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The biological role of pleural fluid PAI-1 and sonographic septations in pleural infection: analysis of a prospectively collected clinical outcome study

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Contributor statement

EOB, NIK and NMR conceived and designed the study. JPC and EOB curated the PILOT database. EOB, NIK and YZ performed the laboratory processing and analyses. EOB and NMR analysed the data. MH, RA, RMM, AS, DNA, VG, RB and DM contributed clinical data. EOB wrote the first draft of the manuscript. TD provided materials. RFM, TD and AMC provided expert knowledge. All authors reviewed and approved the final manuscript. EOB, NIK and NMR verified the underlying data and jointly act as guarantors.

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Abstract

Rationale

Sonographic septations are assumed to be important clinical predictors of outcome in pleural infection but the evidence for this is sparse. The inflammatory and fibrinolysis-associated intrapleural pathway(s) leading to septation formation have not been studied in a large cohort of pleural fluid (PF) samples with confirmed pleural infection, matched with ultrasound and clinical outcome data.

Objectives

To assess the presence and severity of septations against baseline PF Plasminogen-Activator Inhibitor-1 (PAI-1) and other inflammatory and fibrinolysis-associated proteins as well as to correlate these with clinically important outcomes.

Methods

We analysed 214 pleural fluid samples from the PILOT study, a prospective observational pleural infection study, for inflammatory and fibrinolysis-associated proteins using the Luminex platform. Multivariate regression analyses were utilised to assess association of pleural biological markers with septation presence and severity (on ultrasound), and clinical outcomes.

Results

PF PAI-1 level independently predicted presence of septations ($p=<0.001$) and was the only protein associated with septation severity ($p=0.003$). PF PAI-1 levels predicted longer length of hospital stay ($p=0.048$) and increased 12 month mortality ($p=0.003$), whereas septations did not predict clinical outcomes.

Conclusions

In a large and well characterised cohort, this is the first study to associate pleural biological parameters with a validated sonographic septation outcome in pleural infection, and demonstrates pleural biology predicts clinically important outcomes. While PF PAI-1 plays an integral role in driving septation formation, it is associated with longer length of stay and worse mortality at 12 months, which septations themselves are not. These novel findings now require prospective validation.

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Introduction

Fibrin is not present in the normal pleural space, yet disordered fibrin turnover and aberrant extravascular fibrin deposition are key components of pleural injury (1). Pleural injury is characterised by fibrin accumulation and a marked suppression of fibrinolysis resulting in the formation of fibrinous strands known as pleural septations, or loculations when they form closed networks that sequester inflammatory fluid and impair pleural drainage. Plasminogen-derived plasmin is the main mediator of fibrinolysis, however despite the presence of endogenous plasminogen in the injured pleural space, plasminogen activity (and thus fibrinolysis) is inhibited by significantly elevated levels of plasminogen activator inhibitor 1 (PAI-1) (2).

In pleural infection, significant variation has been observed in levels of endogenous pleural fluid (PF) PAI-1 in samples from participants recruited to the MIST-2 trial (3). However, the degree of septation and loculation in these patients was not known as MIST-2 took place prior to the widespread use of bedside thoracic ultrasound (3, 4).

There is a paucity of evidence directly linking the presence of sonographic septations to clinically important outcomes. It has been suggested that the sonographic presence of pleural septation at diagnosis may be a prognostic indicator based on small retrospective studies (5, 6) yet clinicians frequently use the presence of septations to alter treatment (specifically, larger chest tube insertion and/or upfront surgery or intrapleural therapy early in treatment). If septations and loculations are truly important predictors of clinical outcome, personalised therapy based on evaluation of the components of the fibrinolytic system in pleural fluids at baseline could be of clinical value, and PF PAI-1 or other established proteins in the inflammatory and fibrinolysis pathways are thus potentially important candidate biomarkers. Moreover, sonographic septations, which are easily detectable given the now commonplace use of thoracic ultrasound, may be an accurate radiological surrogate.

The aim of this study was to explore the inflammatory and fibrinolysis-associated intrapleural pathway(s) leading to formation of septations in the infected pleural space by measuring a number of proteins from real life human samples, with key roles in the development and progression of pleural infection. Combined with

matched ultrasound septation data and known clinical outcomes, the aim was to test the following hypotheses:

1. Septation formation is dependent on endogenous PF PAI-1 levels at baseline
2. PF PAI-1 is superior to conventional serum/pleural fluid biomarkers of pleural infection in predicting development of septations
3. PAI-1 and sonographic septation presence / severity is associated with clinically important outcomes

Methods

The recently published Pleural Infection Longitudinal Outcome Study (PILOT) was an international multicentre prospective observational cohort study which enrolled adult patients with pleural infection (n=546; 29 sites). Participants were managed according to published guidelines (adapted for usual local practice). Details of the study inclusion and exclusion criteria are outlined in the PILOT manuscript (7). Baseline pleural fluid samples were collected from all patients who met the inclusion criteria from participating sites; Perth (processed locally) and select sites in the UK only to allow prompt receipt and processing by the central trial site (Oxford Respiratory Trials Unit, University of Oxford) and stored as per a trial specific procedure (TSP) (see supplement). Thus 243 samples were available for analysis, and baseline and clinical outcome data was available for all these patients. The PILOT study demonstrated that a clinical baseline score (RAPID) accurately predicted clinical outcome at 12 months.

Pleural fluid analysis

Protein measurement assays were performed using a commercially available Luminex bead-based multianalyte profiling kit (Luminex® High Performance Assays, R&D Systems). Luminex assays were chosen over ELISA for the protein measurements due to increased precision, time efficiency and cost-effectiveness (8). Absolute expression of total antigenic PAI-1, chemokine (C-C motif) ligand 2/monocyte chemoattractant protein-1/ (CCL2/MCP-1), urokinase type plasminogen activator (uPA), D-dimer, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) were measured in the pleural fluid samples. These were selected based on existing knowledge of the procoagulant and inflammatory pathways involved in pleural

infection. A spectrophotometer (NanoDrop™, ThermoFisher Scientific, UK) was used to measure the total protein expression in each sample. PF concentrations were normalised relative to total protein expression in pleural fluid. Only samples with a complete protein profile were used in the biomarker analysis.

Clinical outcome data

The primary endpoint of the PILOT study was all-cause mortality at 3 months with a data completion rate (DCR) of 542/546 (99.3%). Secondary endpoints included all-cause mortality at 12 months (DCR 542/546; 99.3%), length of hospital stay (LOS), and need for surgical drainage over 12 months (DCR 546/546; 100%). Use of combination intrapleural fibrinolytic and enzyme therapy (IET) was not a specific outcome of the PILOT study but as recorded on case report forms (CRFs), it was included in the analysis of this study (DCR 546/546; 100%).

Ultrasound septation score

An objective thoracic ultrasound septation score reflecting the sonographic extent of pleural fluid septation has been developed and validated, as described previously (9). This score categorises heterogeneously septated pleural effusions based on the maximum number of septations per image field of view into one of the following groups: non-septated; mildly septated (<2 septations per field); moderately septated (2-4 septations per field); or severely septated (>4 per field).

As part of the PILOT protocol, participants underwent ultrasound assessment prior to pleural intervention by a respiratory (or other) physician holding Royal College of Radiology Thoracic Ultrasound level 1(10) competency equivalent or above. Study CRFs documented septation score, with visual scales of ultrasound images included to guide the ultrasound operator in grading (Figure S1).

Baseline ultrasound data on the presence or absence of septations were available in 462/546 (84.6%) participants, with septation severity data in 434/462 (93.9%). The clinical population was divided and compared according to the septation status thus defined.

Funding and ethical approval

The analysis of the PILOT samples was funded by Oxford NIHR Biomedical Research Centre and included in the PILOT ethics approval (Oxford B Research Ethics Committee Ref:13/SC/0204).

Statistical analysis

Patient data are reported as the median/interquartile range (+/- range) for continuous variables. Chi squared statistics were used to compare differences in proportions between groups. Correlation between pleural fluid protein measurements, conventional biomarkers, septation presence and severity was assessed using Spearman's rank correlation coefficient (CC) with $p < 0.05$ used to define statistical significance. For outcome assessments, protein measurements and other biologically plausible conventional pleural fluid and serum biomarkers were analysed as independent variables in multiple (univariate) regressions with septation status as the dependent variable. Those with statistical significance $p < 0.2$ were exported into a stepwise multivariate regression model. The accuracy of statistically significant predictors of septation status was assessed using area under the curve (AUC) statistics with 95% confidence intervals (95% CI). When analysing the six biomarkers against each other, the p-value cut-off for statistical significance was $0.05/6 = 0.0083$

For clinical outcomes, Chi squared statistics were used to determine the proportions of requirement for IET and surgery, 12-month readmission rates and mortality (3 and 12 months) between the different septation groups. LOS was analysed using Mann Whitney U and Kaplan Meier. Linear regression was used to assess whether statistically significant biomarkers of septation status could predict clinical outcomes. A Cox regression for survival analysis was conducted between septation and biomarker groups, as categorical variables. Statistical analysis was performed using SPSS 27.0 (IBM).

Results

Baseline demographics

Baseline demographic and infection characteristics were similar between septated and non septated groups (Table 1). Specifically, there were no differences in baseline RAPID category (11) and chest tube size initially used. There was a higher proportion of macroscopically purulent fluid in the non-septated group (43.6% vs 21.7%; χ^2 1df 10.66; p=0.001) and a higher incidence of baseline tachycardia (HR >100bpm) in the septated group (36% vs 24%, χ^2 1df 4.57; p= 0.03). Assessment of the association between baseline variables and septation severity demonstrated no significant correlations (table S1).

Incidence of septations

The incidence of sonographic septation at diagnosis overall was 368/462 (79.7%). Detailed breakdown of septation severity, based on recorded septation score, is presented in Table S2.

Correlations between serum / PF biomarkers and fibrinolysis-associated proteins

Complete PF protein profile data was available in 214/243 (88%) samples. There were no statistically significant associations between any PF fibrinolysis-associated proteins and RAPID score or number of comorbidities. PF uPA was correlated with all 3 commonly used PF indicators of cell death/activity; PF pH (CC -0.29; p<0.001), PF glucose (CC -0.45; p<0.001), and cell turnover; PF LDH (CC 0.39; p<0.001). PF IFN-γ had a weak but statistically significant correlation with PF glucose (CC -0.27; p=0.044). PF PAI-1 had no correlation with PF pH, LDH or glucose.

With regards to conventional serum indicators of infection [serum C-reactive protein (CRP), peripheral blood white cell count (WCC) and platelets], modest correlations were seen between PF PAI-1 and CRP (CC 0.22; p=0.007), and PF CCL2/MCP-1 and WCC (CC -0.26; p<0.001).

Septations and PF fibrinolysis-associated proteins

Paired complete protein profile and ultrasound data were available for 166 patients. Due to the smaller size of this analysis cohort compared to the overall PILOT

population, baseline demographics were compared with the entire PILOT population, to ensure this was a representative cohort. The analysis and overall groups were well matched, with the only statistically significant difference being an increased proportion of community acquired infections in the analysis groups (92.5% vs 86.9%, χ^2 1df 4.89; p=0.03) (Table S3).

Of all assessed parameters, PF PAI-1 was the only protein to show a statistically significant correlation with septation status and severity (Table S4). Median values of PF proteins were compared by degree of septations (none, mild, moderate, heavy) (Table 2). Overall, concentrations of PF PAI-1 were significantly higher compared to other proteins. PF PAI-1 was the only parameter that independently discriminated septated and non-septated effusions (Figure S2). This finding was consistent across septation severity, with increased PAI-1 levels associated with worsening septation severity (Figure 2).

PF-PAI-1 as a biological predictor of septation

Conventional serum and pleural fluid biomarkers were analysed with pleural fluid biomarkers to assess for predictors of septation status (Table S5). In the multivariate model, PF PAI-1 was the only independent predictor of septation presence (p<0.001) (table S6). From the Receiver Operating Characteristic (ROC) curve analysis, a PF PAI-1 cut-off of 1700ng/ml had an AUC of 0.70 (95% CI 0.61-0.79). The AUC values for the remaining experimental proteins analysed are shown in the online supplement (table S7).

Septations and PF-PAI-1 as predictors of clinical outcome

The presence of septations at baseline independently predicted use of IET [19.6% vs 9.6%; p=0.023 (OR 2.30 95%CI 1.10-4.78)]. However, baseline septations were not associated with surgery, mortality, readmission or length of stay (Table 3). This was consistent when analysed for septation severity (Table 4). Using binary logistic regression and adjusting for use of IET, presence of septations did not predict length of hospital stay (p=0.67), need for surgery at 3 months (p=0.25), mortality at 3 months (p=0.44) or 12 months (p=0.49). The Kaplan-Meier (KM) curve for time-to-discharge is shown in the supplementary materials (Figure S3). There was no

statistical difference in time-to-death between baseline presence or absence of septations (Figure 3a).

PF-PAI-1 levels did not predict use of IET (p=0.62) or surgery at 3 months (p=0.26). In a linear regression, higher PF-PAI-1 predicted longer length of stay; t(1,214)=1.99, p=0.048). In terms of mortality there was a trend towards, but not reaching, significance with death at 3 months (p=0.07). PAI-1 was converted into a categorical variable using the covariate mean (1974 ng/mL) to classify all cases into 'PAI-1-low' and 'PAI-1 high' and in a KM survival analysis, the latter was predictive of time-to-death at 12 months (Figure 3b). This result was consistent when repeated within the septated population alone.

Macroscopic pleural fluid purulence

Given the increased representation of frank purulence in the non-septated cohort (45% vs 21%) (Table 1), further analysis was performed to explore the composition of inflammatory and fibrinolysis-associated proteins in fluid samples comprising frank pus versus non-purulent macroscopic appearances. The only significant difference in patient characteristics between the two groups was a significantly greater proportion of poor dental hygiene in the purulent group (60.3% vs 44.3%, χ^2 1df 11.1; p=0.009) (Table S8).

The frequency of pleural infection diagnoses based on the aspiration of frank pus in the PILOT study overall was 151/537 (28.1%) (Figure S4) and in 65/214 (30.3%) (χ^2 1df 0.38; p=0.54) of the pleural fluid samples with complete protein profile data used in this study. The pleural fluid protein compositions were completely different with purulent samples containing significantly higher median levels of IFN-ɣ, TNF-α and uPA with significantly lower levels of PAI-1, MCP-1/CCL2, and D-dimer (Table 5). The PF proteins were entered into a stepwise multinomial regression model and low PAI-1 and high uPA were the only proteins independently predictive of a purulent fluid outcome (Table S9).

Comparing conventional pleural infection serum and pleural parameters of cell death turnover, patients with frank purulence had a higher serum platelet count, lower serum albumin concentration and a 3.5-fold higher median pleural fluid LDH (Table S10).

Discussion

Using human biological samples from the largest prospective observational pleural infection cohort to date in the world literature, this study confirms the findings of studies using animal models and smaller retrospective clinical studies in demonstrating that levels of endogenous PF PAI-1 in pleural infection are considerably elevated intrapleurally (12–14). These data infer that despite multiple factors being associated with a general increased level of endogenous PF PAI-1 (15–17), the differences in concentrations of PF PAI-1 and the presence or severity of septations in this study population were independent of any pre-existing patient factors such as age or co-morbidity.

Endogenous PF PAI-1 appears to play an integral role in the biological development and progression of septations. PF PAI-1 had a stronger association with septations than any other conventional serum, blood, or pleural fluid parameter as well as the inflammation and fibrinolysis-associated proteins measured in this study. Of particular interest was the finding that pro-inflammatory cytokines (CCL2/MCP-1, IFN-γ and TNF-α) measured from patients with active pleural infection were not associated with septation severity. This observation is intriguing and may suggest that once pleural injury induces suppression of the fibrinolytic system, inflammation has a lesser role than PAI-1 in driving septation progression. This may explain why septation severity was not associated with differences in fever or tachycardia or markers of pleural and systemic inflammation (PF LDH, serum CRP, blood WCC).

This large prospective cohort is the first to show that approximately 4 in 5 patients with pleural infection will present with some degree of sonographic septation, this incidence having been previously unknown. One in three will have a severely septated appearances. Detection of septations is often used by clinicians as a decision-making parameter, or a tool to select treatment, based on assumptions such as reduced likelihood of successful pleural drainage. However, these assumptions are challenged by the presented evidence; there was no increased need for more invasive intervention compared to standard treatment with an ultrasound-guided optimally placed chest tube with regular saline flushes. Whether subjects with sustained elevations in PF PAI-1 are more predisposed to florid septation and failed drainage was not examined in this study.

Two retrospective studies (5, 6) have previously suggested septations are associated with poorer clinical outcomes and need for more invasive intervention. However, due to sample size and methodology, these data are likely to be flawed. The data from our study demonstrate an increased septation severity is associated with a greater use of IET and surgical referral at 3 months. However, neither septation state nor severity at baseline, in isolation, predicted a need for surgery at 3 months, longer length of hospital stay, or likelihood of readmission at 12 months. This being the case, it is not possible to exclude the possibility that septation detection results in different behaviour by clinicians, as demonstrated by their independent predictive ability of IET use in the multivariate model.

In most cases, the diagnosis of pleural infection is straightforward based on well-established conventional blood, serum and PF biomarkers, and initial management of pleural infection is focused on early chest tube drainage and prompt antibiotics. In a cohort of 93 patients with parapneumonic effusions, Arnold and colleagues demonstrated that high PF soluble urokinase plasminogen activator receptor (suPAR) predicted pleural fluid pH and subsequent chest tube insertion (18). This represented a step forward in our understanding of the biology of pleural infection progression. Albeit in a smaller cohort, pleural fluid suPAR concentrations were higher in patients with loculated collections (graded as absent/present), which likely represented two ends of the septation spectrum. In the present study, using a *pre hoc* definition and a validated method for quantification of septation severity, we have now demonstrated PF PAI-1 to be an accurate biological correlate of a radiological outcome across the spectrum of septation development.

Importantly, this is the first study to demonstrate that pleural biology predicts clinically important outcomes in pleural infection. PF PAI-1 was a predictor of length of stay and mortality, a finding thus far not demonstrated by suPAR (18). A recent study by Hoshino et al examining sepsis biomarkers and coagulation/fibrinolysis markers on ICU admission found serum PAI-1 to be the only independently predictor of 28-day mortality in sepsis patients (19). Schmitt et al found that acute fibrinolysis shutdown, judged by raised serum PAI-1 levels, occurred early in sepsis and was associated with increased morbidity and mortality in septic shock (20). The underlying pathomechanisms and specific temporal kinetics of PAI-1 in the pleural space are yet to be fully understood but it is plausible that this process is

exaggerated, or occurs more rapidly, within the confines of the pleural space prior to significant systemic compromise reflecting the increased mortality associated with this condition.

Septations are an attractive radiological biomarker, particularly with bedside ultrasound becoming routinely used in clinical practice by respiratory physicians. However, despite data from both this study and that by Arnold et al demonstrating that biomarkers of fibrinolysis inhibition such as PF-PAI-1 and PF suPAR predict the development of septations, this large prospective cohort to our knowledge provides the strongest evidence that septations do not, in isolation, predict clinical outcomes. We therefore hypothesize that septations are likely an epiphenomenon in the progression of pleural sepsis, and this may be the reason that lone fibrinolytic therapy (in the absence of DNase) does not result in improved clinical outcomes in randomised trials of adult pleural infection (3).

Treatment with IET is based on activation of endogenous plasminogen providing sustained fibrinolytic activity that degrades intrapleural fibrin. Several factors may be associated with treatment outcome including the rate of intrapleural inactivation of a fibrinolytic, levels of endogenous plasminogen, a higher level of active PAI-1 and extracellular DNA, and potentially the formation of biofilms (21). These may collectively, or synergistically contribute to poor outcomes in pleural infection (22).

The analysis of purulent pleural fluid samples adds important insight into pathogenesis. It has not yet been fully explained why some patients present with unilocular purulent collections, while other develop more complex, septated effusions. In this study, purulent collections were shown to contain higher levels of pro-inflammatory cytokines, cell death and turnover as demonstrated by the higher levels of IFN-gamma, TNF-alpha and PF LDH with relatively suppressed intrapleural levels of fibrinolysis inhibition (PAI-1). These data suggest that lesser inhibition of plasminogen activator activity associated with reduced pleural fluid levels of PAI-1 may thereby favour intrapleural purulence. The inhibition of fibrinolysis favours intrapleural organization with septation, but whether it reduces intrapleural inflammation remains unclear (13). Therefore, is a surge in fibrinolysis inhibition an intrinsic host defence mechanism to reduce inflammation and sepsis? The significance of cross-talk between bacteria and an inflammatory pleural environment

remains unclear. One may infer that the invading pathogen(s) plays a significant role judging by the increased proportion of patients with poor dental hygiene who appear to develop purulent collections, and a detailed analysis of microbiology and its association with septation is now required.

This study has some limitations. PAI-1 testing is complex. Total PAI-1 antigen assays measure the sum of active PAI-1, tPA/PAI-1, and latent PAI-1 (23). PAI-1 levels, but not its activity were measured in this study. Whether PAI-1 is cleaved in pleural fluid by proteases or if a proportion of PAI-1 reverts to its latent form remains unknown. As pleural infection is a one-off event where sampling occurs at baseline followed by urgent drainage being clinically required, intermittent drainage and repeated sampling (e.g. via an indwelling pleural catheter) was not feasible (or ethical) to assess for diurnal variability in PAI-1 or measure how levels progressed with treatment. To the best of our knowledge, no other studies have addressed these limitations and they should be prioritised in future studies. Ideally, raid centrifugation followed by immediate storage of the cell-free fluids at -80C is required to reliably perform these analyses. However, in a large scale, multicentre study such as PILOT, this was not feasible. The sample collection and processing protocol applied is standardised within our group to ensure samples are sent promptly, received and processed centrally in a timely and uniform fashion and has been used with success in other studies for proteomics analyses (24). The validated septation score method allowed assessment of septation severity but is not immune to a degree of inter-operator variability (as septation appearance can differ depending on angle of the probe against the rib space) but operators were asked to specifically use the maximal degree of septation to quantify severity, which should have minimised this. Furthermore, it should be clear that final clinical outcome was not knowable at the time the ultrasound images were taken and scored by clinicians.

The size of the analysis cohort paired with complete biological samples and ultrasound data was smaller than the PILOT population as a whole but nonetheless we have demonstrated that this was a representative cohort based on similar patient demographics and baseline characteristics (table S3). Despite this, the current study still represents the largest analysis using human samples associating baseline parameters of inflammation and fibrinolysis in pleural fluid with radiological and predefined clinical outcomes. Secondly, as the PILOT study did not collect blood

samples, measurement of serum PAI-1 was not possible and a correlation of these levels with PF PAI-1 may have enabled a more complete understanding of its role in the pathogenesis of pleural infection. The most commonly used plasminogen activator (tPA) is rapidly inactivated by PAI-1 in the pleural space. We are here unable to assess whether PAI-1 levels were associated with tPA treatment failure due to the small number of patients in the PILOT study who received IET, as the majority of trial recruitment occurred prior to IET becoming commonplace. Nonetheless our data suggest that PAI-1 has some influence on the outcome of pleural injury and may dampen the ability of fibrinolytics to activate plasminogen.

Conclusion

In summary, this is the first study to associate pleural biological parameters with a validated sonographic septation outcome as well as clinically important outcomes. Within a large cohort of patients with confirmed pleural infection, increased levels of endogenous PF-PAI-1 was associated with more severe sonographic septation, longer hospital stay and reduced survival at 12 months. Plasminogen activation suppression with downstream suppression of local fibrinolysis appears to have more dominant role compared to the pro-inflammatory state in driving septation development and progression. Increasing severity of septations was associated with a higher rate of clinician-driven intervention with IET and surgery, but were not independently predictive of clinical outcomes. These signals require prospective validation before the utility of PF-PAI-1 in pleural infection prognostication and management can be fully elucidated.

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Figures and tables

Table 1. Baseline demographics by septation status.

	Not septated (n=94)	Septated (n=368)	p-value
Age, yr, median (IQR)	69 (54-75)	68 (53-77)	0.65
Male, n, (%)	72 (76.6)	256 (69.6)	0.18
Community acquired, n (%)	86 (91.5)	321 (87.2)	0.18
Poor dental hygiene, n (%)	44 (46.8)	168 (45.7)	0.99
Antibiotic use before diagnosis, n (%)	56 (59.6)	228 (62)	0.52
Fluid purulence, n (%)	43 (45.7)	79 (21.7)	0.01
Micro positive	58 (61.7)	223 (60.6)	0.87
Small bore drains (<=14F); n, (%)	52 (55.3)	191 (51.9)	0.32
Fever (T>37.8C)	20 (21.7)	77 (21.5)	0.96
Tachycardia (HR >100bpm)	22 (24.2)	129 (36)	0.03
RAPID category			
Low	24 (25.5)	111 (30.2)	0.72
Medium	37 (39.4)	139 (37.8)	
High	20 (21.3)	73 (19.8)	
Comorbidities (%)			
0: n, (%)	37 (39.4)	145 (39.4)	0.934
1 to 2: n, (%)	45 (47.9)	168 (45.7)	
3 or more: n, (%)	12 (12.8)	51 (13.9)	

Key: proportions were compared using χ^2 statistics and medians were compared using independent K samples median test.

Table 2. Pleural fluid protein levels by septation severity.

PF protein	Septation Score				p-value
	Nil (n=46)	Mild (n=28)	Moderate (n=39)	Severe (n=53)	
PAI-1, ng/mL; median (IQR)	725.2 (182-1480)	1104.1 (513-1685)	1464.9 (696-1893)	1573.7 (1212-2111)	0.003
MCP-1/CCL2, ng/mL; median (IQR)	0.59 (0.14-2.55)	1.77 (0.54-8.93)	3.83 (0.78-8.64)	2.02 (0.56-5.03)	0.16
IFN-γ, ng/mL; median (IQR)	0.02 (0.017-0.049)	0.02 (0.019-0.039)	0.02 (0.016-0.030)	0.02 (0.017-0.032)	0.79
uPA, ng/mL; median (IQR)	0.55 (0.19-2.05)	0.34 (0.21-1.19)	0.28 (0.18-0.57)	0.35 (0.20-1.17)	0.64
TNF-α, ng/mL; median (IQR)	0.05 (0.03-0.09)	0.06 (0.03-0.11)	0.06 (0.04-0.14)	0.05 (0.04-0.09)	0.59
D-dimer, ng/mL; median (IQR)	9.09 (6.21-14.98)	9.83 (7.72-11.39)	10.02 (8.55-13.96)	10.90 (8.85-14.63)	0.34

Key: The p-value represents the statistical significance in difference between the means of the four groups.

Table 3. Outcomes according to baseline presence or absence of septations.

Outcome	Non-septated (n=94)	Septated (n=368)	p-value
IET; n (%)	9 (9.6)	72 (19.6)	χ^2 (1df) 5.17, p=0.023
Surgery within 12 months; n (%)	20 (21.2)	81 (22.0)	χ^2 (1df) 0.01, p=0.92
Readmission within 12 months; n (%)	11 (11.7)	59 (16)	χ^2 (1df) 1.09, p=0.29
Length of stay (days); median (IQR)	14 (9-21)	15 (10-22)	Mann Whitney p=0.31
3 month mortality*; n (%)	6 (6.4)	40 (10.9)	χ^2 (1df) 1.68, p=0.19
1 year mortality; n (%)	10 (10.7)	69 (18.7)	χ^2 (1df) 3.13, p=0.07

Key: *Primary outcome of the PILOT study.

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Table 4. Outcomes according to baseline septation score.

Outcome	Septation score (n=434)				p-value
	Non-septated (n=94)	Mild (n=72)	Moderate (n=125)	Severe (n=143)	
IET; n (%)	9 (9.6)	11 (15.1)	31 (24.8%)	26 (18.3%)	Ordinal χ^2 (1df) 4.02; p= 0.045
Surgery within 12 months; n (%)	20 (21.2)	14 (19.2)	21 (16.8)	37 (26.1)	Ordinal χ^2 (1df) 3.63; p= 0.41
Readmission within 12 months; n (%)	11 (11.7)	16 (21.9)	19 (15.2)	21 (14.8)	Ordinal χ^2 (1df) 3.61, p=0.31
Length of stay (days); median (IQR)	14 (9-21)	13	15	15	Mann Whitney p=0.73
3 month mortality; n (%)	6 (6.4)	10 (13.9)	13 (10.4)	15 (10.5)	Ordinal χ^2 (1df) 1.26, p=0.74
1 year mortality; n (%)	10 (10.7)	9 (12.5)	27 (21.6)	23 (16.1)	Ordinal χ^2 (1df) 1.19, p=0.27

Key: p values represent results of ordinal χ^2 test (linear by linear association)

Table 5. Pleural fluid protein levels based on presence/absence of frank purulence

PF Protein	Grand Median	Purulent (n=65)	Non-purulent (n=149)	p-value
PAI-1, ng/mL; median (IQR)	1330.28	565.29 (133.43 – 1329.29)	1559.80 (904.51 – 2019.49)	<0.001
MCP-1/CCL2, ng/mL; median (IQR)	2.05	0.86 (0.08 – 3.21)	4.48 (1.31 – 10.10)	0.034
IFN-γ, ng/mL; median (IQR)	0.022	0.027 (0.016 – 0.043)	0.021 (0.018 – 0.034)	0.009
D-dimer, ng/mL; median (IQR)	9.85	9.07 (4.10 – 11.35)	10.14 (8.69 – 14.73)	0.001
TNF-α, ng/mL; median (IQR)	0.058	0.078 (35.5 – 140.8)	0.052 (41.2 – 113.8)	0.012
uPA; ng/mL; median (IQR)	0.39	1.06 (0.41 – 2.24)	0.27 (0.16 – 0.46)	<0.001

Key: proportions were compared using χ^2 statistics and medians were compared using independent K samples median test.

Figure 1 – Study flow diagram

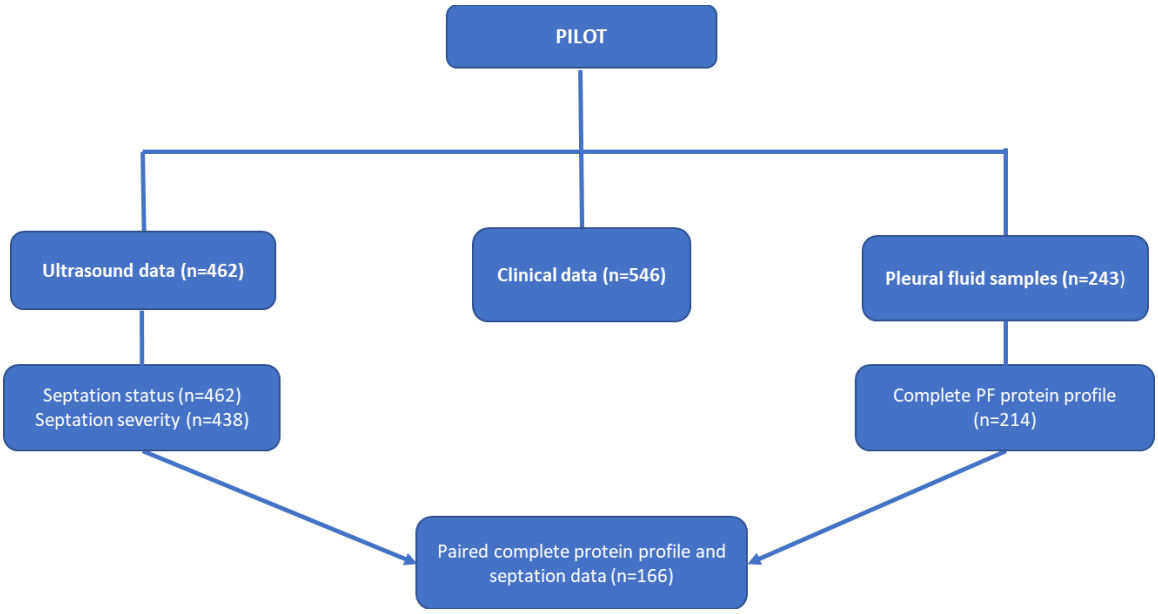
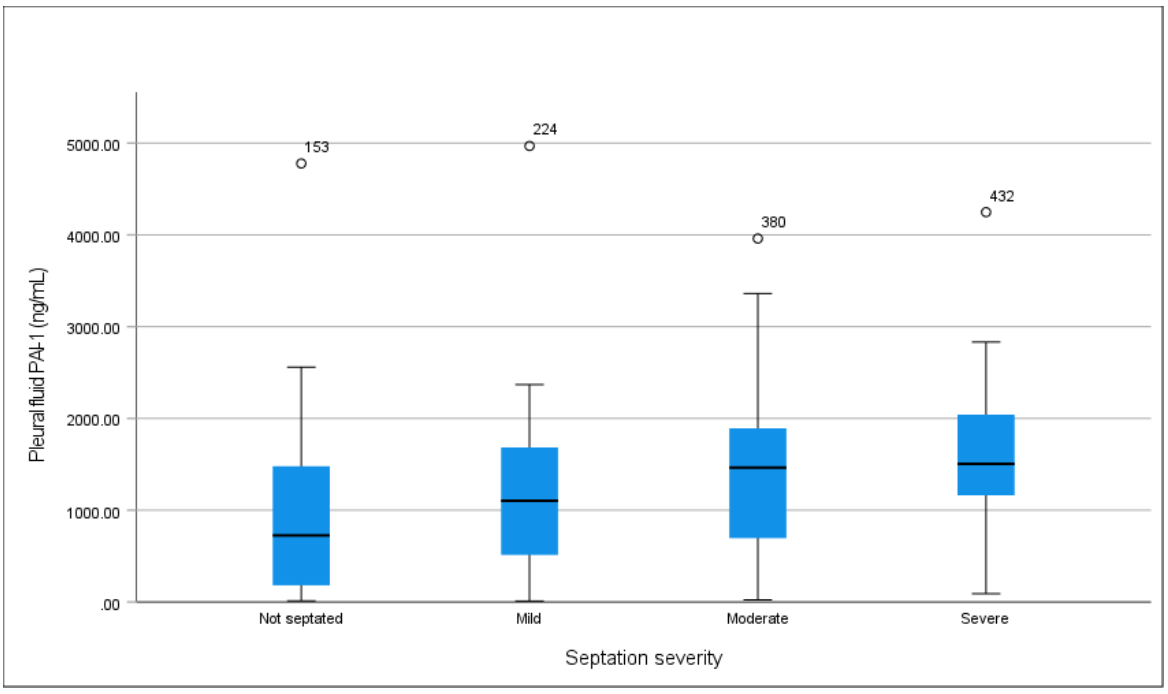
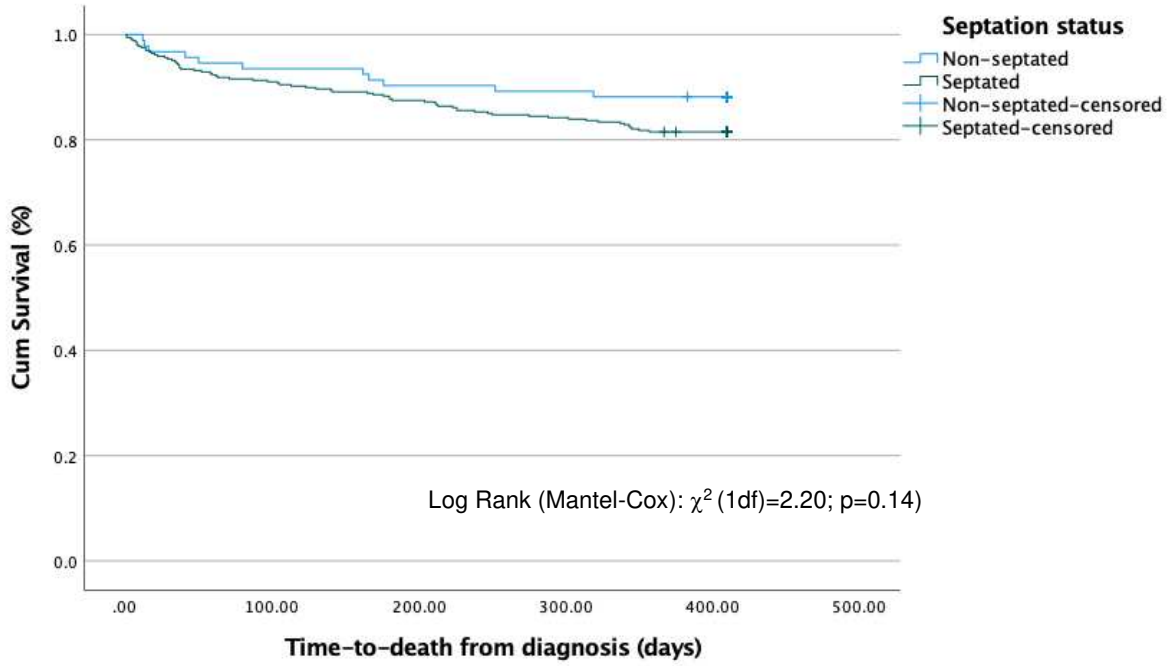


Figure 2. Box and whisker plot of pleural fluid PAI-1 by septation severity.



The numbers over the boxes represent the study identifier for the outlier cases (1 case in each septation group)

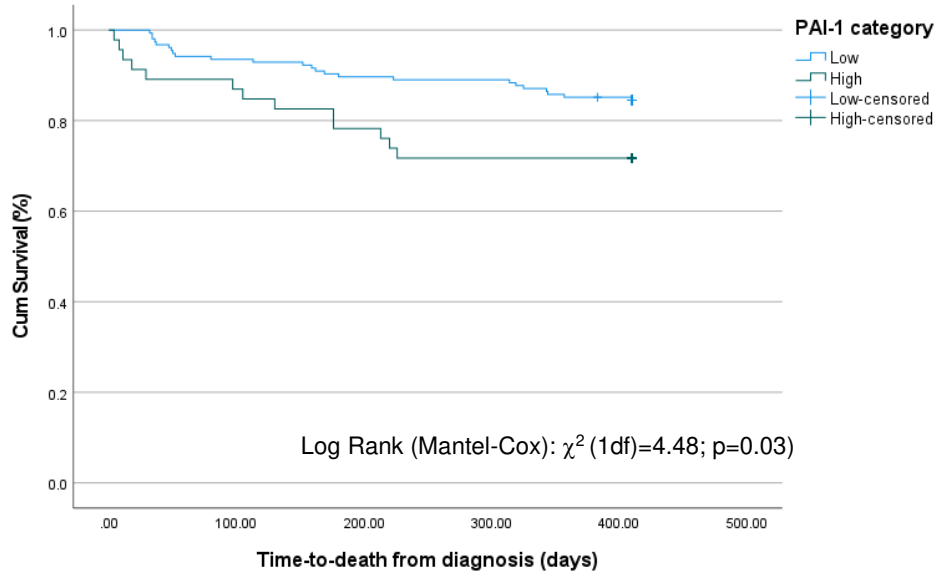
Figure 3a. Kaplan Meier survival curves presenting one-year mortality for septated and non septated cases



Days	0	100	200	300	400
Non-septated	94	88	86	84	83
Septated	368	335	322	310	298

Numbers at risk

Figure 3b. Kaplan Meier survival curves presenting one-year mortality PAI-1 high and PAI-1 low cases (Multivariate Cox regression P value)



Days	0	100	200	300	400
PAI-1 low	164	153	146	145	138
PAI-1 high	50	43	38	34	34

Numbers at risk