III secretion system are represented by black circles and those without are marked by white circles.

Figure 5. Outcome of *Pseudomonas aeruginosa* ventilator-associated pneumonia according to the ratio of MMP-9/TIMP-1. * indicates $p < 0.05$

Table 1

Characteristics of the study population

	TTSS ⁺ (n=29)	TTSS ⁻ (n=12)	Control (n=8)
Age, years	64.4±12.4	63.1±10.1	56±4.9
Gender (Male/Female)	21/8	7/5	3/5
Days of mechanical ventilation prior to VAP, days	15.9±5.1	11.9±2.9 [*]	0
Previous antibiotics, n (%)	17 (59)	7 (58)	0
APACHE II score	26.0±5.3	22.6±5.8	14.4±3.2 [†]
CPIS score	7.8±0.6	7.5±0.5	2.1±0.9
Comorbid diseases			
Coronary artery disease	11 (38)	3 (25)	0
Diabetes Mellitus	5 (17)	2 (29)	1 (13)
Renal disease	6 (21)	1 (8)	0
Miscellaneous	8 (28)	2 (29)	1 (13)

^{*} p<0.05 comparing TTSS⁺ and TTSS⁻

[†] p<0.05 comparing TTSS⁺ and TTSS⁻ to control

Table 2
Bronchoalveolar lavage cellularity in *P. aeruginosa* ventilator-associated pneumonia

	Day 1 TTSS ⁺	TTSS ⁻	Day 4 TTSS ⁺	TTSS ⁻	Day 8 TTSS ⁺	TTSS ⁻
BALF recovery (%)	76	68	66	71	73	67
Total BAL cell count ($\times 10^5$ /ml BAL)	36 $\pm$ 1.4	29 $\pm$ 1.1	25 $\pm$ 0.4	18 $\pm$ 1.1	17.2 $\pm$ 0.5	11 $\pm$ 0.8
Neutrophils (%)	87	83	67	28	34	16