



ORIGINAL ARTICLE

# The value of total protein in guiding management of infectious parapneumonic effusion by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry



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## KEYWORDS

Biomarkers;  
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*Background/Purpose:* Infectious parapneumonic effusion (PE) contains proteins originating from circulation as well as proteins locally released by inflammatory pulmonary cells. The purpose of this study was to investigate the value of total protein analysis in guiding management of infectious PE by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

*Methods:* Fifty-seven children with pneumonia followed by PE were consecutively enrolled into our study. Protein profiles generated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry after fractionating samples with functionalized magnetic

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beads (C8) were used for differentiating complicated PE (CPE) from non-CPE. A training set was used to generate classification models and the clinical efficacy of these models in detecting CPE and the need for intervention was then evaluated in an independent set.

**Results:** The MS spectra derived from PE were analyzed, and classification models were constructed in the training set. A total of 123 mass/charge ( $m/z$ ) values were identified and 23  $m/z$  values which were significant with  $p < 0.05$  were used as classifiers. An optimized genetic algorithm model containing enforced selection of three significant downregulated  $m/z$  values (2127, 2232, and 2427) was able to classify CPE with 100% positive predictive value and predict the need of aggressive therapeutic intervention with 77% positive predictive value.

**Conclusion:** A diagnostic model construction comprising three potential biomarkers can predict CPE and need for surgical intervention rapidly and precisely. Pleural fluid proteins downregulated during the progression of pneumonia could potentially guide the management of infectious PE.

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## Introduction

Community-acquired pneumonia is a common infectious illness in children and complicated parapneumonic effusion (CPE) is a well-recognized complication of bacterial pneumonia.<sup>1</sup> Failing adequate therapy to control the pleural inflammation may lead to progressive pleural thickening and fibrin deposition, resulting in organizing empyema. The therapeutic strategies for parapneumonic effusion (PE) vary from a conservative approach with adequate antibiotic therapy, expedient drainage including thoracentesis, tube thoracostomy, or surgical drainage.<sup>2</sup> However, the optimal time of intervention for PE and empyema in pediatric patients remains a challenge in clinical practice.

Infectious pleural fluid accumulation is posited to be a continuing process of pleural inflammation and is mainly a result of inflammatory response caused by pneumonia. Clinically, biochemical analysis of pleural effusion plays an important role in the management of pleural effusions.<sup>3</sup> In general practice, pleural infection is indicated by acidosis associated with raised lactate dehydrogenase (LDH) and low glucose levels. The indicators of  $\text{pH} \leq 7.2$ ,  $\text{LDH} \geq 1000 \text{ U/L}$  and  $\text{glucose} \leq 40 \text{ mg/dL}$  are characteristics of CPE that are more likely to require aggressive interventions.<sup>3,4</sup> Despite this, several inflammatory mediators such as tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , and interleukin-6 have been reported to have a significant role in pyogenic infections in the pleural space.<sup>5–7</sup> A recent study has also shown that not only pleural fluid proinflammatory cytokines but also anti-inflammatory cytokines are accurate in discriminating complicated effusions.<sup>8</sup> It is therefore believed that proteins in pleural fluids could potentially be useful as markers of CPE for guiding clinical management.

Use of proteomics techniques to identify disease-specific protein biomarkers is a powerful tool for defining prognosis of disease.<sup>9,10</sup> The discovery of biomarkers, proteins that change in concentration or state in association with a specific biological process can help to gain deep insights into disease mechanisms in which proteins play a major role. Several studies have shown that pleural effusion contains proteins originating from circulation as well as proteins locally released by inflammatory or epithelial

cells.<sup>11</sup> The pleural exudates from a patient with severe pneumonia have been investigated and contain proteins of potential diagnostic and therapeutic value.<sup>12</sup> A systematic identification of the dynamic changes of proteins involved in inflammation in the pleural cavity, however, has not been well clarified. An improved understanding in the pattern recognition of proteins in pleural fluids is potentially able to classify CPE for clinical management. The aim of this study was to investigate the proteomics profiling data of infectious parapneumonic effusions obtained using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), hopefully to obtain some potential diagnostic value and even guide the timing of surgical intervention.

## Materials and methods

### Patients and supernatant specimens of pleural fluid

The study population consisted of patients aged  $<18$  years who had community-acquired pneumonia requiring hospitalization in Chang Gung Children's Hospital (Taoyuan, Taiwan) between January 2005 and January 2007. Patients with PE were consecutively enrolled into our study and all underwent real-time chest sonography and thoracentesis irrespective of size of pleural effusion. This study was approved by the Ethic Committee of Chang Gung Memory Hospital. Informed written consent was obtained from the parents of all study participants.

Pleural fluid was collected using a standard thoracentesis technique after chest sonography prior to intervention procedures. Pleural fluid was immediately analyzed for pH, total cell counts, and differential cell count, and for protein, glucose, and LDH concentrations. Pleural fluid for pH analysis was collected anaerobically with heparin and measured in a handheld analyzer (i-STATA Portable Clinical Analyzer; i-STATA Corporation; East Windsor, NJ, USA). A 4- $\mu\text{L}$  sample of specimen was mixed with 3.2% sodium citrate solution in a ratio of 9:1 pleural fluid to citrate, which were immediately immersed in ice separately and centrifuged at 1500g for 10 minutes. The cell-free supernatant from each sample was stored

at  $-70^{\circ}\text{C}$  after centrifugation for further MALDI-TOF MS analysis (Bruker Daltonics, Bremen, Germany).

Patients were classified into two groups, either with CPE or non-CPE, based on the biochemical characteristics of the fluid. Patients with CPE were diagnosed if the fluid met at least two of the following criteria: a pleural fluid pH of  $\leq 7.2$ , a LDH level of  $\geq 1000$  U/L, and a glucose level of  $\leq 40$  mg/dL. Otherwise, patients with non-CPE were defined as those whose pleural fluid met only one or none of the above criteria. In clinical practice, antibiotic selection covered the likeliest organisms and was adjusted after culture results were obtained. Intervention procedures for intercostal drainage including tube thoracostomy or video-assisted thoracic surgery were considered if patients had persistent fever  $>39^{\circ}\text{C}$ , dyspnea, and sepsis despite appropriate antibiotic therapy. *Streptococcus pneumoniae* infection was defined by a positive result in blood or pleural fluid culture or the detection of antigens in the pleural fluid by latex agglutination testing. Acute pneumococcal infection was also included for patients who had necrotic lung parenchyma with a positive urine test for *S. pneumoniae* (Binax, Portland, ME, USA).

### Sample preparation using hydrophobic magnetic beads

For protein/peptide purification, each sample was prepared automatically using a robot for magnetic beads assisted extraction (ClinProt; Bruker Daltonics). The robotic workflows are virtually identical to the workflows performed manually for preparing serum samples,<sup>13</sup> apart from the inherent differences due to the switch-over to automation (simultaneous spotting, marginally shorter time frames, etc.). Three purification kits characterized by hydrophobic interaction chromatography (HIC) including C3, C8, and C18, were tested to identify the best functionalized magnetic beads (MB), which can obtain abundant protein spectra. Briefly, 10  $\mu\text{L}$  of pleural effusion were mixed well with 5  $\mu\text{L}$  of magnetic beads and 15  $\mu\text{L}$  of MB-HIC binding buffer. Magnetic beads bound by interacted proteins were then separated using a magnetic bead separator and were washed for 20 seconds three times in 100  $\mu\text{L}$  of MB-HIC washing solution. Bound peptides or proteins were eluted with 10  $\mu\text{L}$  eluting buffer containing 50% acetonitrile and prepared for mass spectrum by MALDI-TOF MS.

### Mass spectrometry

A ground steel plate (Bruker Daltonics) was used for extracted sample spotting. One  $\mu\text{L}$  of each eluted sample was diluted in 9  $\mu\text{L}$  matrix solution containing  $\alpha$ -cyano-4-hydroxycinnamic acid (7 mg/mL) in 70% acetonitrile with 0.1% trifluoroacetic acid and then spotted 1  $\mu\text{L}$  to each well on the ground steel plate. Four analytical replicates were prepared and spotted onto the MALDI target. All preparations were performed at room temperature.

Positive ion mass spectra were obtained on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics) with linear mode after the sample was dried at room temperature. A 337-nm nitrogen laser irradiated and ionized the samples at a shot rate of 25 Hz. Each spectrum had a summation of

1500 laser shots with a mass range of 1000–25,000 Da, which was controlled by FlexControl acquisition software (Version 3.0; Bruker Daltonics). Mass calibration was achieved using a mixture of standard peptides and proteins (angiotensin II, ACTH, insulin, and myoglobin) to maintain mass accuracy at  $>500$  ppm.

### Data analysis

To analyze the mass spectra patterns of pleural fluids, ClinProTools software (version 2.0.365, Bruker Daltonics) was firstly employed for peak definition (signal/noise  $> 3$ ), integration (end-point level), mass recalibration (0.2 % maximal peak shift), area normalization (against total ion count), and statistical analysis (Wilcoxon/Kruskal–Wallis test). Each corresponding peak throughout the spectra within each studied set was carefully inspected.

For rapid classification, the quick classifier (QC), support vector machine, and genetic algorithm (GA) methods embedded in the ClinProTools software (Bruker Daltonics) were implemented for model generation. To reduce classifier complexity, mass/charge ( $m/z$ ) values with  $p < 0.05$  (Wilcoxon/Kruskal–Wallis test) were selected for model generation across all experiments. In GA classification, the K-nearest neighbor text categorization method was used to select classifiers and validate their recognition rates by the leave-one-out method for cross validation. The performance of the classification models was evaluated by recognition capability (RC) and cross validation achievement (CVA). The RC is essential to internally evaluate the fitness of the classification models, whereas CVA is crucial to measure robustness of the resulting classification models.

In the initial model construction stage, the data from the training set were used to establish models as well as perform cross-validation procedures. For external validation, an independent data set was employed to examine the robustness of the established models. The selected model for classification was then used for predicting the need of advanced interventional procedure. The positive predictive value (PPV) and negative predictive value (NPV) were used to evaluate the performance and reliability of the models.

### Statistical analysis

Statistical analysis was performed using SPSS version 10.0 for Windows (SPSS Inc., Chicago, IL, USA). A  $p$  value  $< 0.05$  was considered to indicate statistical significance. Parametric data were compared using analysis of variance followed by Bonferroni's multiple comparison test. When the data were not normally distributed, or were nonparametric, the Kruskal–Wallis test was used as appropriate. Categorical data were analyzed using contingency table analysis and the Chi-square or Fisher's exact test.

## Results

### Patient characteristics and work flow of the study

Fifty-seven children with pneumonia followed by PE were consecutively enrolled into our study. The mean age was

5.5 ± 4.3 years. Patients received intervention procedures within 24 hours after thoracentesis, and pleural fluid met all three criteria of CPE (pH ≤ 7.2, LDH ≥ 1000 U/L, and glucose ≤ 40 mg/dL) in 13 (23%) patients. There were also 13 patients (23%) whose fever subsided within 24 hours after thoracentesis and the fluid met none of the criteria of CPE. These 26 well-characterized patients with or without CPE ( $n = 13$  in each group) were used for the construction of the classification models. The comparisons of pleural variables and characteristics of 26 well-characterized patients with and without CPE for model generation are shown in Table 1. An independent set contained another 31 PEs that did not meet all three criteria of CPE or non-CPE was then used for external validation of the constructed classification models. The prediction for the need of surgical intervention was also evaluated by using selected classification models. Fig. 1 illustrates the workflow of this study.

### Sample preparation and C8-functionalized MB

Fifty-seven mass spectra generated by MALDI-TOF MS after fractionating samples with each functionalized MB were used for analysis in subsequent studies. Three different purification kits (MB-C3, MB-C8, and MB-C18) are commercially available from Bruker Daltonics. After comparing the protein profiles from the use of these three kits, C8-functionalized MB (MB-C8) were identified to result in rich peak contents of the spectra and the highest reproducibility.

### Clinical efficacy of the analytical strategy

The MS spectra of pleural fluids derived from 26 well-characterized patients in the training set were analyzed,

and classification models were constructed via several statistical approaches including quick classifier, support vector machine, and GA. A total of 123 peaks were identified from the MS spectra of pleural fluids. Among them, 23 peaks which were significant with  $p < 0.05$  via Wilcoxon/Kruskal–Wallis test were used as classifiers. A series of models were constructed and representative ones including their RC and CVA are listed in Table 2. Three  $m/z$  peaks (2127, 2232, and 2427) were significantly downregulated and were selected as classifiers (Fig. 2). Model 7, containing the above-mentioned three significant peaks, has 73% sensitivity and 100% specificity, and was able to classify CPE with a PPV of 100% and an NPV of 73% (11/15). In the classification analysis for requiring subsequent aggressive intervention, compared to the PPV of 100% using biochemical markers, this model still had a remarkable PPV of 77% (13/17) despite its NPV of 50% (7/14).

### Discussion

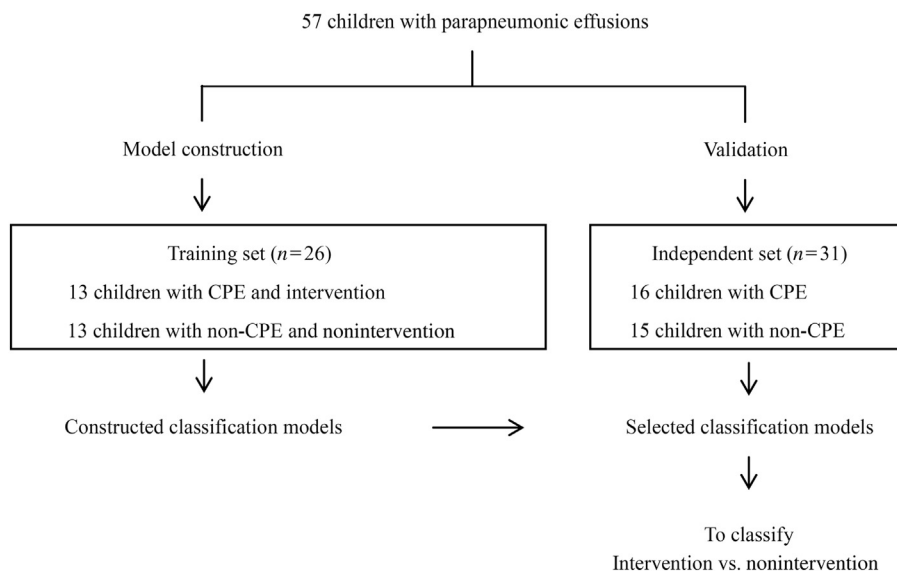
Pleural effusion, an accumulation of pleural fluid, contains proteins originating from plasma filtrate and parenchymal interstitial spaces of lungs especially when lung tissues are damaged. In pleural exudates, most of the abundant proteins were matched with the proteins in plasma, such as albumin, microglobulin, haptoglobin, glycoprotein, apolipoprotein, and immunoglobulin.<sup>11,14</sup> Low abundance proteins, cystatin C, transthyretin,  $\beta$ -2-microglobulin, and transferrin, detected in pleural exudates associated with pneumonia have also been reported in the literature to be found in plasma.<sup>12,15</sup> A C8 adsorbent is an ideal tool for purification of both peptides and proteins. In addition, MB-C8 have been reported to enrich a specific subset of plasma proteins based on their absorption by this hydrophobic functionality.<sup>16,17</sup> In this study, the proteomic profiling of

**Table 1** Comparison of characteristics of patients and pleural variables in complicated parapneumonic effusion (CPE) and non-CPE

Characteristics	Non-CPE ( $n = 13$ )	CPE ( $n = 13$ )	$p$
Age, y	4.5 ± 1.4	4.4 ± 4.1	0.225
Sex			
Male	6 (46)	7 (54)	0.695
Female	7 (54)	6 (46)	
Days of fever elapsed prior to thoracentesis	5.0 ± 3.6	7.5 ± 4.2	0.118
Pathogen			
<i>Streptococcus pneumoniae</i>	9 (69)	10 (77)	1.000
Unknown	4 (31)	3 (23)	
Hemogram			
WBC, × 10 <sup>9</sup> /L	12.4 ± 6.6	22.4 ± 6.7	0.001
Hb, g/dL	10.3 ± 1.3	9.9 ± 2.7	0.574
Platelet, × 10 <sup>9</sup> /L	277.1 ± 132	376.2 ± 211	0.152
CRP, mg/L	163.1 ± 104	192.5 ± 80	0.295
Pleural effusion			
pH	7.42 ± 0.10	6.89 ± 0.26	<0.001
Glucose, mg/dL	73.6 ± 18.1	11.6 ± 16.9	<0.001
LDH, IU/L	546.2 ± 422.8	7,762 ± 9,768	<0.001
Hospital stay, d	9.9 ± 3.2	15.3 ± 4.7	0.002

Data are presented as  $n$  (%) or mean ± standard deviation.

CRP = C-reactive protein; Hb = hemoglobin; LDH = lactate dehydrogenase; WBC = white blood cell.



**Figure 1.** Flowchart of the study.

pleural exudates achieved by using MB-C8 was shown to result in rich peak contents of the spectra and the highest reproducibility, indicating that the proteins/peptides obtained using C8 can not only represent the characteristics of pleural exudates, but also accurately reflect various pathological processes.

Several proteomic studies on pleural effusions by using a combination of two-dimensional gel electrophoresis and MS have been reported.<sup>11,18–20</sup> The changes of protein in the pleural effusions during the process of diseases, however, could not be established precisely due to the subtleties of the changes and high variability between individuals. By contrast, protein profiling by MALDI-TOF MS after sample fractionation with magnetic beads is a robust, precise, and rapid technique for the investigation of protein pattern changes and has been applied in various disease research for biomarker discovery.<sup>16,21</sup> Unlike the research published so far, the dynamic changes of protein during the process of pleural infection caused by pneumonia was well established in this study by using MALDI-TOF MS combined with C8

magnetic beads. Furthermore, a well-recognized protein pattern also provides an alternative approach for management of PE.

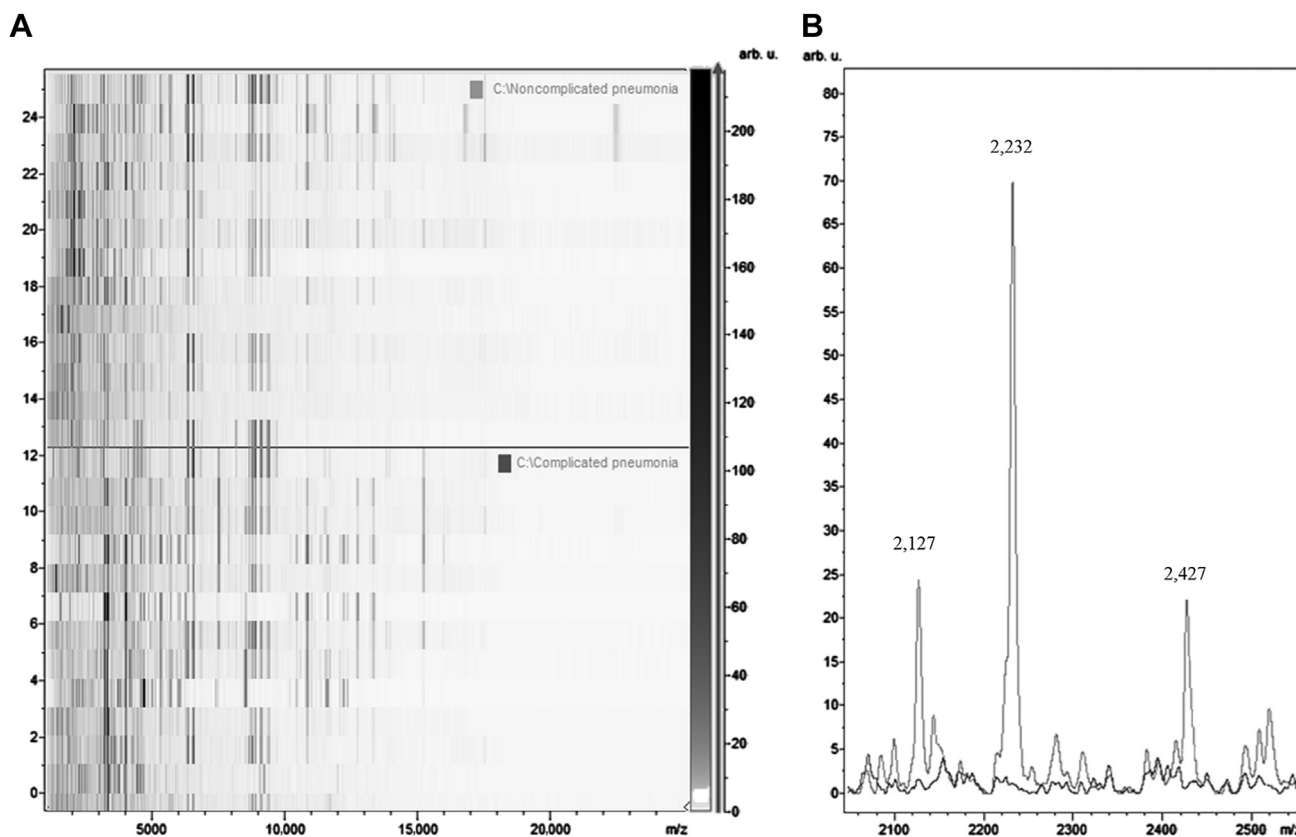
In this study, the data of proteomic spectra generated from PE were analyzed and pattern recognition models were successfully generated by ClinProTools software (Bruker Daltonics) for classification and prediction. The existence of 23 differential proteomic peaks between CPE and non-CPE indicates that broad pathological changes in proteins are involved in the progression of pneumonia. In addition, instead of a single protein type, a group of proteins contributes to disease progression in pneumonia. A pattern of three combined potential biomarkers effectively distinguished CPE from non-CPE in this study. Three peaks located at  $m/z$  2127, 2232, and 2427 were, however, all downregulated. Downregulated proteins that play major roles in the activation of innate immune response to bacterial infection have also been reported in a comparative proteomic study of plasma from children with pneumococcal pneumonia.<sup>22</sup> These findings indicate that proteins

**Table 2** Comparison of recognition capability (RC), cross-validation achievement (CVA), and positive predictive value of an independent set for the classification models

Model	Algorithms	No. peaks	CVA (%)	RC (%)	PPV of independent set for CPE (%)	PPV for intervention (%)
1	QC	14	77.3	95.5	93.8	76.5
2	SVM	21	83.9	100	43.8	35.3
3	GA	3	74.0	100	56.3	52.9
4	GA	5	72.0	100	50.0	41.2
5	GA	10	74.8	100	50.0	47.1
6	GA	15	71.7	100	56.3	47.1
7	Optimized GA <sup>a</sup>	5	95.5	95.5	100	76.5

<sup>a</sup> Optimized GA, enforced selection of specific  $m/z$  values with intensity >5% of total and  $p < 0.05$  via Wilcoxon/Kruskal–Wallis test during GA model construction.

CPE = complicated parapneumonic effusion; GA = genetic algorithm; PPV = positive predictive value; QC = quick classifier; SVM = support vector machine.



**Figure 2.** Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry spectra of 26 samples for model construction. (A) The gel view showing the spectra of two classes of parapneumonic effusions (top, noncomplicated parapneumonic effusion; bottom, complicated parapneumonic effusion). (B) A region of mass spectra from 2100 to 2500 is enlarged to illustrate the three significant peaks (gray) that were enforced selected by genetic algorithm. Three significant downregulated  $m/z$  values (2127, 2232, and 2427) are highlighted. x axis, mass/charge in Daltons [ $m/z$  (Da)]; y axis, intensity [arbitrary units (arb.u.)].

related to downregulation of immunity against bacterial infections may play an important role in the pathogenesis of pneumonia, and potentially become novel therapeutic targets.

The model of pattern recognition established in this study can classify CPE with 100% PPV and predict the need of subsequent surgical intervention with 77% PPV. Despite the high specificity of this model, there were still 4 of 15 non-CPE that could not be correctly classified. In clinical practice, chest sonography can monitor the progression of parapneumonic effusions by the demonstration of fibrin and formation of fibrin septations.<sup>23</sup> Furthermore, the process of pleural infection is shown to be rapid and may progress within one day.<sup>24</sup> The high misclassification rate in this study may therefore be due to the high variability of timing for thoracentesis in the independent set. In addition, the hesitation of parents for their children to receive invasive intervention is believed to make the results of low PPV for predicting the need of surgical intervention.

In conclusion, a combination of MB-based sample preparation and MALDI-TOF MS analysis can effectively profile pleural effusion proteome. In children with PE-associated pneumonia, clinical proteomics analysis of PE can detect changes in protein profiles reflecting disease states. Despite the limitation of the small sample size in this study, the application of MALDI-TOF MS in combination with a

diagnostic model construction comprising three downregulated biomarkers provides an alternative approach for predicting CPE and need for subsequent surgical intervention. These findings indicate that pleural fluid proteins that are downregulated during the progression of pneumonia could potentially guide the management of infectious PE.

## Conflicts of interest

All contributing authors declare no conflicts of interest.

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