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Original Research

CRITICAL CARE

Ventilator-Associated Pneumonia Is Characterized by Excessive Release of Neutrophil Proteases in the Lung

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Background: **Ventilator-associated pneumonia (VAP) is characterized by neutrophils infiltrating the alveolar space. VAP is associated with high mortality, and accurate diagnosis remains difficult. We hypothesized that proteolytic enzymes from neutrophils would be significantly increased and locally produced inhibitors of human neutrophil elastase (HNE) would be decreased in BAL fluid (BALF) from patients with confirmed VAP. We postulated that in suspected VAP, neutrophil proteases in BALF may help identify "true" VAP.**

Methods: **BAL was performed in 55 patients with suspected VAP and in 18 control subjects. Isolation of a pathogen(s) at $> 10^4$ colony-forming units/mL of BALF dichotomized patients into VAP (n = 12) and non-VAP (n = 43) groups. Matrix metalloproteinases (MMPs), HNE, inhibitors of HNE, and tissue inhibitors of matrix metalloproteinases (TIMPs) were quantified. Plasminogen activator (PA) activity was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and zymography.**

Results: **Neutrophil-derived proteases HNE, MMP-8, and MMP-9 were significantly increased in cell-free BALF from patients with VAP as compared with those without VAP (median values: HNE, 2,708 ng/mL vs 294 ng/mL, $P < .01$; MMP-8, 184 ng/mL vs 5 ng/mL, $P < .01$; MMP-9, 310 ng/mL vs 11 ng/mL, $P < .01$). HNE activity was also significantly increased in VAP (0.45 vs 0.01 arbitrary units; $P < .05$). In contrast, no significant differences were observed for protease inhibitors, TIMPs, or PAs. HNE in BALF, at a cutoff of 670 ng/mL, identified VAP with a sensitivity of 93% and specificity of 79%.**

Conclusions: **Neutrophil proteases are significantly elevated in the alveolar space in VAP and may contribute to pathogenesis. Neutrophil proteases appear to have potential in suspected VAP for distinguishing true cases from "non-VAP" cases.** ***CHEST 2012; 142(6):1425–1432***

Abbreviations: α_1-PI = α_1 protease inhibitor; BALF = BAL fluid; BSA = bovine serum albumin; cfu = colony-forming units; ELISA = enzyme-linked immunosorbent assay; HNE = human neutrophil elastase; MMP = matrix metalloproteinase; PA = plasminogen activator; PAI = plasminogen-activator inhibitor; PBS = phosphate-buffered saline; ROC = receiver operating characteristic; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; SLPI = secretory leukocyte protease inhibitor; TIMP = tissue inhibitor of metalloproteinases; t-PA = tissue-type plasminogen activator; u-PA = urinary-type plasminogen activator; VAP = ventilator-associated pneumonia

Ventilator-associated pneumonia (VAP) arises as a complication in 15% to 30% of patients who are intubated and mechanically ventilated in ICUs.[1-3] VAP is typically associated with mortality rates of 20%.[4,5]

Histologically, VAP is characterized by neutrophilic alveolitis. Recent evidence suggests VAP drives increased alveolar expression of the inflammatory cytokines IL-1β and IL-8.[6] However, the neutrophils recruited appear to be dysfunctional.[7]

Neutrophils contain proteases, including human neutrophil elastase (HNE), matrix metalloproteinase (MMP)-8 (neutrophil collagenase), and MMP-9

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(gelatinase B). These enzymes are normally contained in granules and are thought to contribute to bacterial killing.[8-10] If degranulation or necrosis occurs, these

proteases are ordinarily neutralized by a relative excess of antiproteases in pulmonary epithelial-lining fluid. HNE is inhibited by α_1 protease inhibitor (α_1-PI) and by locally secreted "antimicrobial antiproteases," such as secretory leukocyte protease inhibitor (SLPI) and elafin.[11] MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs).[12] Despite this, several inflammatory lung conditions are characterized by an excess of neutrophil proteases.[13-20]

In addition to neutrophil-associated proteases, fibrinolytic enzymes may be active in inflammatory lung disease. Alveolar fibrin deposition is a characteristic of several inflammatory lung conditions, and associated fibrin degradation suggests activity of the fibrinolytic enzymes tissue-type plasminogen activator (t-PA) and/or urinary-type plasminogen activator (u-PA).[21-23] t-PA is produced by endothelial cells, although small amounts of u-PA may be found in neutrophil granules.[8,24] In general, however, conditions such as acute lung injury favor continued fibrin deposition.[21,23,25,26]

We hypothesized that VAP would be specifically associated with increased activity of neutrophil-associated proteases in the alveolar space and with reduced expression of SLPI and elafin. We further postulated that fibrinolytic activity would be undetectable in the alveoli.

A final aim of this study was to determine whether alveolar proteases or antiproteases might potentially allow patients with suspected VAP to be reliably separated into those with and without VAP. Definitive diagnosis of VAP generally requires microbiologic confirmation using good quality samples from the lower respiratory tract. Standard microbiologic cultures and sensitivities typically take 48-72 h to return. As a consequence, there is a tendency to prescribe empirical antibiotics and modify these as appropriate when microbiology results return. A problem with this approach is that the majority of patients with clinically suspected VAP do not have confirmed infection,[1,4,27,28] and a significant proportion of patients, therefore, receive unnecessary antibiotics. Emerging evidence suggests that empirical use of antibiotics in this setting does not reduce (and may potentially increase) mortality and is associated with the emergence of antibiotic-resistant pathogens.[29,30] A more rapid means of reliably confirming or excluding VAP would be of considerable practical benefit.

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Materials and Methods

Patients and Control Subjects

Patients and control subjects described here have been studied previously to address distinct, separate questions.[6] The current study involved a cohort of 55 patients from the original study, which contained 73 patients. Briefly, patients from ICUs of two, general, teaching hospitals were included if they fulfilled criteria for suspected VAP, that is, intubation and mechanical ventilation for >48 hours, new infiltrates on chest radiograph, and at least two of the following: temperature $>38°C$, WBC count $>11 \times 10^9$/L of peripheral blood, or purulent tracheal secretions. Patients with suspected VAP who also fulfilled predefined criteria for ARDS were eligible.[31]

Patients underwent bronchoscopy and BAL according to a standardized protocol.[6] Quantitative culture was performed on BAL fluid (BALF) and growth of a pathogen(s) at $>10^4$ colony-forming units (cfu)/mL BALF was taken to confirm a diagnosis of VAP.[32] Patients with no growth or with growth at $<10^4$ cfu/mL were considered to belong to a "non-VAP" group. Remaining BALF was centrifuged at 700 *g* for 10 min at room temperature. Proteases and antiproteases were measured in cell-free BALF supernatant.

Ambulant, nonhospitalized control subjects within an age range typical for patients with suspected VAP were also included.[6] The protocol described here was followed with the exception that control subjects were not intubated or mechanically ventilated, and bronchoscopy was performed through an oral approach with topical 2% lidocaine and optional sedation.

The study was approved by Lothian Research Ethics Committee (LREC/2002/8/19 and 06/S1101/50). Informed consent was provided by the next of kin or guardian of all patients, in accordance with the Adults with Incapacity (Scotland) Act 2000, and ethics committee approval. Informed written consent was obtained from all control subjects.

Human Neutrophil Elastase

HNE concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (Hycult Biotechnology b.v.) recognizing HNE in free form and in complex with native inhibitors. Activity was estimated by incubating BALF with the fluorogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (Sigma-Aldrich Co), excited at 360 nm and measuring emission at 480 nm. Undiluted BALF was incubated with the substrate at 37°C for 12 hours, with the assay calibrated using activity from known concentrations (0-2 ng/mL) of purified HNE (Sigma-Aldrich Co LLC).

Inhibitors of HNE

α_1-PI in BALF was quantified using an in-house ELISA relative to a commercially available standard (The Binding Site Group Ltd). Rabbit antihuman α_1-PI (100 μL) (The Binding Site Group Ltd) in 0.05 M sodium carbonate/bicarbonate pH 9.6 was added to a MaxiSorp (Thermo Fisher Scientific Inc) microtiter plate and incubated overnight (4°C). The plate was washed with phosphate-buffered saline (PBS) containing 0.05% (volume/volume) Tween 20. Unoccupied sites were blocked with 5% bovine serum albumin (BSA) and incubated for 60 min at 21°C.

A standard curve was made using doubling dilutions of α_1-PI, using 1% (weight/volume) BSA in PBS as diluent. Samples were diluted using the same diluent. Standard/sample (100 μL) was added to the plate for a 2-hour incubation at room temperature. The plate was washed with PBS containing 1% (volume/volume) Tween 20. Antihuman α_1-PI-peroxidase conjugate (100 μL) (The Binding Site Group Ltd) in PBS containing 1% (weight/volume) BSA was added for 2 hours at room temperature. After further washing, 100 μL of tetramethylbenzidine substrate solution (Sigma-Aldrich Co) was added for 10 min at 25°C. The reaction was stopped with 100 μL of 1M sulfuric acid. The plate was read at 450 nm and α_1-PI concentrations read from the standard curve. SLPI and elafin were quantified using commercially available ELISAs (R&D Systems, Inc and Hycult Biotechnology b. v., respectively) per manufacturers' instructions.

Matrix Metalloproteinases

BALF was diluted in Calibrator Diluent (RD5-37; R&D Systems, Inc) and MMPs measured using a multi-analyte array (R&D Systems, Inc) according to the manufacturer's instructions.[20] Dilution factors were based on our previous data for BAL samples from intubated patients with VAP or ARDS. Samples beyond the limits of the standard curve were arbitrarily assigned the value of the upper or lower standard. The array measures pro-, active, and TIMP-complexed MMPs.

MMP-9 Activity

BALF was diluted and estimated using the active MMP-9 fluorokine assay (R&D Systems, Inc). Analysis was performed according to the manufacturer's instructions.[20]

TIMP-1 and TIMP-2 ELISA

TIMP-1 and TIMP-2 were quantified using DuoSet ELISA kits (R&D Systems, Inc) according to the manufacturer's instructions.[20] When a sample fell beyond the range of the standard curve, it was assigned the value of the upper or lowest standard.

Proteases and antiproteases in BALF were quantified as above. In addition, the concentration of urea was measured in serum and BALF. Urea equilibrates freely across the alveolar-capillary membrane and can be used to correct for the "diluting" effects that BAL has on epithelial-lining fluid.[33] Urea-corrected values were therefore derived for all antigenic proteases and antiproteases in BALF.

Fibrinolytic Activity

Identification of plasminogen activators (PAs) in free or complexed form was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with zymography.[34] BALF (20 μL) was loaded onto SDS-PAGE gels, then agarose "detector" gels containing fibrin and plasminogen were applied to the SDS-PAGE gels. The presence of PA was indicated by a clear region of lysis in the opaque detector gel.

Statistics

Comparisons between groups were made using the Kruskal-Wallis test with Dunn post hoc analysis. Categorical data were analyzed by χ^2 test. Statistical significance was set at the level $P = .05$. Diagnostic utility was assessed by construction of receiver operating characteristic (ROC) curves, using the Youden index[35] to derive optimal cutoff values. All analyses were performed using Prism version 4.0 (GraphPad Software, Inc).

Results

Fifty-five patients were studied. Twelve (22%) had confirmed VAP, the other 43 comprised a non-VAP group. Eighteen control subjects were included to place patients' results in the context of normal values. Clinical and demographic features are described in Table 1, and pathogens isolated from BAL fluid in Table 2. Although there was a trend toward a higher proportion of women in the non-VAP group, this did not reach statistical significance. Within the VAP group, two patients (17%) also fulfilled diagnostic criteria for ARDS, while the corresponding number was 18 (42%) patients in the non-VAP group (not statistically significant).

HNE was significantly higher in BALF from the VAP group as compared with the non-VAP group (Fig 1A). The differences remained significant when the non-VAP group was subdivided into ARDS and non-ARDS groups (ie, HNE levels remained higher in the VAP group than in either the ARDS

Table 1—*Clinical and Demographic Features of Patients and Control Subjects*[a]

Characteristics	VAP (n = 12)	Non-VAP (n = 43)	Control Subjects (n = 18)
Age, mean (range), y	59 (32-78)	58 (26-83)	58 (24-78)
Male patients	73	42	72
APACHE II score, mean (95% CI)	23 (18-27)	21 (16-26)	NA
Ventilation prior to study enrollment, median (IQR), d	8 (6-14)	7 (5-10)	NA
ICU mortality	36	33	NA
Patients with surgical diagnosis on admission	73	58	NA
Patients receiving immunosuppressant drugs (including corticosteroids)	9	12	NA
Patients with acute lung injury/ARDS	17	42	NA
Patients with ≥ 1 comorbidity	64	51	NA

Data are shown as % unless otherwise indicated. APACHE = Acute Physiology and Chronic Health Evaluation; IQR = interquartile range; NA = not applicable.

[a]No significant differences were detected for any of the comparisons made.

Table 2—*Organisms Isolated From BAL Fluid in Patients With and Without VAP*

Organism	VAP, No.	non-VAP, No.
Staphylococcus aureus	2	2
Methicillin-resistant *S aureus*	2	4
Coagulase-negative staphylococci	1	1
Escherichia coli	3	1
Citrobacter koseri	0	1
Enterobacter cloacae	1	1
Acinetobacter baumannii	0	1
Pseudomonas aeruginosa	0	1
Serratia marcescens	0	1
Klebsiella pneumoniae	0	3
Candida albicans	1	2
Aspergillus fumigatus	1	1
Anaerobes	1	0

By definition, organisms were isolated at $>10^4$ cfu/mL in the VAP group and at $<10^4$ cfu/mL in the non-VAP group. cfu = colony-forming units; VAP = ventilator-associated pneumonia.

or non-ARDS groups; data not shown). Proteolytic activity attributable to HNE was detectable, and significantly higher, in BALF from patients with VAP as compared with patients in the non-VAP group (Fig 1B). Again, the difference was maintained when the non-VAP group was divided into patients with ARDS and those without ARDS (data not shown). In contrast, no significant differences were detected when comparing any inhibitors of HNE in the VAP and non-VAP groups (Figs 1C-E).

Concentrations of MMP-8 and MMP-9 were significantly higher in the VAP group than in the non-VAP group (Fig 2). These differences persisted when the non-VAP group was divided into ARDS and non-ARDS subgroups (data not shown). However, MMP-9 activity was not significantly higher in patients from the VAP group as compared with the non-VAP group (Fig 2C). No significant differences were observed for concentrations of MMP-1, MMP-2, MMP-3, MMP-7, TIMP-1, or TIMP-2 when comparing the VAP and non-VAP groups (Table 3).

No fibrinolytic species were detected in BALF from control subjects. Among the patients with VAP, three had detectable free t-PA, none had free u-PA, and one had evidence of a PA/plasminogen-activator inhibitor (PAI) complex. In contrast, the non-VAP group contained six patients with free u-PA, two with free t-PA, and 13 with a PA/PAI complex (four of whom also had free u-PA).

We also sought to determine the potential for the markers described above to distinguish VAP from among the population of patients with suspected VAP. The results of the analysis by area under ROC curve are shown in Table 4, with all three curves demonstrating diagnostic potential.

Discussion

This study suggests a consistent trend for an extracellular excess of the neutrophil-derived proteases HNE, MMP-8, and MMP-9 in the alveolar space of carefully characterized patients with microbiologically

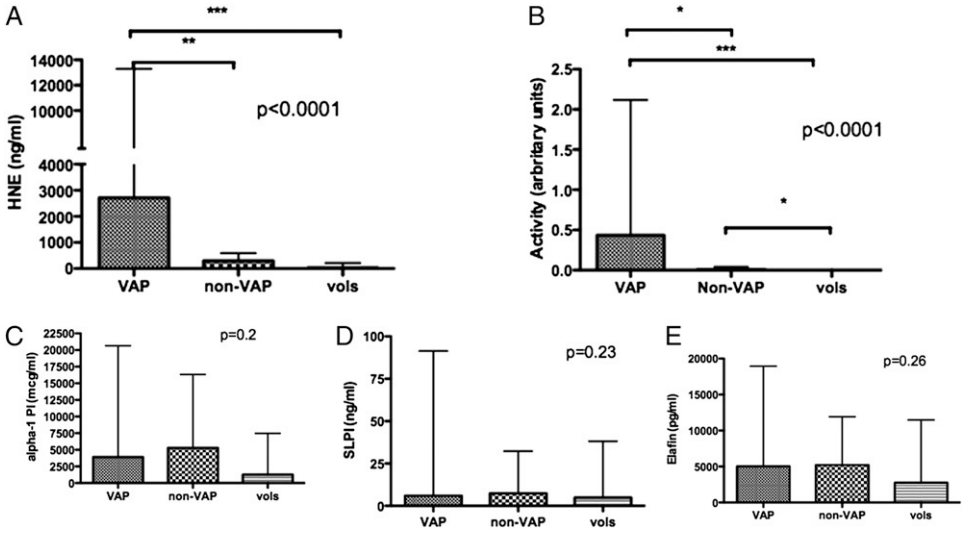

Figure 1. HNE and its natural inhibitors in BALF. A, HNE was measured by ELISA. B, HNE activity was measured by fluorogenic activity assay. C-E, Concentrations of alpha-1 PI, SLPI, and elafin were determined by ELISA. All values shown are corrected for dilution as described in the "Materials and Methods" section. Data are presented as median and interquartile range. Overall *P* value (by Kruskal-Wallis test) is shown in the panel. $*P<.05$, $**P<.01$, $***P<.001$ by Dunn post hoc test. alpha-1 PI = α_1 protease inhibitor; BALF = BAL fluid; ELISA = enzyme-linked immunosorbent assay; HNE = human neutrophil elastase; SLPI = secretory leukocyte protease inhibitor; VAP = ventilator-assisted pneumonia; vols = volunteer control subjects.

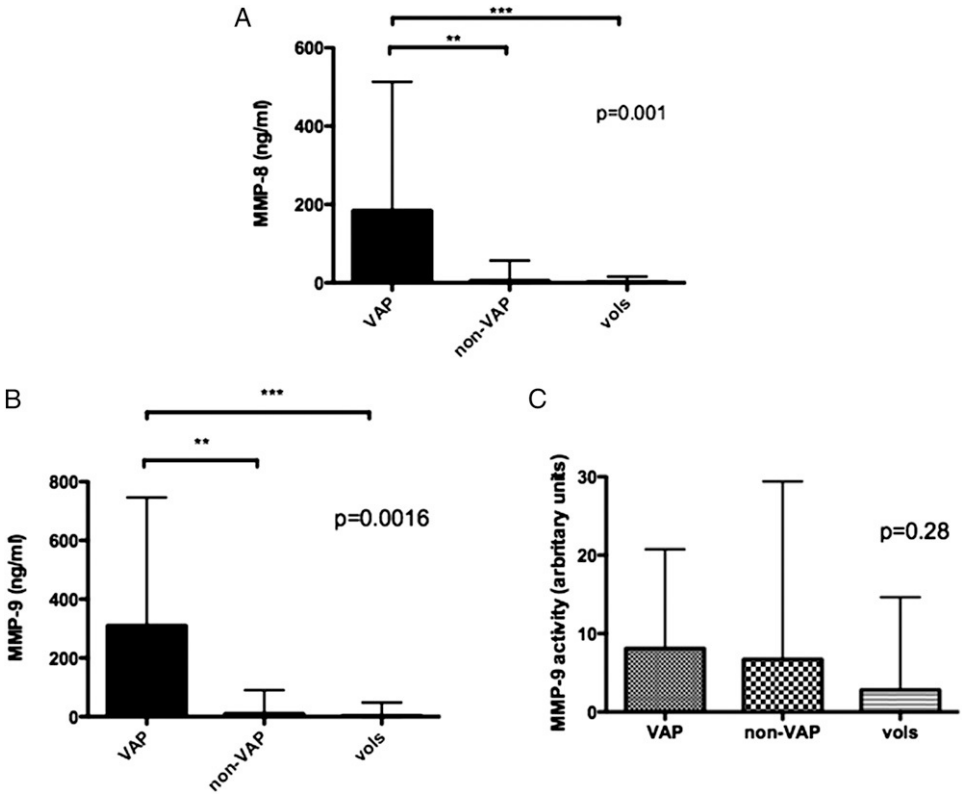

FIGURE 2. MMP-8 and MMP-9 in BALF. A, MMP-8 and B, MMP-9 antigen levels were measured by ELISA. C, MMP-9 activity was measured using a fluorogenic assay.[20] Data are presented as median and interquartile range. Overall *P* value (by Kruskal-Wallis test) is shown in the panel. $^{**}P < .01$, $^{***}P < .001$ by Dunn post hoc test. MMP = matrix metalloproteinase. See Figure 1 legend for expansion of other abbreviations.

confirmed VAP. However, we did not observe the hypothesized reduction in expression of the antimicrobial antiproteases elafin and SLPI in VAP. The implication is that acquisition of pneumonia in patients who are mechanically ventilated (who, by definition, are already ill) leads to an uncompensated release of proteolytic enzymes from neutrophils in the lung. In parallel with strikingly elevated concentrations of MMP-8, MMP-9, and HNE in BALF, we found corresponding evidence of proteolytic activity for HNE in VAP. This is particularly notable given the clear evidence for an apparently large excess of α_1-PI in all groups (Fig 1). This observation of excessive HNE activity is consistent with the description of elastin degradation products in VAP.[36]

To our knowledge, this study is the first to analyze neutrophil proteases in "unselected" VAP. Elevated BALF HNE has been described in patients with suspected VAP/ARDS (ie, those without microbiologic confirmation),[37] and increased MMP-8 and MMP-9 concentrations were found in patients with suspected hospital-acquired pneumonia (some of

Table 3—*Concentrations of MMPs and TIMPs in BAL Fluid*

Molecule	VAP	non-VAP	Control Subjects	*P* value for Kruskal-Wallis ANOVA
MMP-1, pg/mL	142 (47-310)[a]	116 (36-256)[a]	U/D[b]	.0001
MMP-2, pg/mL	3,908 (3,276-8,566)[a]	4,707 (1,400-14,652)[c]	885 (432-1,917)	.0021
MMP-3, pg/mL	408 (87-866)[c]	115 (35-403)	77 (51-80)	.048
MMP-7, pg/mL	3,350 (832-14,390)	1,065 (413-6,742)	3,305 (1,055-8,998)	.078
TIMP-1, ng/mL	58 (21-160)[c]	30 (13-98)[c]	4 (2-41)	.01
TIMP-2, ng/mL	6 (2-10)	3 (1-6)	1 (1-5)	.06

Data are expressed as median (IQR). ANOVA = analysis of variance; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinases; U/D = undetectable. See Table 1 legend for expansion of other abbreviations.

[a] $P < .001$ by Dunn post hoc test for difference between patient group and control subjects.

[b] Undetectable in all 18 control subjects.

[c] $P < .05$.

Table 4—*Diagnostic Potential of BALF Levels of HNE, MMP-8, and MMP-9*

Characteristic	HNE,[a] 670 ng/mL	MMP-8,[a] 13 ng/mL	MMP-9,[a] 22 ng/mL
Area under ROC curve (95% CI)	0.87 (0.78-0.96)	0.81 (0.69-0.93)	0.79 (0.66-0.92)
Sensitivity, %	93	91	82
Specificity, %	79	63	63

BALF = BAL fluid; HNE = human neutrophil elastase; ROC = receiver operating characteristic. See Table 2 legend for expansion of other abbreviations.

[a]The cutoff value was derived by Youden index[35] applied to ROC curves.

whom were not mechanically ventilated).[38] MMP-9 activity was significantly higher in patients with hospital-acquired pneumonia that was microbiologically confirmed using a mini-BAL than in control subjects.[39] However, only 41% of patients in that study were mechanically ventilated. In patients with well-characterized VAP attributable to *Pseudomonas aeruginosa*, MMP-8 and MMP-9 were significantly elevated in BALF,[40] and HNE levels correlated significantly with apoptotic neutrophils in BALF.[41]

The pattern described here adds to evidence for neutrophil dysfunction in VAP. Our previous data suggest that neutrophils from patients with suspected VAP are less capable of phagocytosing microbial particles or killing bacteria and generate lower levels of superoxide on stimulation, but more readily damage pulmonary epithelial membranes.[7] When pneumonia develops in critically ill patients, an excess of dysfunctional neutrophils is recruited to the lung, and the present data suggest that these liberate active proteolytic enzymes into the alveolar space.

We found no clear evidence of impaired fibrinolysis in VAP as compared with the non-VAP group. The only conclusion we can draw is that u-PA was detectable by zymography in, at the most, one patient with VAP, but between 6 and 15 patients in the non-VAP group. Taken together, our results suggest a trend toward impaired fibrinolysis (and/or a potential reduction in u-PA generation) in the alveolar space in VAP, but further work is required. Elevated BALF PAI-1 and impaired fibrinolytic activity have been described previously in well-characterized VAP in adults,[42-44] and in VAP diagnosed clinically in the pediatric ICU.[45]

One important issue to consider when studying VAP is the potential for confounding effects of ARDS, which itself has been associated with elevated HNE, MMP-8, and MMP-9 in BALF.[16,19,20,46] Clearly, the small numbers of patients with ARDS in this study limit any firm conclusions. However, if anything, the proportion of patients simultaneously fulfilling criteria for ARDS was higher in our non-VAP group. Furthermore, a subtly different pattern has been described in ARDS, with significantly elevated levels of antiproteases such as SLPI, PAI-1, and TIMP-1 in BALF,[19,20,23,25,47,48] a phenomenon not observed here.

In addition to providing insights into pathogenesis, our findings may have some diagnostic potential. Our previous findings suggested that low levels of IL-1β in BALF exclude VAP, with promising levels of accuracy.[6] The present study suggests that HNE, MMP-8, and MMP-9 also have some discriminatory potential in this setting. In particular, HNE gave equivalent sensitivity, higher specificity, and higher area under the ROC curve compared with IL-1β.[6] MMP-8 had broadly similar sensitivity, specificity, and area under the ROC curve compared with IL-1β. The assays used to quantify HNE, MMP-8, and MMP-9 are potentially available within 4 to 6 hours.

While these findings are encouraging, caution is required in their interpretation, and the results require validation in a larger cohort of patients across multiple centers (eg, our study contains a relatively high proportion of postoperative patients). One limitation is that we performed a post hoc analysis on a small cohort of 55 patients from an original study including 73 patients.[6] The smaller numbers reflect an absence of remaining BALF in 18 patients, raising the possibility that we selected out a group of patients in whom BAL yielded good returns. However, we believe our cohort to be representative, as the proportion of patients with proven VAP was almost identical to that in our original study (22% vs 23%), and patient demographics were also extremely similar.

In summary, microbiologically confirmed VAP was associated with extracellular neutrophil-derived proteases in the alveoli. The biologic and diagnostic relevance deserves closer attention in future, larger studies.

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Dr Wilkinson: contributed to study design, data collection and analysis, and manuscript preparation.
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