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# Pleural Fluid suPAR Levels Predict the Need for Invasive Management in Parapneumonic Effusions

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**Author contributions**

DA, FH, RB and NM wrote the manuscript. DA and SF conceived and planned the study. DA and KE carried out the experiments. DA and FH performed the statistical analysis. NZE and SP lead the recruitment of patients, curation of the database and storage of samples. NM, AM and RB supervised the project and the primary observational study. All authors discussed the results and contributed to the final manuscript.

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The suPARnostic™ ELISA kits were gifts from ViroGates (Birkeroed, Denmark)

**Clinical Impact Statement:** The ability to predict the clinical course of parapneumonic effusions would be invaluable to physicians when making management decisions at diagnosis. In this prospectively collected cohort a raised pleural suPAR was highly predictive of patients who went on to receive more invasive management of parapneumonic effusions and added value to conventional biomarkers.

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**ABSTRACT**

**Rationale**: Parapneumonic effusions have a wide clinical spectrum. The majority settle with conservative management but some progress to complex collections requiring intervention. For decades, physicians have relied on pleural fluid pH to determine the need for chest tube drainage despite a lack of prospective validation and no ability to predict the requirement for fibrinolytics or thoracic surgery.

**Objectives**: To study the ability of soluble urokinase Plasminogen Activator Receptor (suPAR), a potential biomarker of pleural fluid loculation, to predict the need for invasive management compared to conventional fluid biomarkers (pH, glucose, LDH) in parapneumonic effusions.

**Methods**: Patients presenting with pleural effusions were prospectively recruited to an observational study with biological samples stored at presentation. Pleural fluid and serum suPAR levels were measured using the suPARnostic™ double-monoclonal antibody sandwich ELISA on 93 patients with parapneumonic effusions and 47 controls (benign and malignant effusions).

**Measurements and Main Results**: Pleural suPAR levels were significantly higher in effusions that loculated versus non-loculated parapneumonic effusions (median 132ng/ml vs 22ng/ml, $p<0.001$). Pleural suPAR could more accurately predict the subsequent insertion of a chest tube with an AUC of 0.93 (95%CI. 0.89-0.98) compared to pleural pH (AUC 0.82, 95%CI. 0.73-0.90). suPAR was superior to the combination of conventional pleural biomarkers (pH, glucose and LDH) when predicting the referral for intrapleural fibrinolysis or thoracic surgery (AUC 0.92 vs 0.76).

**Conclusions**: Raised pleural suPAR was predictive of patients receiving more invasive management of parapneumonic effusions and added value to conventional biomarkers. These results need validation in a prospective multicenter trial.

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**INTRODUCTION**

The clinical spectrum of pleural effusions related to infection is wide. From simple parapneumonic effusions that settle with conservative management, through to grossly septated fibropurulent collections needing chest tube drainage, intrapleural fibrinolytics or thoracic surgery for resolution. The process by which an effusion progresses down this cascade has been the subject of much research but the ability to predict which patients will require more aggressive intervention remains elusive (1). In 1980, Light et al proposed the use of a pleural fluid pH cut-off of 7.2 to indicate the need for chest tube drainage on the basis that as bacteria metabolise and neutrophils phagocytose the pleural pH falls. (2) This cut off is referenced in the majority of international guidelines despite never being prospectively validated (3-5).

A defining feature in the spectrum of parapneumonic effusions is the dysregulation of the fibrinogenesis/fibrinolytic cascade and the subsequent development of loculations within the effusion. Loculations prevent adequate chest tube drainage, impede source control, can result in long term respiratory compromise and might even reduce the effectiveness of antibiotics (6). Pleural fluid pH, although a mainstay of initial management decisions, does not predict the development of loculations. A biomarker called soluble urokinase Plasminogen Activator Receptor (suPAR) is theoretically a more appropriate guide for management. suPAR is the soluble form of uPAR which, once bound to endogenous urokinase (uPA), catalyses the conversion of plasminogen to plasmin (a potent fibrinolytic). Originally documented in the plasma, serum and urine of patients with human immunodeficiency virus (HIV), pneumonia, sepsis, tuberculosis and various solid-tumours (7), more recent studies have shown suPAR also rises in infected ascitic and pleural fluid (8-11).

We aimed to assess the potential role of pleural fluid suPAR in the investigation and subsequent management of parapneumonic effusions using a prospectively collected cohort of patients.

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Some of the results of this study have been previously reported in the form of an abstract (12).

## METHODS

### *Patients*

Between 2009 to 2016, patients presenting to a UK tertiary pleural service with undiagnosed pleural effusions requiring a diagnostic thoracentesis were prospectively recruited to an observational study (08/H0102/11). All had routine serum and pleural fluid analysis including a full blood count, serum C-reactive protein (CRP) and pleural fluid pH, glucose and lactate dehydrogenase (LDH). At the time of pleural fluid sampling, pleural ultrasound was performed by a physician at least Level 1 BTS ultrasound trained (or equivalent) with the presence of loculations documented. Repeat ultrasounds or computed tomography (CT) scans were performed if clinically indicated and the development of loculations was recorded. Patients gave consent for storage of their baseline pleural fluid and serum samples in a -70$^{o}$C freezer for future analysis.

Patients were followed up at 1 year to ascertain the final diagnosis of their pleural effusion, which was decided by two independent respiratory consultants based on pre-specified criteria (see Appendix 1). Patients were otherwise treated as per standard care, see Appendix 2 for local guidelines on parapneumonic effusion management.

### *suPAR testing*

Pleural fluid and serum samples were analysed from patients with an effusion secondary to infection. Those with frank pus on thoracentesis were excluded on the grounds that management for those cases is unequivocal. All patient samples were handled in accordance with a standardized study protocol; see Appendix 3 for full sample processing details and validation experiments of different sample preparation

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methods. suPAR levels were analysed in duplicate (mean value presented, with high correlation observed ($R^2$>0.99)) using the suPARnostic® AUTO Flex ELISA assay according to the manufacturer's protocol (Virogates, Denmark). This assay detects free suPAR (as well as domains II and III), it does not capture suPAR-scuPA (suPAR bound to single chain urokinase) or suPAR-scuPA-PAI-1 (suPAR-scuPA bound to plasminogen activator inhibitor-1) complexes (13). As per protocol, samples were diluted until they fell within the workable range of the assay (0.2-15 ng/ml).

In order to explore suPAR levels in other aetiologies, selected controls from the same cohort were also analysed including;

1. transudative effusions secondary to congestive cardiac failure or hepatic hydrothorax,
2. non-loculated malignant effusions,
3. loculated malignant effusions,
4. malignant effusions that were simple at baseline but became loculated at later timepoints receiving intrapleural fibrinolytics (urokinase).

*Statistical analysis*

Patient data are reported as the median/interquartile range (IQR)/range for continuous variables. The statistical differences between groups were analysed using a non-parametric Mann–Whitney U-test.

The correlation between serum/pleural suPAR and conventional biomarkers (including serum CRP and neutrophils, pleural pH, LDH, glucose and protein) was assessed using Spearman's rank correlation coefficient (with $p<0.05$ used to define statistical significance). Multivariable binomial logistic regression was used to compare clinical outcomes to biochemical markers. The accuracy of suPAR and other conventional markers as diagnostic tests was assessed using standard sensitivity, specificity, positive and

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negative likelihood ratios (PLR and NLR) and area under the curve (AUC) statistics with 95% confidence intervals. DeLong's test was performed to compare the differences in AUCs. Statistical analysis was performed using SPSS 24.0 statistical software (Chicago, IL, USA), receiver operating characteristic (ROC) curve graphs were generated using RStudio 3.6.1 (R Foundation for Statistical Computing, Vienna).

**RESULTS**

Between 2009 to 2016, 93 patients presenting to our centre with pleural effusions secondary to infection (excluding frank pus) were recruited and had biological samples stored. As controls, 31 cases of malignant effusions and 16 transudative effusions were also included in this analysis. The median age of patients with parapneumonic effusions was 66 and there was a male predominance. Full patient demographics by aetiology are represented in Table 1.

*Pleural suPAR levels in all effusions*

The median pleural suPAR of pleural effusions varied significantly by aetiology, with parapneumonic effusions having significantly higher levels than malignancy and transudative effusions at baseline (p= <0.01). Pleural suPAR was strongly correlated with the commonly used pleural fluid indicators of infection: pH (Correlation coefficient (CC) -0.576 p<0.01), glucose (CC -0.632 neg p<0.01) and LDH (CC 0.596 p<0.01), but not pleural fluid protein (CC 0.057 p= 0.59) across all aetiologias.

*Pleural suPAR in parapneumonic effusions*

Table 2 shows the levels of pleural and serum suPAR from patients with parapneumonic effusions alongside routine pleural fluid and serum tests depending on the presence/absence of fluid loculation during hospital admission. Levels of pleural suPAR were significantly higher in loculated versus non-loculated effusions (p<0.01). Using a cut-off of 35 ng/ml pleural suPAR had a 100% sensitivity (95% C.I.

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91-100) for predicting pleural fluid loculations with a specificity of 91% (95% C.I. 80-97), PLR of 12.3 and NLR of 0.0. This compared to pleural pH which, using the conventional cut-off of 7.2, had a sensitivity of 52% (95% C.I. 37-68), specificity of 84%(95% C.I. 70-93), PLR of 3.2 and NLR of 0.57, see Figure 1. In a multivariable analysis model, including all the analytes presented in Table 2, pleural suPAR was the only independent predictor of pleural effusion loculation during hospital admission, see Appendix 4.

In 9 patients where the initial ultrasound was simple, loculations developed on subsequent pleural ultrasound and/or CT scans at a median of 5 days (range 3-10). The baseline pleural suPAR was significantly higher in parapneumonic effusions that subsequently loculated (median 139.6 ng/ml, IQR 41.9-312.8) compared to those that remained non loculated (median 22.3, IQR 14.0-28.1) and was equivalent to effusion that were loculated from baseline (median 131.0, IQR 52.7-223.8) ($p<0.01$).

*Serum suPAR in parapneumonic effusions*

Paired serum suPAR levels were not correlated with pleural suPAR within parapneumonic effusions (CC 0.170 p=0.11), nor any other pleural fluid marker or fluid loculation. Serum suPAR was correlated with serum CRP (CC 0.268 $p< 0.01$) and serum neutrophils (CC 0.233 p=0.03) but not clinical outcomes.

*Pleural suPAR and chest tube insertion for parapneumonic effusions*

Of the conventional pleural fluid markers for predicting chest tube insertion (pH, glucose and LDH), pleural pH was the most accurate (AUC 0.82 C.I. 0.73-0.90, sensitivity 54%, specificity 95%, PLR 10.5 and NLR 0.5 using 7.2 as a cut-off). Pleural suPAR (alone) was superior to pleural pH (alone) at predicting the insertion of a chest tube for drainage of infected pleural effusions (AUC 0.93, C.I. 0.89-0.98, p=0.01 using DeLong's test). Using a cut-off of 35ng/ml, pleural suPAR had an 83% sensitivity, 92% specificity, PLR 10.8 and NLR 0.2 (see Figure). In a multivariable logistic regression, pleural pH (p=0.02), pleural LDH (p=0.05), a

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neutrophilic effusion (p=0.05) and pleural suPAR (p=0.01) were significant indicators for chest tube insertion, see Appendix 4).

*Pleural suPAR and referral for medical/surgical rescue therapies*

Pleural suPAR was superior to all other conventional markers combined at predicting the need for rescue therapies (intrapleural fibrinolytics or thoracic surgery) with an AUC of 0.92 (0.87-0.98, p=0.02 using DeLong's test). Using a cut-off of 65ng/ml, pleural suPAR was 94% sensitive and 84% specific (PLR 6.0, NLR 0.1) at predicting the referral for these therapies (16 of the 93 patients), see Table 3.

The combination of markers that are conventionally used to define a complex parapneumonic effusion (including pleural pH < 7.2 OR pleural glucose ≤ 3.0 mmol/L (≤ 55mg/dL) OR pleural LDH >1000 IU/L)(4) had an AUC of 0.76 (0.71-0.81) for predicting rescue therapies, see Figure 3. Pleural suPAR was the only significant baseline predictor of rescue therapies (p =0.01), see Appendix 4.

*Pleural suPAR in malignant effusions*

Pleural suPAR levels were significantly higher in malignant effusions that were loculated at the time of pleural fluid analysis (p< 0.01). We carried out a further analysis to assess whether baseline pleural suPAR levels could predict future malignant loculations. The 'Delayed Loculation' group included effusions that started out non-loculated (simple) and became loculated (over a period of 4-6 months). Baseline pleural suPAR levels were non-significantly higher in the delayed loculation group compared to those that remained non-loculated (p-0.19).

**DISCUSSION**

In this prospectively recruited cohort of patients presenting with parapneumonic effusions, high pleural suPAR could predict the insertion of a chest tube with an AUC of 0.93 (95% CI. 0.89-0.98). It could predict

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the presence or development of loculations with considerable accuracy. A high pleural suPAR was also indicative of the referral for intrapleural fibrinolytics and/or thoracic surgery and was superior to conventional pleural fluid biomarkers.

The optimal management of parapneumonic effusions is contentious and the topic of several ongoing research studies. Much of the uncertainty relates to the difficulty predicting which patients require formal drainage of their effusion and which will resolve with conservative management (antibiotics) alone. Guidelines recommend formal drainage in the case of frank pus or a positive gram stain/culture of pleural fluid (3, 4). Given low culture rates of fluid from complex parapneumonic effusions (14), formal drainage is also recommended if the pleural fluid pH is less than 7.2. This threshold was first suggested by Light in 1980 after a case series of 90 patients showed that low pH effusions (n=10) tended to need chest tube drainage (2). In 1995, Heffner and colleagues performed an elegant meta-analysis of the studies relating to the topic of using pleural pH, glucose or LDH in distinguishing complicated and uncomplicated parapneumonic effusions (5). From the 7 included studies (251 patients) they concluded that pleural pH was the best performing analyte at a cut-off of 7.21. However, they also recognised that given the observational nature of the 7 studies, and the fact that this analyte had become 'entrenched in clinical practice', it required 'validation in well-designed prospective studies'. pH falls due to lactic acid and carbon dioxide production by bacteria within the pleural space (15, 16). Although indicating the presence of bacteria it is prone to both false positive and negatives in the need for invasive pleural management and has never been prospectively validated in this regard (17).

A crucial factor in the management of parapneumonic effusions is the development of pleural thickening, septations and loculations. The tendency for loculation development is not only associated with more severe infection; it also reduces the likely success of simple fluid drainage versus the need for more invasive medical (e.g. intrapleural fibrinolytics) or surgical therapies. Again, a low pleural pH is more likely

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in loculated effusions (18) but is inaccurate as it is a sequalae of numerous biochemical reactions, not simply the derangement of normal fibrinolysis (19) so cannot be used as an indicator for fibrinolytics or surgery. Other markers to predict which patients might require more invasive management of their parapneumonic effusion have been elusive. Pleural fluid biomarkers such as procalcitonin (20), C-reactive protein (21) and calprotectin (22) have been tested in parapneumonic effusions but given these markers focus on neutrophilic activation and/or general increased in chemo-cytokine activity they are no more specific than pH in prognostication. Recent studies have tested cytokines involved in the production of pleural fluid (23) but less have focused on those related to loculation development for which suPAR seems a more specific target.

Loculations develop due to derangement of the normal fibrinolysis cascade mediated by the urokinase-type plasminogen activator system, see Figure 5. This is composed of a proteinase called urokinase-type plasminogen activator (uPA), a cell bound uPA receptor (uPAR) and suPAR. suPAR, the soluble form of uPAR, is a glycoprotein with a molecular weight of 55–60 kDa. uPAR is cleaved from its glycosylphosphatidylinositol (GPI) anchor by various proteases related to infection and inflammation. The uPA system is involved in pericellular proteolysis, cell migration and tissue remodeling. Most notably, uPA once bound to uPAR, catalyzes the conversion of plasminogen into plasmin, a potent endogenous fibrinolytic. It has been demonstrated in both animal models and humans that the development of pleural loculations is related to levels of plasminogen activator inhibitor-1 (PAI-1) which is released by pleural mesothelial cells (24). PAI-1 inhibits uPA and therefore the conversion of plasminogen as well as suppressing the activity of several other endogenous and therapeutic fibrinolytics. Given these pathological roles it is logical that PAI-1 itself could serve as a biomarker of pleural organization; however, this is limited by the instability and variation of the enzyme (25). We have shown that in parapneumonic effusions pleural suPAR is dramatically raised in the presence or even future development of pleural loculations. The biological role of suPAR in pleural fluid are less well understood. While neutrophil bound

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uPAR is inversely correlated with suPAR levels in critically ill patients, ((26) binding of single chain uPA (scuPA) to suPAR increases its PA activity suggesting that suPAR can augment pleural fluid plasminogen activator activity and plasmin generation (27). This interaction could localize plasminogen activation within pleural fluid, similar to that which occurs at cell surfaces(28). Measurement of suPAR could potentially provide a method to assess the capacity of pleural fluids to support uPA-related plasminogen activator activity. The MIST-2 trial demonstrated that the combination of intrapleural alteplase and DNase improved radiographic appearance, reduced hospital length of stay and surgical referral rates in pleural infection (29). However, uncertainty persists around patient selection and optimal timing of both fibrinolytics and surgical intervention given the difficulty of predicting the course of pleural infection at baseline. Given its ability to predict the development of complicated effusions, suPAR may be an opportunity to use biomarkers in lung precision medicine to identify which patients are likely to require admission for drainage and early rescue treatments, addressing a “specific clinical unmet need” (30).

The higher levels of pleural suPAR in loculated effusions of a malignant aetiology compared to non-loculated suggest that malignant locule development follows a similar fibrinogenesis cascade. Levels were lower in loculated malignant effusions compared to loculated parapneumonic effusions suggesting the process is more subacute in malignancy. However, there were several cases where malignant effusions had pleural suPAR levels similar to loculated parapneumonic effusions limiting its utility as a diagnostic test in the sometimes-challenging clinical situation of distinguishing an advanced malignant effusion from infection. We also tested the ability of pleural suPAR to predict the development of loculations within malignant effusions that were simple at baseline. Levels were non-significantly higher in the delayed loculation group and given the small numbers involved this relationship needs further investigation.

This study has some limitations that may affect the generalisability of its findings. Although suPAR levels were done en-bloc and therefore researchers were blind to the results, the other biochemical results (pH,

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LDH, glucose) were part of clinical care so would have affected a physician's management. This is a weakness of all research that has attempted to study the true utility of pleural pH and may actually strengthen the conclusions of the suPAR results. The decision to insert a chest tube is influenced by many different biochemical and radiological factors and may vary according to the treating physician. Despite this, pleural suPAR was the most accurate baseline variable (including all biochemical and radiological markers), at identifying patients who went on to have a chest tube or rescue therapies. Secondly, some of the clinical outcomes such as fibrinolytic use and surgical referral may have been confounded by other factors not related to the pleural infection alone. The recently presented PILOT study has demonstrated that a significant proportion of patients with the most serious pleural infection do not go on to have surgery due to frailty and/or comorbidity (31). Additionally, the routine use of the fibrinolytic agents tPA/DNase was not adopted for several years into this study's recruitment. Both factors may explain why many patients with a high pleural suPAR did not have fibrinolytics or surgery, although the biomarker was highly specific for these rescue therapies. Thirdly, suPAR levels were measured on clinical samples that had been frozen for up to 10 years (median 6 yrs). However, serum suPAR levels have been shown to be resistant to up to 8 freeze-thaw cycles and stable over a 5 year period limiting the impact on this analysis (32, 33). Finally, the urgent nature of pleural infection treatment means that any biomarker should be able to be analysed rapidly. This study used the commercial suPARnostic® ELISA which would not fulfil this requirement. However, more rapid analytical platforms are available including a suPARnostic® Quick Triage point of care device or turbidimetric assay (suPARnostic® TurbiLatex).

In conclusion, the management of parapneumonic effusions has been dictated by crude measures of inflammation and bacterial replication for decades. The urokinase-type plasminogen activator system plays a key role in the development of pleural loculations and is a theoretically promising target of study. This prospective cohort study demonstrated that high pleural fluid suPAR levels are strongly correlated with the development of loculations in parapneumonic effusions as well as subsequent invasive

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management including chest tube drainage, fibrinolytics and thoracic surgery. A comprehensive assessment of the utility of pleural suPAR in parapneumonic effusions requires a prospective multicenter trial of suPAR guided management versus standard care.

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**FIGURE LEGENDS**

**Figure 1. Pleural fluid pH against pleural suPAR by fluid loculation (intercepts at pH = 7.2 and suPAR 35ng/ml).**

**Figure 2. ROC curves of pleural markers to predict insertion of a chest tube, plus boxplot of pleural suPAR and insertion of chest tube.**

**Figure 3. ROC curves of conventional pleural biomarkers combined (pH, glucose, LDH) and the additional benefit of pleural suPAR at predicting the use of fibrinolytics/surgery, plus boxplot of pleural suPAR and use of fibrinolytics/surgery.**

**Figure 4. Boxplot of pleural suPAR levels in malignant effusions.**

**Figure 5. The biology of suPAR and the urokinase-type plasminogen activator system.**

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**TABLES**

**Table 1. Patient demographics, baseline biochemistry and pleural suPAR levels**

| | **Parapneumonic N=93** | **Malignant N=31** | **Transudative N=16** |
|---|---|---|---|
| Age, median (IQR) | 66 (46-78) | 68 (61-79) | 74 (62-86) |
| Male/Female (%) | 57/36 (61/39) | 19/12 (61/39) | 10/6 (63/37) |
| *Serum, median (IQR)* | | | |
| Neutrophils (x$10^9$/L) | 8.50 (6.45-12.09) | 6.0 (4.46-6.94) | 4.41 (2.88-5.69) |
| CRP (mg/L) | 119.0 (56.5-210.9) | 29.0 (5.9-72.4) | 20.5 (8.3-55.2) |
| *Pleural fluid, median (IQR)* | | | |
| pH | 7.32 (7.06-7.41) | 7.41 (7.32-7.47) | 7.53 (7.43-7.71) |
| Protein (g/L) | 44 (36-51) | 45 (32-50) | 20 (13-27) |
| LDH (IU/L) | 679 (432-1493) | 476 (309-768) | 176 (137-217) |
| Glucose (mmol/L) | 5.3 (3.5-6.5) | 5.5 (3.3-6.7) | 7.3 (6.4) |
| Pleural suPAR (ng/ml) | 36.9 (20.2-124.1) | 15.0 (9.4-26.7) | 12.0 (8.2-13.8) |
| [range] | [9.1-644] | [3.0-68.0] | [8.2-18.3] |

IQR- Interquartile range, CRP- C reactive protein, LDH- Lactate dehydrogenase, suPAR- soluble urokinase Plasminogen Activator Receptor.

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**Table 2. Loculated versus non-loculated parapneumonic effusions and biochemical markers.**

| | **Non-loculated (n-49)** | **Loculated (n- 44)** | **p- value (univariable analysis)** |
|---|---|---|---|
| **Median (IQR)** | | | |
| Pleural pH | 7.4 (7.28-7.44) | 7.14 (6.88-7.33) | <0.01 |
| Pleural Protein (g/L) | 45 (38-51) | 40.0 (34.3-50.0) | 0.98 |
| Pleural LDH (IU/L) | 516 (330-747) | 1276 (657-2794) | <0.01 |
| Pleural Glucose (mmol/L) | 5.7 (4.95-6.90) | 3.45 (0.2-5.3) | <0.01 |
| Pleural suPAR (ng/ml) | 22.3 (14.0-28.1) | 132.2 (52.3-229.2) | <0.01* |
| [range] | [9.1-42.3] | [36.9-614.0] | |
| Serum Neutrophils ($x10^9$/L) | 7.00 (5.51-10.32) | 10.1 (7.56-13.77) | <0.01 |
| Serum CRP (mg/L) | 96.3 (46.0-150.3) | 139.1 (75.1-247.2) | 0.01 |
| Serum suPAR (ng/ml) | 4.64 (3.66-6.41) | 6.12 (3.95-7.96) | 0.22 |
| [range] | [2.02-16.90] | [1.94-20.9] | |

LDH- Lactate Dehydrogenase, suPAR- soluble urokinase Plasminogen Activator Receptor, CRP- C reactive protein.

*Significant on multivariable analysis, see Appendix 4.

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**Table 3. Median pleural suPAR and conventional biomarker levels by clinical outcomes**

| **Median biomarker levels (IQR)** | **Conservative management (n=39)** | **Chest Tube (n=54)** | **Fibrinolytics and/or surgery (n=16)** |
|---|---|---|---|
| Pleural pH | 7.40 | 7.14 | 6.93 |
| (IQR) | (7.35- 7.47) | (6.89-7.35) | (6.80-7.29) |
| Pleural LDH (IU/L) | 451 | 1004 | 1119 |
| (IQR) | (317-906) | (565-2645) | (203-4657) |
| Pleural Glucose (mmol/L) | 6.2 | 4.1 | 0.6 |
| (IQR) | (5.0-7.1) | (0.3-5.6) | (0.2-5.4) |
| Pleural suPAR (ng/ml) | 19.7 | 65.9 | 218.7 |
| (IQR) | (13.3- 27.9) | (38.4-218.3) | (141.8-312.1) |

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**Table 4. Pleural pH and suPAR levels in malignant effusions**

| | **Non-loculated** **n- 12** | **Delayed loculation** **n- 9** | **Loculated** **n- 10** |
|---|---|---|---|
| *Median (IQR)* | | | |
| Pleural pH | 7.46 (7.43-7.50) | 7.39 (7.32-7.44) | 7.33 (7.18-7.53) |
| Pleural suPAR (ng/ml) | 10.7 (7.3-14.0) | 17.4 (12.3-25.2) | 36.5 (21.9-51.3) |

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**Figure 1. Pleural fluid pH against pleural suPAR by fluid loculation (intercepts at pH = 7.2 and suPAR 35ng/ml).**

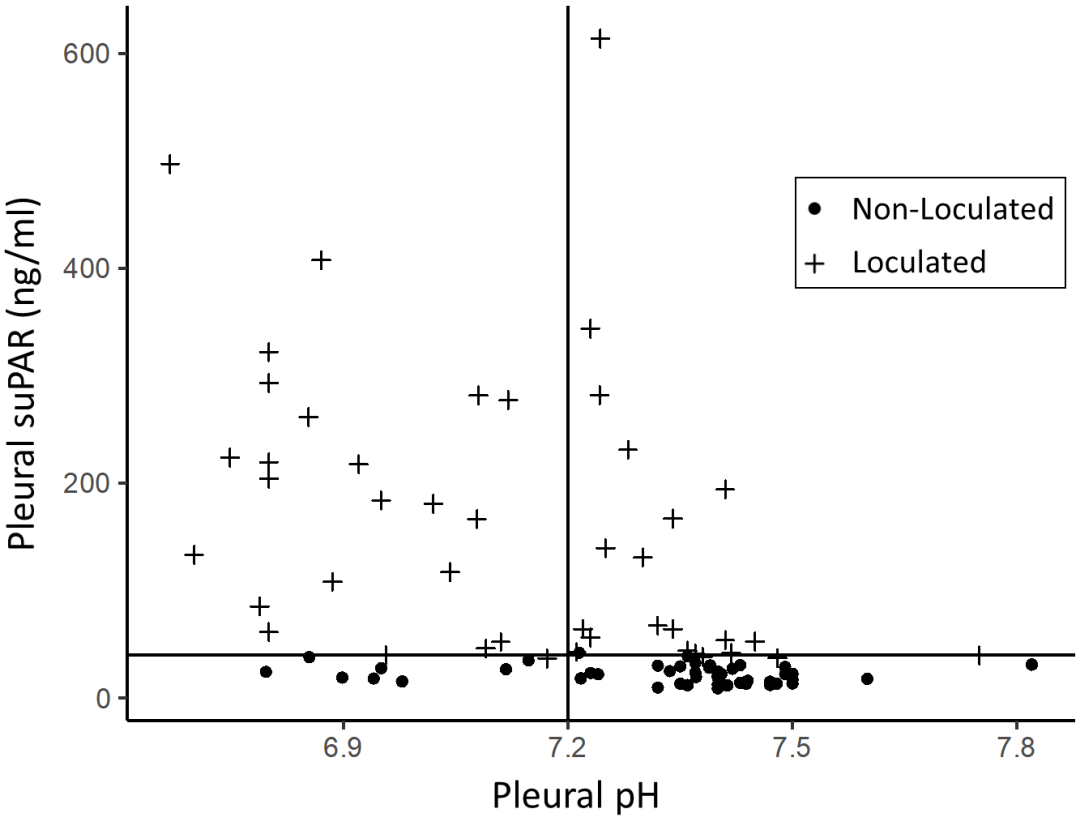


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**Figure 2. ROC curves of pleural markers to predict insertion of a chest tube, plus boxplot of pleural suPAR and insertion of chest tube.**

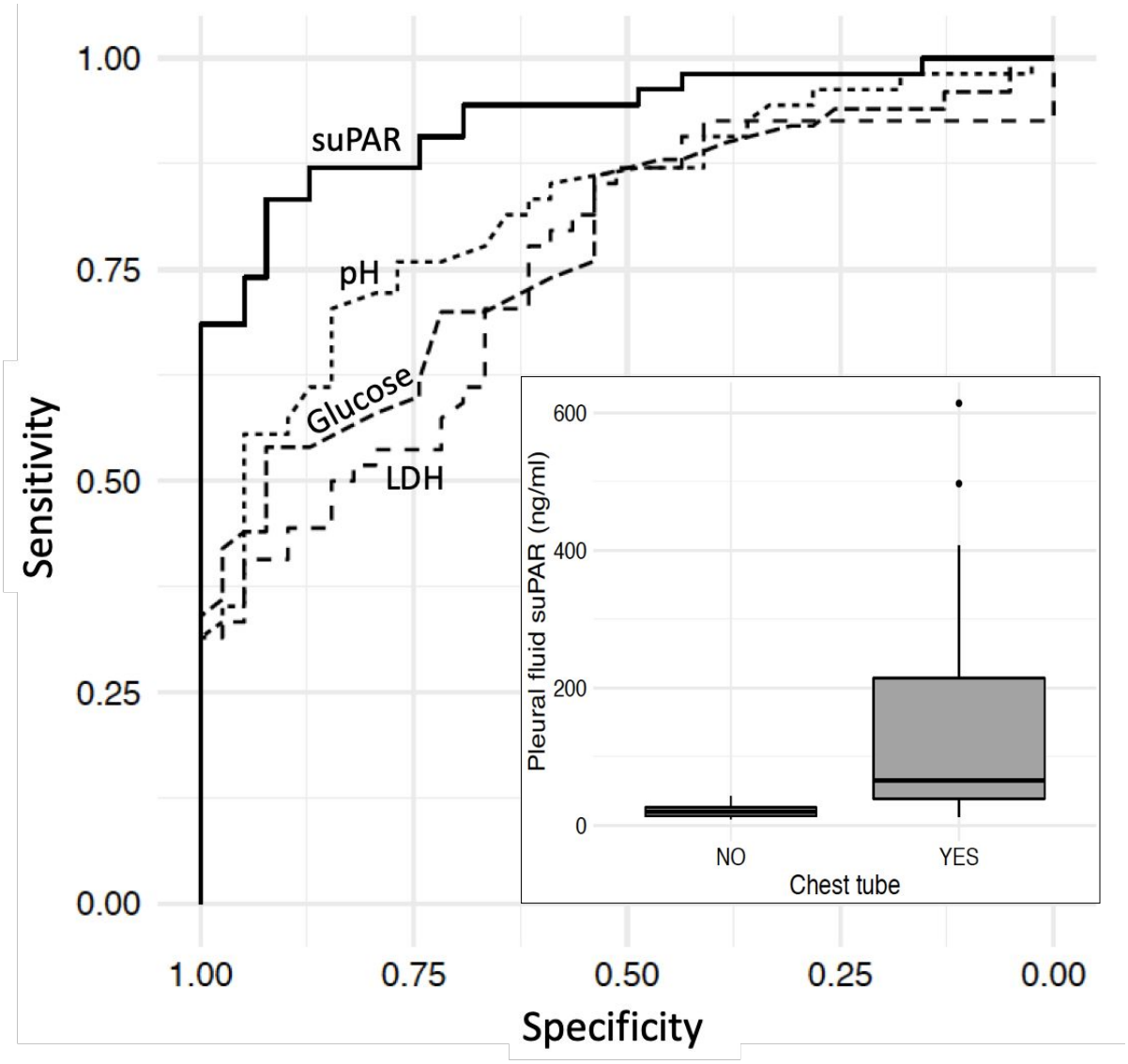


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**Figure 3. ROC curves of conventional pleural biomarkers combined (pH, glucose, LDH) and the additional benefit of pleural suPAR at predicting the use of fibrinolytics/surgery, plus boxplot of pleural suPAR and use of fibrinolytics/surgery.**

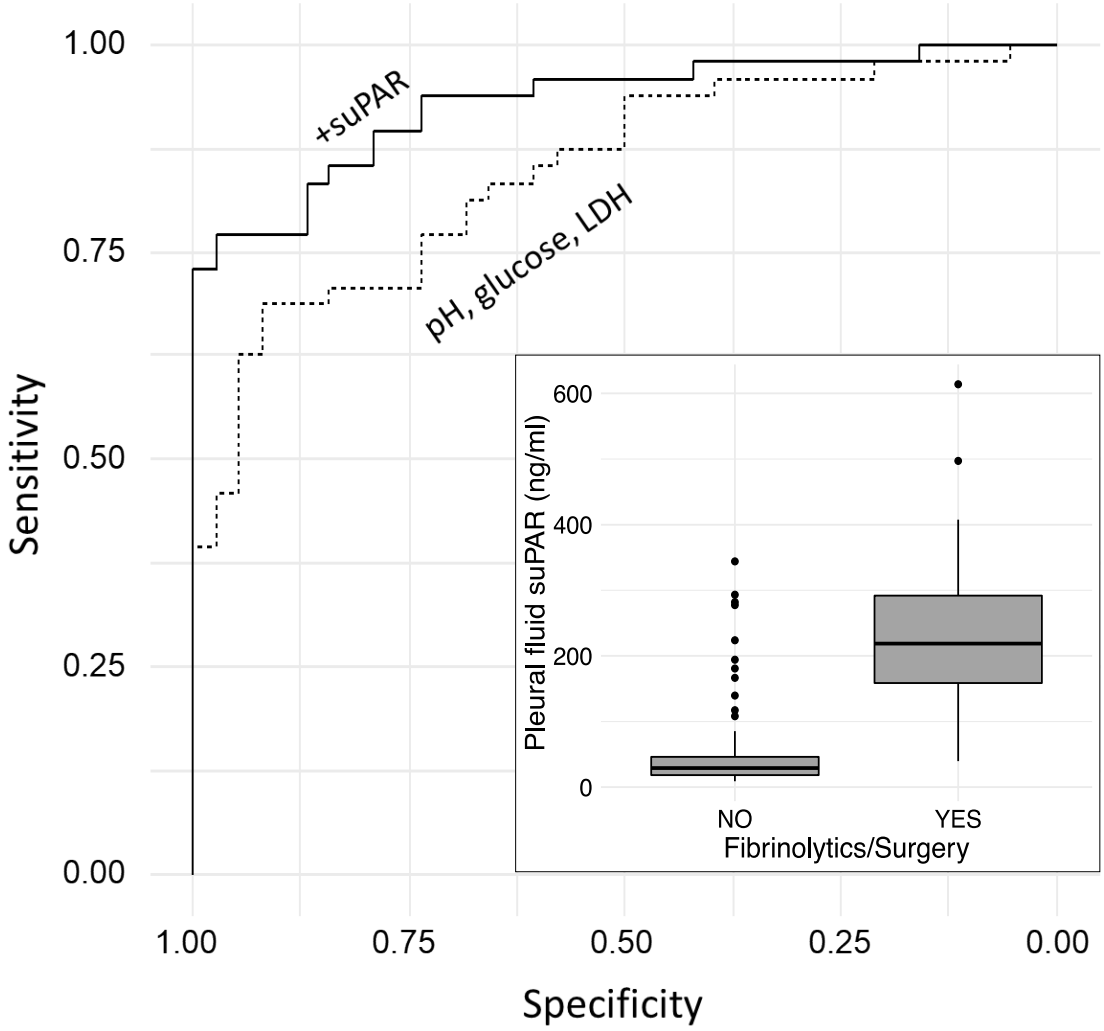


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**Figure 4. Boxplot of pleural suPAR levels in malignant effusions**

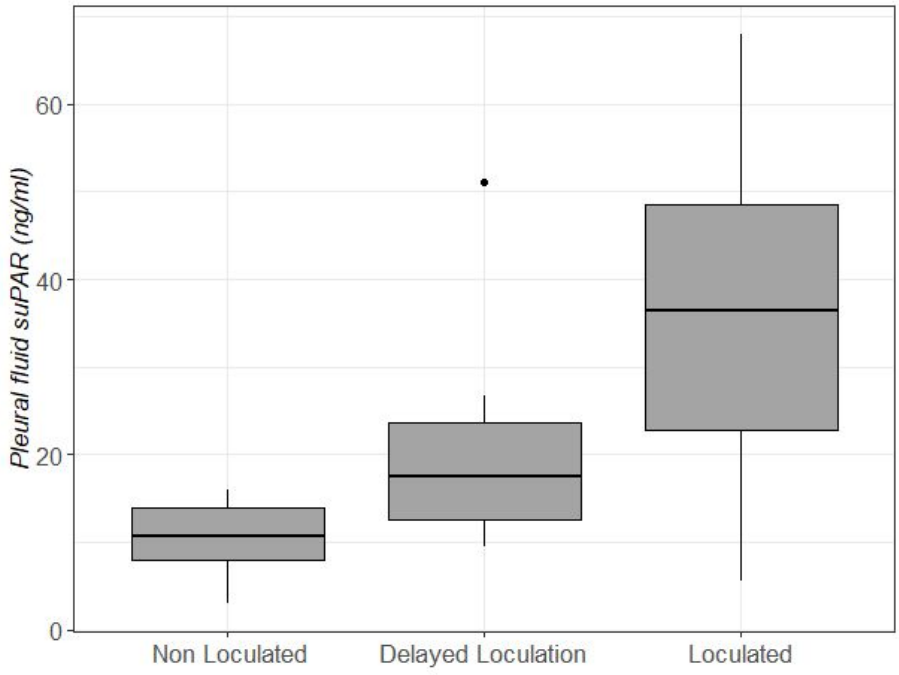


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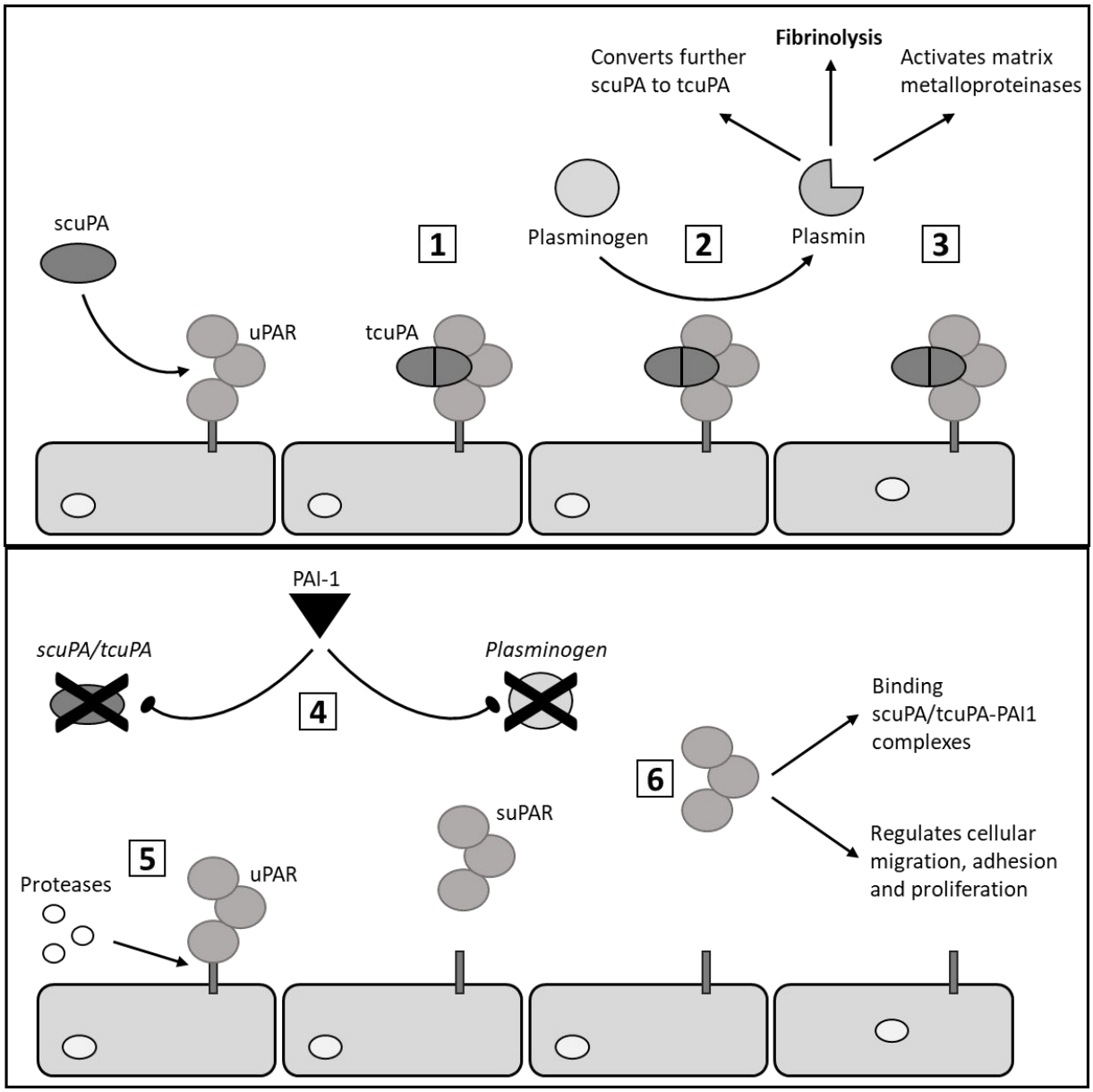
**Figure 5. The biology of suPAR and the urokinase-type plasminogen activator system.**




**Normal physiology**

1.Endogenous single chain urokinase (scuPA) is converted to the more active two chain uPA (tcuPA) by binding to cell bound uPA receptor (uPAR)

2. tcuPA readily converts plasminogen to plasmin

3. Plasmin promotes fibrinolysis, activates matrix metalloproteinases and converts further scuPA to tcuPA.

**Pathological state**

4. In response to proinflammatory stimuli, various cytokines, other mediators and plasminogen activator inhibitor 1 (PAI-1) are upregulated. PAI-1 inhibits scuPA bound to uPAR or suPAR, tcuPA and tissue plasminogen activator (not shown) thereby decreasing local fibrinolysis.

5. Inflammatory proteases cleave the glycosyl phosphatidylinositol (GPI) anchor to generate soluble uPAR (suPAR).

6. suPAR exerts several functions including binding of scuPA or tcuPA or their complexes with PAI-1, regulation of cellular migration, adhesion and proliferation.

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**Online Data Supplement**

**Appendix 1; Standardised Diagnostic criteria for pleural effusions (North Bristol Pleural Investigation Study (08/H0102/11)). Online Data Supplement.**

***Malignant***

Malignant pleural fluid cytology or biopsy

or

Histologically confirmed pulmonary/extra-thoracic malignancy with radiographic evidence of metastasis to ipsilateral pleura on CT.

or

Radiological changes meeting Leung's criteria which have progressed in keeping with malignancy on interval CT scan in the correct clinical context (1).

or

Autopsy confirming pleural malignancy

***Empyema***

Clinical presentation suggestive of sepsis/infection

And (one or more of the following)

a/. Pleural fluid gram stain or culture positive

or

b/. Frank pus on pleural aspiration

***Complicated parapneumonic effusion (CPE)***

Clinical presentation suggestive of sepsis/infection (and follow up for at least 6 months inconsistent with pleural malignancy)

And (one or more of the following)

a/. Pleural fluid pH ≤7.2

or

b/. Pleural fluid glucose $\leqslant$ 3.0 mmol/L

or

b/. Pleural fluid LDH > 1000 IU/L (upper limit of serum LDH is 480 U/L)

***Simple Parapneumonic effusion (SPE)***

Clinical presentation suggestive of sepsis/infection with appropriate chest radiology and pleural fluid which does not fulfil the criteria for empyema or CPE (above)

And

Resolution of effusion on CXR after antibiotics

***Connective tissue disease (including RA)***

Systemic features or known diagnosis of connective tissue disease

And

chest radiology (including CT imaging) showing benign features (eg doesn't meet any of Leung's criteria) with at least 6 months follow-up and /or pleural biopsy negative for malignancy.

***Pulmonary embolism***

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Evidence of PE on CTPA

And

No alternative explanation for pleural effusion on cross sectional imaging or pleural fluid analysis. (where the CT shows no evidence of pleural thickening – which would suggest another cause)

***BAPE or diffuse pleural thickening due to asbestos***

History of asbestos exposure or evidence of pleural plaques on CT

And

a/. Stable or improving CT appearances with follow-up for at least 12 months.( The development of enfolded lung is allowed)

or

b/. Negative thoracoscopy (benign pleural biopsy)

***Congestive Cardiac Failure (CCF)***

History and examination features of CCF

or

Evidence of at least moderate LV systolic or diastolic failure or severe valvular disease on echo

or

Improvement of effusion and symptoms with diuretic therapy

***Coronary artery bypass graft (CABG) effusion***

CABG in 3 months prior to development of pleural effusion in the absence of an alternative cause

***Hepatic hydrothorax***

Known history or clinical presentation consistent with liver disease

And

Recurrent transudative pleural effusion

And

Negative cytology

***Renal failure or hypoalbuminaemia***

Biochemical confirmation of renal failure or hypoalbuminaemia in the absence of clinical, radiological or pleural fluid analysis suspicious of an alternative cause.

***TB pleuritis***

Culture or Acid-alcohol-fast-bacilli (AAFB) positive sputum, pleural fluid or pleural tissue

And

Resolution of pleural effusion with anti TB therapy at 6 month follow-up.

***Inflammatory pleuritis (Non-specific pleuritis)***

Demonstration of non-specific inflammatory pleuritis on pleural biopsy

And

Presentation not in keeping with parapneumonic effusion (see above)

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And

Follow-up for 12 months without progression that would suggest a malignant cause.

**Undiagnosed**

Exhaustive investigations including 12 months follow-up with interval CT scans has not demonstrated a diagnosis

or

Patient unfit for further investigation and follow up

or

Patient died without definitive diagnosis and no post mortem examination conducted

**Reference**

1. Leung, A.N., N.L. Muller, and R.R. Miller, CT in differential diagnosis of diffuse pleural disease. AJR Am J Roentgenol, 1990. 154(3): p. 487-92.

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## Appendix 2. Parapneumonic effusion management

### *Indication for chest tube insertion for parapneumonic effusions*

Chest tube insertion should be considered for the following indications;

Clinical presentation consistent with parapneumonic effusion AND fulfilling at least one of the following criteria;

- Purulent pleural fluid
- Pleural fluid pH ≤ 7.2
- Pleural fluid glucose ≤ 3.0 mmol/L
- Pleural fluid lactate dehydrogenase (LDH) > 1000 IU/L
- Pleural fluid gram stain and/or culture positive for bacteria

If, despite not meeting the above criteria, the patient fails to respond to initial medical therapy with ongoing markers of infection/sepsis then chest tube drainage should be reconsidered.

### *Indication for intrapleural fibrinolytics*

Intrapleural fibrinolytics (tPA 5mg and DNase 5mg delivered via the chest tube) should be considered after a minimum of 24-48hours chest tube drainage.

Assuming no contraindications to treatment, intrapleural fibrinolytics should be considered when:

- Thoracic ultrasound imaging has revealed pleural fluid septation or loculation which is felt to be clinically important
- The chest tube has stopped draining but there remains a clinically important residual effusion on chest radiograph/CT scan/pleural ultrasound
- The patient continues to show clinical or biochemical signs of infection despite good chest tube drainage and appropriate antibiotic therapy

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***Indication for thoracic surgical referral***

Referral to the thoracic surgeons, if appropriate, should be considered if:

- intrapleural fibrinolytics have failed to result in clinical improvement
- intrapleural fibrinolytics are contraindicated in the context of failed chest tube drainage (above criteria)
- the degree of fluid complexity precludes the use of chest drainage or fibrinolytic therapy

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**Appendix 3. Sample handing protocol and additional experiments on the effect of sample collection tubes and centrifugation.**

***Sample handling protocol for main analysis***

*Pleural fluid samples*

- Pleural fluid collected at diagnostic thoracentesis into plain (non-heparinized/non-citrated) collection tubes
- Pleural fluid centrifuged for 20minutes at 1000G
- Supernatant pipetted into 1.5ml Eppendorf tubes (pellet discarded)
- Samples stored at -70oC for future analysis or immediately processed.

*Blood samples*

- Blood samples collected by venepuncture at the time of diagnostic thoracentesis into serum separator gel bottle (yellow-top).
- Blood sample left to rest upright for 30 mins
- Blood sample centrifuged for 20minutes at 1000G
- Following centrifugation, the liquid supernatant (serum) is transferred into 1.5ml Eppendorf tubes
- Samples stored at -70oC for future analysis or immediately processed.

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***Impact of collection tube type on suPAR results***

A subset of 8 patients had pleural fluid and blood collected using plain (gold top serum separator tubes for blood), sodium-citrate (blue) and EDTA (lavender) tubes.

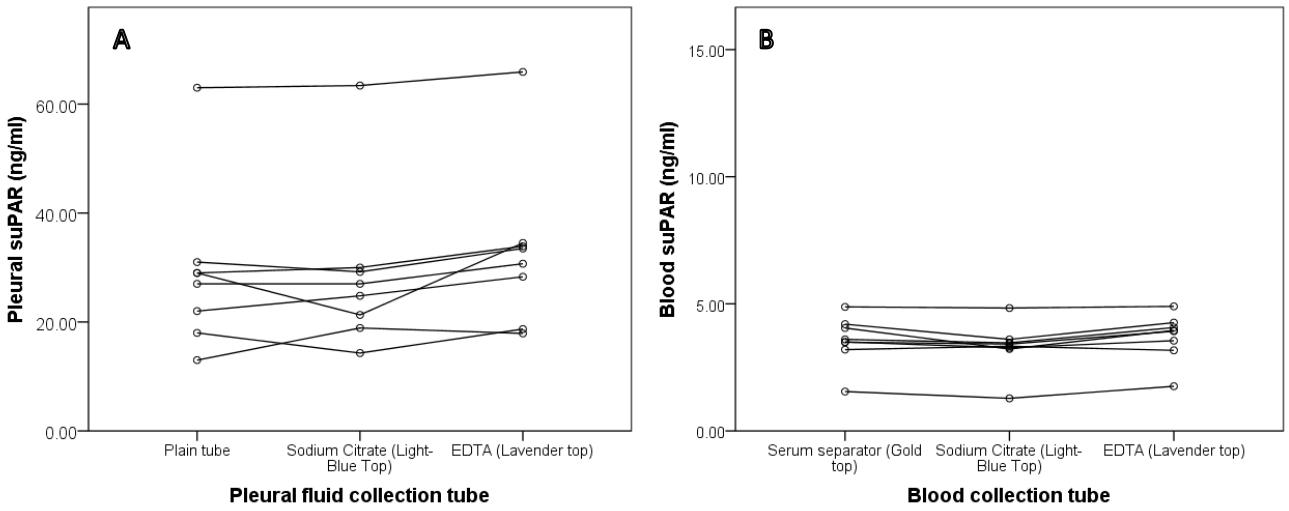
Blood samples were handled as per tube manufacturers protocol;

https://www.thermofisher.com/uk/en/home/references/protocols/cell-and-tissue-analysis/elisa-protocol/elisa-sample-preparation-protocols/plasma-and-serum-preparation.html

Pleural fluid samples were spun at 1000G for 20minutes as per our protocol shown above and the supernatant collected and frozen at -70oc prior to *en-bloc* analysis.

For both pleural fluid and blood samples suPAR levels were analysed using the suPARnostic ELISA in duplicate.

*Figure A&B; Line graphs of suPAR level depending on sample collection tube. A-Pleural fluid, B- Blood*




For pleural fluid there was slight variation in pleural suPAR levels with a slight increase in levels using EDTA tubes.

For blood samples there was no significant variation in suPAR levels depending on the collection tube type, see Figure B.

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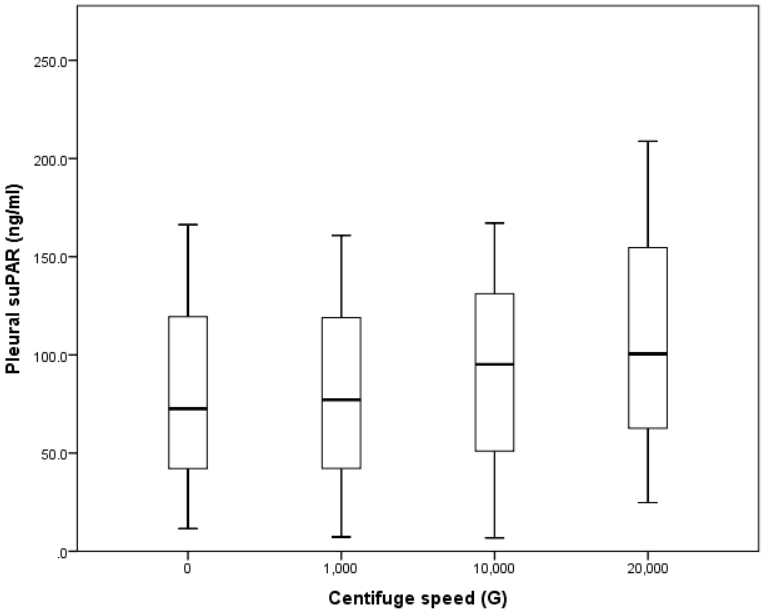
***Impact of centrifuge speed on pleural suPAR results***

To test the impact of varying centrifuge speed on pleural suPAR levels, 3 patients with parapneumonic effusions had pleural fluid collected at diagnosis. Pleural fluid was collectd in plain tubes (as per the protocol for the main analysis) and centrifuged at 3 different speeds for 20 minutes before analysis (as well as a tube that was not spun at all). The supernatant was collected and anlaysed using the suPARnostic ELISA in duplicate.

There was no significant difference between the samples but there was a trend towards increasing suPAR levels as the centriuge spin was increased, see Figure C.

*Figure C; Boxplot of pleural suPAR levels for 3 patients depending on centrifuge speed at sample collection.*

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## Appendix 4. Multivariable binomial logistic regression tables

### *Baseline predictors of parapneumonic effusion loculation development (n=93)*

| Factor | Regression Estimate | Std. Error | z value | p-value |
|---|---|---|---|---|
| Pleural suPAR | 0.5688 | 0.2763 | 2.059 | 0.0395 |
| Pleural Glucose | -0.0531 | 0.4815 | -0.110 | 0.9122 |
| Pleural protein | -0.2707 | 0.2093 | -1.293 | 0.1959 |
| Pleural LDH | -0.0001 | 0.0016 | -0.076 | 0.9397 |
| Pleural pH | 1.9691 | 4.3523 | 0.453 | 0.6509 |
| Serum Neutrophils | -0.3383 | 0.5496 | -0.616 | 0.5381 |
| Serum CRP | 0.0022 | 0.0165 | 0.131 | 0.8956 |
| Serum suPAR | 0.2745 | 0.2894 | 0.949 | 0.3427 |
| suPAR- soluble urokinase Plasminogen Activator Receptor, LDH- lactate dehydrogenase, CRP- C reactive protein | | | | |

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***Baseline predictors of chest tube insertion in parapneumonic effusions (n=93)***

| **Factor** | **Regression Estimate** | **Std. Error** | **z value** | **p-value** |
|---|---|---|---|---|
| Pleural pH | -6.4282 | 2.6101 | -2.463 | 0.0138 |
| Pleural Protein | 0.04990 | 0.0600 | 0.831 | 0.4061 |
| Pleural LDH | -0.0025 | 0.0012 | -2.005 | 0.0450 |
| Pleural Glucose | -0.1338 | 0.1888 | -0.709 | 0.4786 |
| Neutrophilic effusion* | 4.3911 | 2.2373 | 1.963 | 0.0497 |
| Pleural suPAR | 0.2965 | 0.1214 | 2.442 | 0.0146 |
| Serum neutrophils | -0.2042 | 0.1768 | -1.155 | 0.2482 |
| Serum CRP | 0.0121 | 0.0076 | 1.581 | 0.1139 |
| Serum suPAR | 0.2023 | 0.2743 | 0.737 | 0.4609 |
| Effusion size on chest radiograph (over 50% of hemithorax) | 2.3041 | 1.4473 | 1.592 | 0.1114 |
| Loculation on baseline ultrasound | 2.7099 | 2.0081 | 1.349 | 0.1772 |
| suPAR- soluble urokinase Plasminogen Activator Receptor, LDH- lactate dehydrogenase, CRP- C reactive protein, * Defined as >50% neutrophils on pleural differential cell count | | | | |

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***Baseline predictors of rescue therapy (fibrinolytics or surgery) in parapneumonic effusions (n=93)***

| **Factor** | **Regression Estimate** | **Std. Error** | **z value** | **p-value** |
|---|---|---|---|---|
| Pleural pH | -1.2451 | 3.1952 | -0.390 | 0.6968 |
| Pleural Protein | 0.1028 | 0.0669 | 1.537 | 0.1243 |
| Pleural LDH | 0.0001 | 0.0001 | 0.449 | 0.6537 |
| Pleural Glucose | -0.1338 | 0.2618 | -0.511 | 0.6094 |
| Neutrophilic effusion* | -4.0691 | -2.4064 | -1.691 | 0.0908 |
| Pleural suPAR | 0.0157 | 0.0062 | 2.540 | 0.0111 |
| Serum neutrophils (x10^9) | 0.0230 | 0.0796 | 0.289 | 0.7727 |
| Serum CRP | 0.0074 | 0.0075 | 0.979 | 0.3278 |
| Serum suPAR | 0.1810 | 0.1958 | 0.924 | 0.3552 |
| Effusion size on chest radiograph (over 50% of hemithorax) | 0.5352 | 1.0530 | 0.508 | 0.6113 |
| Loculation on baseline ultrasound | 2.7061 | 1.7652 | 1.533 | 0.1252 |
| suPAR- soluble urokinase Plasminogen Activator Receptor, LDH- lactate dehydrogenase, CRP- C reactive protein, * Defined as >50% neutrophils on pleural differential cell count | | | | |

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