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Protocol for the Study Investigating Markers in PLeural Effusion (SIMPLE study): A prospective and double blinded diagnostic study

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Keywords:	Diagnostic accuracy test, Biomarkers, Pleural effusion

Protocol for a Study Investigating Markers in PLeural Effusion (SIMPLE study): A prospective and double-blind diagnostic study

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Abstract

Introduction: Serum and pleural effusion (PE) markers, due to their advantages of low cost, objective result and short turn-around time, are valuable for exploring the etiologies of PE. The diagnostic accuracy of potential markers needs to be rigorously evaluated before their widespread application in clinical practice. Here, we plan to perform a Study Investigating Markers in PLeural Effusion (SIMPLE study).

Methods and analysis: This is a prospective and double-blind study will be performed in the Affiliated Hospital of Inner Mongolia Medical University, China. Adult patients with PE of unknown causes and admitted to our institution between September 2018 and July 2021, will be enrolled. PE and matched serum specimens will be collected and stored at –80°C for research aims. Diagnosis of the included subjects will be proved with imaging, microbiology, cytology and biopsy. The results of investigated markers will be unknown to the clinicians who will make diagnosis and the clinical diagnoses will be unknown to the laboratory technicians who will determine markers. The diagnostic accuracy of investigated markers will be assessed using receiver operating characteristics (ROC) curve analysis, multivariable logistic regression model, net reclassification index (NRI) and integrated discriminatory index (IDI).

Ethics: This study has been approved by the Ethic Committee of the Affiliated Hospital of Inner Mongolia Medical University (NO: 2018011).

Study registration: This study has been registered with the Chinese Clinical Trial Registry platform, with a registration number of ChiCTR1800017449.

Keywords: Biomarkers; Pleural effusion; Diagnostic accuracy test

Strengths and limitations of this study

- A prospective designed study evaluating the diagnostic value of pleural effusion and serum biomarkers in subjects with pleural effusion.
- Double-blinded: the clinician madding diagnosis will be masked to the results of biomarker and the laboratory technician determining biomarker will be masked to the clinical picture of subjects.
- Multiple biomarkers for various target diseases will be studied.
- Multiple etiologies will be considered when making diagnosis.

Introduction

Pleural effusion (PE) is a frequent problem in clinical practice and can be caused by various disorders such as heart failure (HF), malignant diseases, tuberculosis and pneumonia [1,2]. A correct and timely diagnosis is the prerequisite for PE management. Currently, several tools are available for exploring the etiologies of PE, including thoracoscopy, imaging, cytology and bacterial culture [3,4]. Of these tools, thoracoscopy is the most widely-used one and has good diagnostic performance for various disorders. Nevertheless, a previous study indicated that approximately 7% of patients remain undiagnosed after thoracoscopy [2]. Besides, thoracoscopy is an invasive tool and operation-related complications are really a problem. Although some non-invasive diagnostic tools, such as microbiological and cytological examinations, have high specificity for given diseases, their diagnostic sensitivities are unsatisfactory [5–8]. Besides, the diagnostic accuracy of these tools, as well as imaging approaches and thoracoscopy, depends largely on the experience of operator and pathologist [9]. Bacterial culture has high diagnostic specificity for infectious diseases including pneumonia and tuberculosis; however, the long turnaround time (TAT) limits its application in clinical setting.

By contrast, serum and PE biochemistry analyses have some advantages in exploring etiologies, including low cost, short TAT, easy to standardization and less operator or observer variations. Indeed, some markers in PE or serum have shown extremely high diagnostic accuracy in patients with PE. For instance, PE levels of interleukin-27 (IL-27) [10], interferon-gamma [11] and adenosine deaminase (ADA) [12] for tuberculous PE, and N-terminal pro-brain natriuretic peptide (NT-proBNP) for HF [13,14]. However, for malignant diseases and infectious diseases, the diagnostic accuracy of available biomarkers such as tumor markers [14–16], procalcitonin (PCT) [17] and C-reactive protein (CRP) [18] is unsatisfactory. Therefore, further works are needed to identify novel markers and rigorously evaluate their diagnostic accuracy.

To better understand the diagnostic value of PE and serum markers in patients with PE, we plan to perform a prospective, double-blind diagnostic study, named A Study Investigating Markers in PLeural Effusion (SIMPLE study).

Method

Trial registration, foundation and ethic approval

This study has been approved by the Ethic Committee of the Affiliated Hospital of Inner Mongolia Medical University (NO: 2018011). All subjects, or their guardians, will be given a full informed consent before taking part. The study has been registered with the Chinese Clinical Trial Registry platform (http://www.chictr.org.cn/index.aspx. Registration number: ChiCTR1800017449). Currently, this study is not supported by any grant; however, it may be supported by some grants from the Affiliated Hospital of Inner Mongolia Medical University or government in future. The funders will not be involved in study design, sample collection, data analysis, manuscript preparation and decision to publish.

Subject enrollment and specimen collection

Subjects with PE and unknown etiology are eligible for enrollment. The presence of PE will be proved by CT or ultrasound. The exclusion criteria are: (i) age less than 18 years; (ii) with a history of diseases that can cause PE during the last three months; (iii) pregnancy; (iv) refused to sign informed consent; (v) with comorbidities that can prevent PE collection.

PE specimen collection will be initiated after the informed consent process. Approximately 5-10 milliliters PE specimen will be collected in a tube that does not contain any anticoagulant. The specimen will be sent to laboratory within 2 hours and centrifuged at 1200g for 10 minutes. The supernatants of PE specimen will be transferred to 10 Eppendorf tubes (510 μl each tube) and immediately frozen at –80°C for later using. A matched serum sample, which is sent to the laboratory 24 hours before or after PE specimen collection, will be also collected and frozen at –80 °C if available.

All subjects will be enrolled by a respirologist (L Yan). The subjects will be not consecutively enrolled because they may: (i) refuse to sign the informed consent; (ii) be admitted at weekend when Dr. Yan is not on duty; (iii) not be admitted to the Department of Respiratory and Critical Care Medicine of our institution.

The laboratory technician who determines the concentration of investigated markers will be

blinded to clinical picture of the subjects.

Sample size estimation

As this is not a hypothesis-driven research and none target disease or biomarker is proposed, we did not estimate the sample size before subjects' enrollment. The study execute time lasts from September 2018 to July 2021. It is estimated that 200 to 500 subjects will be enrolled.

Final diagnosis

This is an observational study that does not affect the further management of the enrolled subjects. The clinicians decided the further diagnostic or treatment procedures independent of this study. The etiologies of PE are various and the diagnostic criteria for the major diseases are listed in **Table 1**. The final diagnosis will be made by a researcher (L Yan) and the results of investigated biomarkers is unknown when making diagnosis.

Table 1 Diagnostic criteria for major diseases related to PE

Etiology	Diagnostic criteria
Tuberculous	Identification of *Mycobacterium tuberculosis* in the sputum, pleural fluid, or pleural biopsy specimens [19], either by microscopy or cultures. In some cases with adequate clinical context, the diagnosis can be made with granuloma in the parietal pleura, good response to anti-tuberculosis treatment, elevated level of PE adenosine deaminase or positive nucleic acid amplification tests (NAATs) [19–21].
Heart failure	Typical clinical picture of heart failure, including the Framingham score, medical history, the response to diuretic therapy, chest radiography, the echocardiographic evidence of left ventricular systolic dysfunction [13,22–24].
Malignant diseases	Identification of cancer cells in PE, sputum or bronchoalveolar lavage fluid by cytological examination, ultrasound or thoracoscopy-guided

	pleural biopsy [3,23].
Pneumonia	Typical clinical and radiological evidences of pneumonia, or a positive bacterial culture in PE, or good response to antibiotic therapy [3,23].
Pulmonary embolism	Computed tomographic pulmonary angiography (CTPA) [25].

Given that approximately 30% of PEs have more than one etiology and the most common secondary cause is HF [26,27]. Therefore, all patients will be evaluated whether they have HF. For some subjects, the possible etiologies can change during their admission (i.e. HF secondary to pneumonia), only the diagnosis at the time of specimen collection will be used. It is estimated that some subjects' diagnosis remains unknown after discharging. This may be due to the fact that some subjects will refuse to receive invasive approaches, such as thoracoscopy. The number of these subjects will be recorded and reported. The subjects die during hospital will be excluded into analysis.

Patient and public involvement

Figure 1 is a flowchart depicting the study procedure. Subjects who meet the inclusion criteria and do not meet the exclusion criteria will be invited to participate in this study. An informed consent will be signed before their participation. All subjects will not be involved in the recruitment and conduct of the study.

Routine blood and PE analysis will be ordered in these subjects. PE specimens will be obtained for routine laboratory analysis, including cell count and differentiation, tumor markers, biochemistry, bacterial culture, Gram staining, cytology and nucleic acid amplification tests (NAAT). These laboratory test will be ordered by the attending clinicians independent of this study. Approximately 5-10 milliliters PE specimen will be collected simultaneously for research aims. All PE and serum specimens will be collected on admission before diagnosis. The time period between PE specimen collection and final diagnosis will be usually within one weeks, except for some subjects that need follow-up and therapy response to make diagnosis. Imaging (CT, MRI, CTPA), thoracoscopy and bronchoscopy will be ordered if necessary. The results of biomarkers will be disseminated to the

subjects via email or telephone if they require.

Statistical analysis

Normal distribution of continuous data will be tested by Kolmogorov-Smirnov test. For the data with normal distribution, independent t or one-way ANOVA tests will be used for comparison; Otherwise, Mann-Whitney or Kruskal-Wallis tests will be used. Chi-square test will be used to compare categorized data. Receiver operating characteristics (ROC) curve analysis will be used to evaluate the diagnostic accuracy of the investigated marker. Area under ROC curve (AUC) will be used to estimate the overall diagnostic accuracy of a marker. AUCs of markers will be compared by the approach proposed by Delong et al [28]. The optimal threshold will be determined with the method proposed by Pepe et al. [29] or maximum Youden index. Subjects who have multiple etiologies will be categorized into the disease group if the corresponding etiology has been proved, regardless of the etiology is primary or secondary. Multivariable logistic regression model, net reclassification index (NRI) and integrated discriminatory index (IDI) will be used to evaluate whether a given marker provides added diagnostic information [30,31] beyond conventional diagnostic tools. Decision tree approach will be created to evaluate the preferred diagnostic strategy. All analyses will be performed with SPSS 18.0 (IBM Corporation, Chicago, United States), Sigmaplot 12.0 (Systat Software, Inc., San Jose, CA), Graphpad Prism 6.0 (GraphPad Software, La Jolla, CA) and R (http://www.r-project.org).

Discussion

With the advance of omics approaches and basic researches, accumulated markers have been identified for exploring the etiology of PE. Under such a condition, it is valuable to evaluate the diagnostic accuracy of these markers rigorously. Although several studies have been performed on this topic [10,32,33], the result of these study remains needs to be validated. This is because that the diagnostic accuracy of a given marker may be affected by the disease spectrum of study cohort [34]. Besides, majority previous studies only evaluated the diagnostic accuracy of single marker and not compared it with other promising markers. Furthermore, whether multiple markers strategy can improve

the diagnostic accuracy remains largely unknown. The aim of SIMPLE study was to: (i) evaluate the diagnostic accuracy of biomarkers when used alone; (ii) compare the diagnostic accuracy of two or more biomarkers in a head-to-head manner; (iii) verify whether a novel biomarker can provide added diagnostic information beyond traditional promising biomarkers.

Compared with previous studies, SIMPLE study has some strength. First, this is a registered, prospective, double-blind study. Therefore, the results of this study may be more reliable. Second, majority of previous study did not consider the subjects with multiple etiologies and this issue will be considered by SIMPLE study. Third, only limited studies have investigated whether a novel marker could provide added diagnostic information beyond traditional markers. In SIMPLE study, we will investigate this issue with IDI and NRI, two widely-accepted statistical methods.

Some potential biomarkers can be investigated in SIMPLE study. Here, I just give some examples. Previous studies have indicated that presepsin is a useful diagnostic marker for bacterial infected diseases [35,36]; nevertheless, it remains unknown whether presepsin in serum or PE is useful for pneumonia diagnosis in patients with PE. Serum mid-regional pro-atrial natriuretic peptide (MR-proANP) has been reported to have high diagnostic accuracy for HF [37]. However, only one study has investigated the diagnostic accuracy of MR-proANP in PE for HF [22], and the results of this study need to be validated.

SIMPLE study has some limitations. First, this is the single center study and representativeness of study cohort is a limitation. Second, because not all subjects will receive the same reference standard, differential verification bias [38,39] is also a problem. Third, because it is not ethical to let all subjects receiving all diagnostic tools once a diagnosis has been made, partial verification bias can not be avoided. Indeed, establishing one diagnosis does exclude other etiologies.

Taken together, SIMPLE study is a prospective, double-blind diagnostic study aims to investigate the diagnostic accuracy of serum and PE markers. Although it has some limitations, we believe that this study will provide a new insight into the PE etiological diagnosis.

Authors' contributions

Z.D. Hu conceived and designed the study; L Zhang, P.H. Ouyang and P Li provided administrative support; L Zhang, P.H. Ouyang, P Li, L Yan and Y.Q. Han drafted the manuscript. Z.D. Hu critically revised the manuscript; all authors approved the final version of the manuscript.

Competing interests

None declared.

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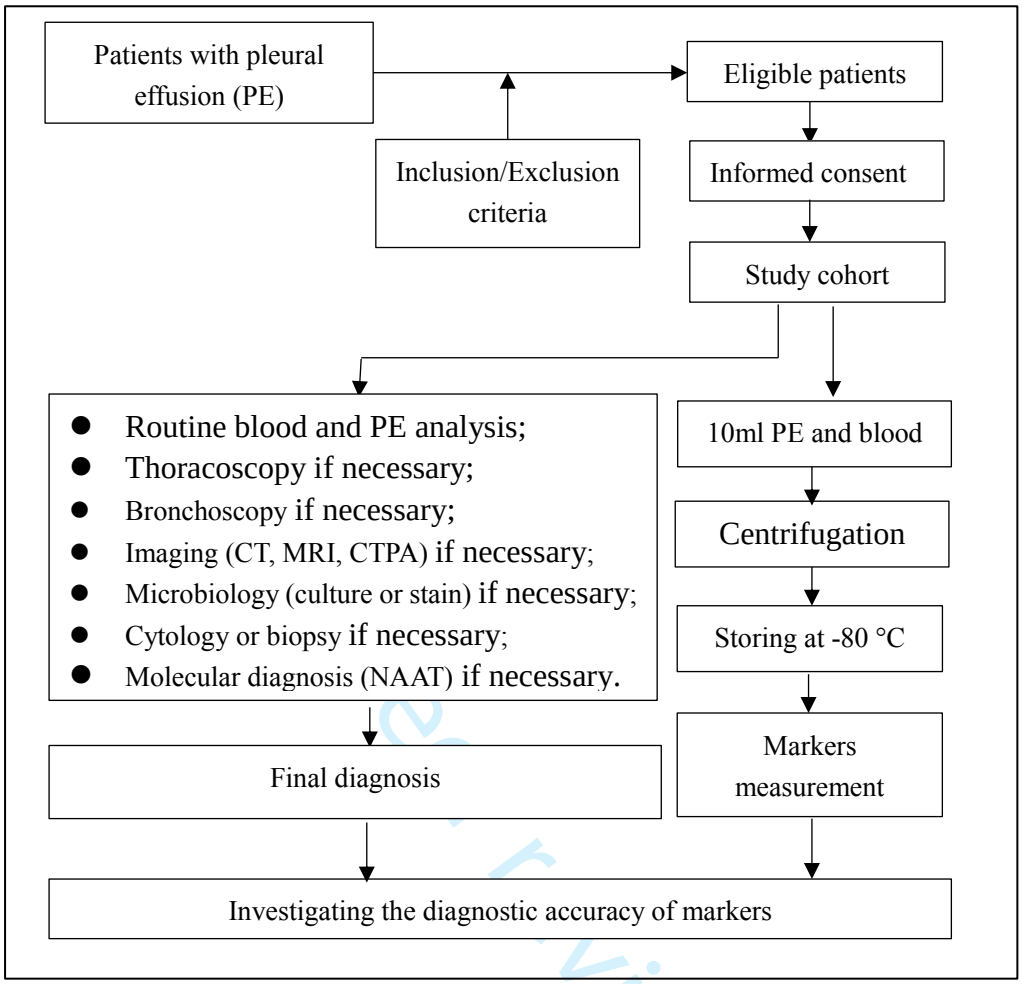
Figure legend

Figure 1. Flowchart of study procedure.



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Abstract

Introduction: Serum and fluid laboratory biomarkers are valuable for exploring the etiologies of pleural effusion because of their relative non-invasiveness, low cost, objective result and short turn-around time. The diagnostic accuracy of these potential biomarkers needs to be rigorously evaluated before their widespread application in clinical practice. Here, we plan to perform a Study Investigating Markers in PLeural Effusion (SIMPLE study).

Methods and analysis: This is a prospective and double-blind clinical trial which is being performed at the Affiliated Hospital of Inner Mongolia Medical University, China. Adult patients admitted for the evaluation of etiology of pleural effusion from September 2018 to July 2021 will be enrolled after informed consent. Pleural fluid and serum specimens will be collected and stored at –80°C for the laboratorial analysis. The final diagnosis will be concurred with further imaging, microbiology, cytology and biopsy if needed. The results of investigated laboratory markers will be unknown to the clinicians who will make diagnosis and the clinical diagnoses will be unknown to the laboratory technicians who will determine markers. The diagnostic accuracy of investigated markers will be assessed using receiver operating characteristics (ROC) curve analysis, multivariable logistic regression model, net reclassification index (NRI) and integrated discriminatory index (IDI).

Ethics: The study is approved by the Ethic Committee of the Affiliated Hospital of Inner Mongolia Medical University (NO: 2018011).

Study registration: The study is also registered with the Chinese Clinical Trial Registry platform, with a registration number of ChiCTR1800017449.

Keywords: Markers; Pleural effusion; Diagnostic accuracy test

Strengths and limitations of this study

- A prospectively designed trial evaluating the diagnostic value of pleural fluid and serum markers in subjects with pleural effusion.
- Double-blinded: the clinicians making diagnosis will be masked to the laboratory results of markers, and the laboratory technician determining markers will be masked to the clinical diagnosis of the subjects.
- Multiple laboratory markers for various target diseases will be studied.
- Multiple differential diagnosis will be considered.
- The main limitations of the study are: single-center design and there is a possibility of patients selection bias.

Introduction

Pleural effusion (PE) is a frequent problem in the clinical practice and can be caused by various disorders such as congestive heart failure (CHF), liver and pancreatic diseases, diseases of lungs such as malignancy, tuberculosis and pneumonia etc. [1,2]. An accurate and timely diagnosis is a prerequisite for PE management to evaluate its cause. Light's criteria, which encompass serum and pleural fluid biochemical analyses, are commonly used in the clinical practice to distinguish between the exudative and transudative pleural effusions [3]. Although Light's criteria has high sensitivity for detecting exudative pleural effusion but occasionally it cannot be used to differentiate the underlying causes, such as infections and malignancies [4]. Currently, several tools are available for exploring the etiology of PE, including thoracoscopy, chest imaging especially CT scan, cytology and bacterial culture [5,6]. Thoracoscopy is one of the most widely-used and has a good diagnostic performance for various thoracic disorders. Nevertheless, a previous study indicated that approximately 7% of patients with PE remain undiagnosed after thoracoscopy [2]. Besides, thoracoscopy is an invasive tool associated with procedure related complications. The microbiological and cytological examinations have high specificity but their diagnostic sensitivities are unsatisfactory [7–10]. Besides, the diagnostic accuracy of these tools is largely operator and pathologist dependent [11]. Bacterial culture has high diagnostic specificity for infectious causes of PE ; however, the long turnaround time (TAT) limits its application in clinical setting.

By contrast, serum and pleural fluid biochemical analyses have some advantages including but not limited to low cost, short TAT, easy standardization with less operator or observer variations. Indeed, some pleural fluid and serum markers have shown extremely high diagnostic accuracy in patients with PE. For instance, interleukin-27 (IL-27) [12], interferon-gamma [13] and adenosine deaminase (ADA) [14] pleural fluid levels for tuberculous PE, and serum N-terminal pro-brain natriuretic peptide (NT-proBNP) for CHF [4,15]. However, for malignant and infectious diseases, the diagnostic accuracy of available markers such as tumor markers [4,16,17], procalcitonin (PCT) [18] and C-reactive protein (CRP) [19] is unsatisfactory. Therefore, further researches are needed to identify novel markers in PE with increased diagnostic accuracy.

Here, we plan to perform a prospective, double-blind diagnostic trial, named A Study Investigating Markers in PLeural Effusion (SIMPLE study). The aim of SIMPLE is to: (i) evaluate the diagnostic accuracy of serum and pleural fluid markers when used alone; (ii) compare the

diagnostic accuracy of two or more markers in a head-to-head manner; (iii) verify whether a novel marker can provide added diagnostic information beyond available traditional markers.

Method

Trial registration and foundation

All subjects, or their guardians, will be provided a full informed consent before inclusion in the study. The study has been registered with the Chinese Clinical Trial Registry platform (http://www.chictr.org.cn/index.aspx. Registration number: ChiCTR1800017449). Currently, this study is not supported by any grant; however, it may be supported by one or more grants from the Affiliated Hospital of Inner Mongolia Medical University or the Chinese government in future. The funders will not be involved in study design, sample collection, and data analyses.

Subject enrollment and specimen collection

Subjects who will be admitted to our hospital for an evaluation of the etiology of the pleural effusion will be eligible for enrollment. The presence of PE will be evaluated first by clinical examination and then further confirmed by chest imaging such as CT scan or ultrasound. The exclusion criteria are: (i) age less than 18 years; (ii) with a known diagnosis of a disease that could cause PE during the last three months; (iii) pregnancy; (iv) refused to sign informed consent; (v) with comorbidities that can prevent pleural fluid collection; (vi) subject dies during hospitalisation without collection of pleural fluid and serum specimens; (vii) death of a subject during hospital stay before the final diagnosis; (viii) patients admitted without PE but developed PE after admission.

Pleural fluid specimen collection will be initiated after obtaining informed consent by the patient. Approximately 5-10 milliliters of pleural fluid specimen will be collected in a tube that does not contain any anticoagulant. The specimen will be sent to laboratory within 2 hours and centrifuged at 1200g for 10 minutes. The supernatants of the specimen will then be transferred to 10 Eppendorf tubes (510 µl tube) and immediately frozen at –80°C for later use. A serum sample will be collected from the same patient within 24 hours before or after the pleural fluid collection to be frozen at –80 °C if available. A case report form will be used to record demographic and clinical details of the subjects, such as age, sex, side of PE (left-, right- or two-sided effusion), smoking history, conventional laboratory tests and microbiological findings.

All subjects will be enrolled by a pulmonologist (L Yan). The subjects will be not consecutively enrolled because they may: (i) refuse to sign the informed consent; (ii) be admitted at weekend when Dr. Yan is not on duty; (iii) not be admitted to the Department of Respiratory and Critical Care Medicine of our institution.

The laboratory technician who determines the concentration of investigated markers will be blinded to clinical presentations of the subjects.

Sample size estimation

As this is not a hypothesis-driven research and no new target disease or marker is proposed, we did not estimate the sample size before subjects' enrollment. The study execute time will last from September 2018 to July 2021. It is estimated that 200 to 300 subjects will be enrolled.

Final diagnosis

This is an observational study that will not affect the further management of the enrolled subjects. The clinicians will decide the further diagnostic, treatment and management independent of this study. The etiologies of PE are diverse and the diagnostic criteria for the major diseases are listed in **Table 1**. The final diagnosis will be made by two researchers independently (L Yan and ZD Hu) and the results of investigated markers will not be known by them when making diagnosis.

Table 1 Diagnostic criteria for major diseases related to PE

Etiology	Diagnostic criteria
Tuberculous	Identification of *Mycobacterium tuberculosis* in the sputum, pleural fluid, or pleural biopsy specimens [20], either by microscopy or cultures. In some cases with adequate clinical context, the diagnosis can be made with presence of granuloma in the parietal pleura, good response to anti-tuberculosis treatment, elevated level of pleural fluid adenosine deaminase or positive nucleic acid amplification tests (NAATs) [20–22].

Congestive heart failure	Typical clinical picture of CHF including the Framingham score, medical history and physical examination, the response to diuretic therapy, typical CHF features on chest X-ray, the echocardiographic evidence of left ventricular systolic dysfunction [15,23–25].
Malignant diseases	Identification of cancer cells in pleural fluid, sputum or bronchoalveolar lavage fluid by cytological examination, ultrasound or thoracoscopy-guided pleural biopsy [5,24].
Parapneumonic effusion	Typical clinical and radiological evidences of pneumonia, or a positive bacterial culture from pleural fluid, or good response to antibiotic therapy [5,24].
Pulmonary embolism	Computed tomographic pulmonary angiography (CTPA) [26].

Given that approximately 30% of PEs have more than one etiology and the most common secondary cause is CHF [27,28]. Therefore, all patients will be evaluated for concomitant presence of CHF. For some subjects, the differential diagnosis can change during their admission only the diagnosis at the time of specimen collection will be used. We also realize that a confirmed diagnosis is not possible in all cases with PE at the time of discharge. This can be due to the fact that some subjects might refuse to receive further diagnostic invasive approaches such as thoracoscopy. The number of these subjects will be recorded and reported. The patients without final diagnosis will be excluded from the final analysis or will be considered as control in the data analysis.

Patient and public involvement

Figure 1 is a flowchart depicting the study procedure. Subjects who will meet the inclusion and exclusion criteria will be invited to participate in this study. An informed consent will be signed before their participation. All subjects will not be involved in the recruitment and conduct of the study.

Routine serum and pleural fluid analysis will be ordered for these subjects. Pleural fluid specimens will be obtained for routine laboratory analysis, including cell count and differentiation, tumor markers, biochemistry, bacterial culture, Gram staining, cytology and nucleic acid amplification tests (NAAT). These laboratory tests will be ordered by the attending clinicians

independent of this study. Approximately 5-10 milliliters of pleural fluid specimen will be collected simultaneously for research aims. All fluid and serum specimens will be collected at the time of admission before final diagnosis. The time period between the pleural fluid specimen collection and final diagnosis will be usually within one weeks, except for some subjects that need follow-up and therapy response to make diagnosis. Imaging (CT, MRI, CTPA), thoracoscopy and bronchoscopy will be ordered if necessary. The results of markers will be disseminated to the subjects via email or telephone upon request after the conclusion of the study.

Dissemination and ethics

The results of SIMPLE will be submitted to international scientific peer-reviewed journals or conferences in laboratory medicine of respiratory medicine, thoracic diseases. This study has been approved by the Ethic Committee of the Affiliated Hospital of Inner Mongolia Medical University (NO: 2018011).

Patient and public involvement statement

All subjects in SIMPLE study will not directly involved in the study design, recruitment and study conduction. There is no public involvement.

Statistical analysis

Normal distribution of continuous data will be tested by Kolmogorov-Smirnov test. For the data with normal distribution, independent t-test or one-way ANOVA will be used for comparison; Otherwise, Mann-Whitney or Kruskal-Wallis tests will be used. Chi-square test will be used to compare categorized data. Receiver operating characteristics (ROC) curve analysis will be used to evaluate the diagnostic accuracy of the investigated markers. Area under ROC curve (AUC) will be used to estimate the overall diagnostic accuracy of a markers. AUCs of markers will be compared by the approach proposed by Delong et al [29]. The optimal threshold will be determined with the method proposed by Pepe et al. [30] or maximum Youden index. When evaluating the diagnostic accuracy of a marker for a given disease, the subjects with this disease will be categorized into a disease group, regardless of whether other etiologies co-occur. Multivariable logistic regression model, net reclassification index (NRI) and integrated discriminatory index (IDI) will be used to evaluate whether a given marker provides added diagnostic information [31,32] beyond conventional diagnostic tools and clinical details (e.g. side information, age, sex, smoking history). Decision tree approach and decision curve analysis [33] will be created to evaluate the preferred

diagnostic strategy. All analyses will be performed with SPSS 18.0 (IBM Corporation, Chicago, United States), Sigmaplot 12.0 (Systat Software, Inc., San Jose, CA), Graphpad Prism 6.0 (GraphPad Software, La Jolla, CA) and R (http://www.r-project.org).

Discussion

Multiple new diagnostic markers have been identified in the evaluation of PE with the advancement in omics approach and basic research. Therefore, it is valuable to evaluate the diagnostic accuracy of these markers rigorously. Although several studies have been performed on this topic [12,34,35], the result of these studies need to be validated. This is because that the diagnostic accuracy of a given marker may be affected by the disease spectrum of a study cohort [36]. Besides, majority of previous published studies evaluated only the diagnostic accuracy of single marker, and did not compare it with other promising markers. Furthermore, whether multi-marker strategy can improve the diagnostic accuracy remains largely unknown.

Compared with previous studies, SIMPLE study has some strength. First, this is a registered, prospective, double-blind study. Therefore, the results of this study are more reliable. Second, majority of the previous studies did not consider the subjects with multiple etiologies of PE and this issue will be considered by SIMPLE study. Third, only limited studies have investigated whether a novel marker could provide added diagnostic information beyond traditional markers. In SIMPLE study, we will investigate this issue with IDI and NRI, although they have some shortcomings [37–40].

Multiple potential serum and fluid markers will be investigated in the SIMPLE study. Previous studies have indicated that presepsin is a useful diagnostic marker for bacterial infection [41,42]; nevertheless, it remains unknown whether presepsin in serum or pleural fluid is useful for the diagnosis of parapneumonic effusion. Serum mid-regional pro-atrial natriuretic peptide (MR-proANP) has been reported to have high diagnostic accuracy for CHF [43]. However, only one study has investigated the diagnostic accuracy of MR-proANP in PE caused by CHF [23], and the results of this study need to be validated. In addition, some novel markers will also be studied, such as soluble Fas ligand [44] and interleukin 27 [12] for tuberculous pleurisy, soluble B7-H4 [45] and human epididymis 4 (HE4) [46] for malignant effusion. The results of SIMPLE study will be reported in accordance with the Standards for Reporting of Diagnostic Accuracy Studies (STARD)

guideline [47]. For researches with multivariable prediction model, the report will comply with Transparent reporting of a multivariable prediction model for individual prognosis or diagnosis (TRIPOD) statement [48].

SIMPLE study has some limitations. First, this is a single center study and representativeness of the study cohort is a limitation. Second, because it is not ethical to let all subjects receiving all diagnostic tools once a diagnosis has been made, partial verification bias can not be avoided. Indeed, establishing one diagnosis does exclude other etiologies. Third, the prognostic value of markers will not be evaluated in this study.

Taken together, SIMPLE study is a prospective, double-blind diagnostic study aims to investigate the diagnostic accuracy of serum and pleural fluid markers. Although it has some limitations, we believe that this study will provide a new insight into the PE etiological field.

Authors' contributions

Z.D. Hu conceived and designed the study; L Zhang, P.H. Ouyang and P Li provided administrative support; L Zhang, P.H. Ouyang, P Li, L Yan and Y.Q. Han drafted the manuscript. Z.D. Hu critically revised the manuscript; all authors approved the final version of the manuscript.

Competing interests

None declared.

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Figure legend

Figure 1. Flowchart of study procedure.

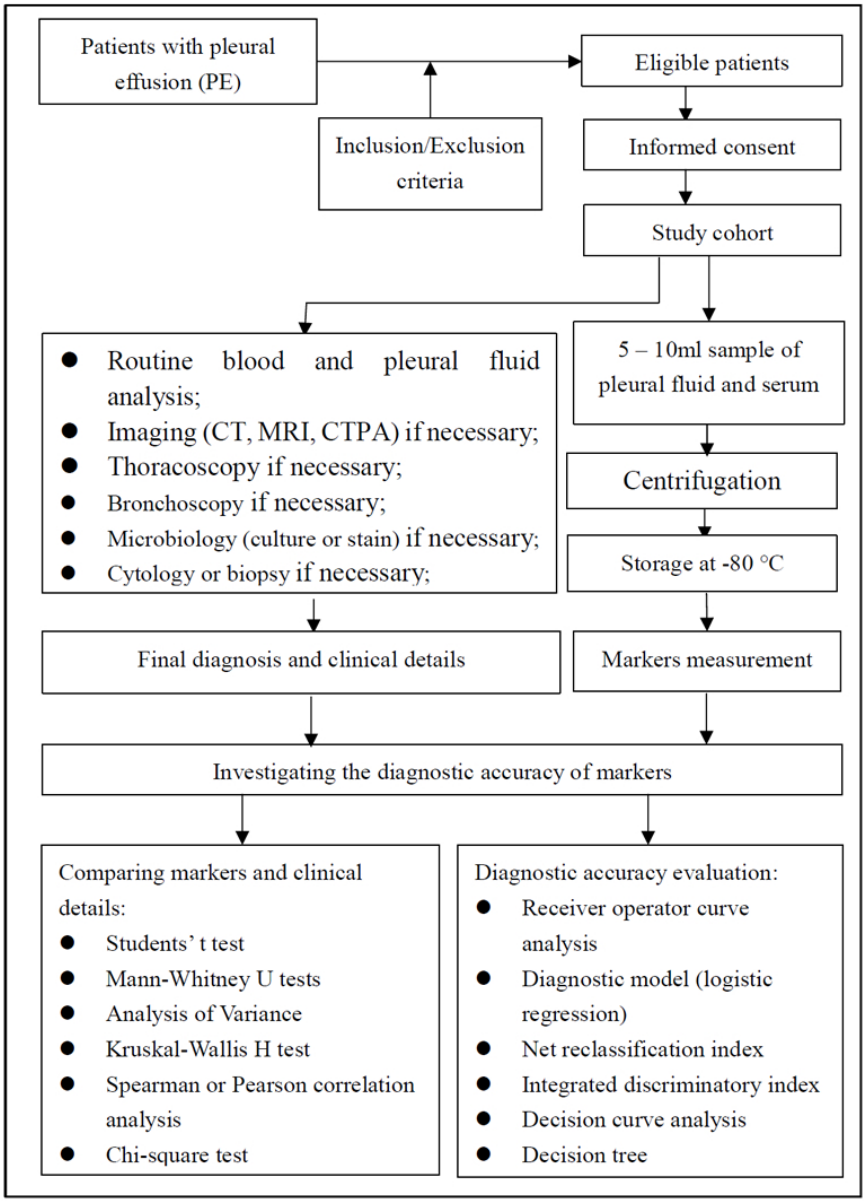

Figure 1. Flowchart of study procedure.

64x90mm (300 x 300 DPI)

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Protocol for a Study Investigating Markers in PLeural Effusion (SIMPLE): A prospective and double-blind diagnostic study

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Protocol for a Study Investigating Markers in PLeural Effusion (SIMPLE): A prospective and double-blind diagnostic study

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Abstract

Introduction: Serum and fluid laboratory markers are valuable for exploring the etiologies of pleural effusion because of their relative non-invasiveness, low cost, objective result and short turn-around time. The diagnostic accuracy of these potential markers needs to be rigorously evaluated before their widespread application in clinical practice. Here, we plan to perform a Study Investigating Markers in PLeural Effusion (SIMPLE).

Methods and analysis: This is a prospective and double-blind clinical trial which is being performed at the Affiliated Hospital of Inner Mongolia Medical University, China. Adult patients admitted for the evaluation of etiology of pleural effusion from September 2018 to July 2021 will be enrolled after informed consent. Pleural fluid and serum specimens will be collected and stored at –80°C for the laboratorial analysis. The final diagnosis will be concurred with further imaging, microbiology, cytology and biopsy if needed. The results of investigated laboratory markers will be unknown to the clinicians who will make diagnosis and the clinical diagnoses will be unknown to the laboratory technicians who will determine markers. The diagnostic accuracy of investigated markers will be assessed using receiver operating characteristics (ROC) curve analysis, multivariable logistic regression model, decision curve analysis (DCA), net reclassification index (NRI) and integrated discriminatory index (IDI).

Ethics: The study is approved by the Ethic Committee of the Affiliated Hospital of Inner Mongolia Medical University (NO: 2018011).

Study registration: The study is also registered with the Chinese Clinical Trial Registry platform, with a registration number of ChiCTR1800017449.

Keywords: Markers; Pleural effusion; Diagnostic accuracy test

Strengths and limitations of this study

- A prospectively designed trial evaluating the diagnostic value of pleural fluid and serum markers in subjects with pleural effusion.
- Double-blinded: the clinicians making diagnosis will be masked to the laboratory results of markers, and the laboratory technician determining markers will be masked to the clinical diagnosis of the subjects.
- Multiple laboratory markers for various target diseases will be studied.
- Multiple differential diagnosis will be considered.
- The main limitations of the study are: single-center design and there is a possibility of patients selection bias.

Introduction

Pleural effusion (PE) is a frequent problem in the clinical practice and can be caused by various disorders such as congestive heart failure (CHF), liver and pancreatic diseases, diseases of lungs such as malignancy, tuberculosis and pneumonia etc. [1,2]. An accurate and timely diagnosis is a prerequisite for PE management to evaluate its cause. Light's criteria, which encompass serum and pleural fluid biochemical analyses, are commonly used in the clinical practice to distinguish between the exudative and transudative pleural effusions [3]. Although Light's criteria has high sensitivity for detecting exudative pleural effusion but occasionally it cannot be used to differentiate the underlying causes, such as infections and malignancies [4]. Currently, several tools are available for exploring the etiology of PE, including thoracoscopy, chest imaging especially CT scan, cytology and bacterial culture [5,6]. Thoracoscopy is one of the most widely-used and has a good diagnostic performance for various thoracic disorders. Nevertheless, a previous study indicated that approximately 7% of patients with PE remain undiagnosed after thoracoscopy [2]. Besides, thoracoscopy is an invasive tool associated with procedure related complications. The microbiological and cytological examinations have high specificity but their diagnostic sensitivities are unsatisfactory [7–10]. Besides, the diagnostic accuracy of these tools is largely operator and pathologist dependent [11]. Bacterial culture has high diagnostic specificity for infectious causes of PE ; however, the long turnaround time (TAT) limits its application in clinical setting.

By contrast, serum and pleural fluid biochemical analyses have some advantages including but not limited to low cost, short TAT, easy standardization with less operator or observer variations. Indeed, some pleural fluid and serum markers have shown extremely high diagnostic accuracy in patients with PE. For instance, interleukin-27 (IL-27) [12], interferon-gamma [13] and adenosine deaminase (ADA) [14] pleural fluid levels for tuberculous PE, and serum N-terminal pro-brain natriuretic peptide (NT-proBNP) for CHF [4,15]. However, for malignant and infectious diseases, the diagnostic accuracy of available markers such as tumor markers [4,16,17], procalcitonin (PCT) [18] and C-reactive protein (CRP) [19] is unsatisfactory. Therefore, further researches are needed to identify novel markers in PE with increased diagnostic accuracy.

Here, we plan to perform a prospective, double-blind diagnostic trial, named A Study Investigating Markers in PLeural Effusion (SIMPLE). The aim of SIMPLE is to: (i) evaluate the diagnostic accuracy of serum and pleural fluid markers when used alone; (ii) compare the diagnostic

accuracy of two or more markers in a head-to-head manner; (iii) verify whether a novel marker can provide added diagnostic information beyond available traditional markers.

Method

Trial registration and foundation

All subjects, or their guardians, will be provided a full informed consent before inclusion in the study. The study has been registered with the Chinese Clinical Trial Registry platform (http://www.chictr.org.cn/index.aspx. Registration number: ChiCTR1800017449). Currently, this study is not supported by any grant; however, it may be supported by one or more grants from the Affiliated Hospital of Inner Mongolia Medical University or the Chinese government in future. The funders will not be involved in study design, sample collection, and data analyses.

Subject enrollment and specimen collection

Subjects who will be admitted to our hospital for an evaluation of the etiology of the pleural effusion will be eligible for enrollment. The presence of PE will be evaluated first by clinical examination and then further confirmed by chest imaging such as CT scan or ultrasound. The exclusion criteria are: (i) age less than 18 years; (ii) with a known diagnosis of a disease that could cause PE during the last three months; (iii) pregnancy; (iv) refused to sign informed consent; (v) with comorbidities that can prevent pleural fluid collection; (vi) subject dies during hospitalization without collection of pleural fluid and serum specimens; (vii) death of a subject during hospital stay before the final diagnosis; (viii) patients admitted without PE but developed PE after admission.

Pleural fluid specimen collection will be initiated after obtaining informed consent by the patient. Approximately 5-10 milliliters of pleural fluid specimen will be collected in a tube that does not contain any anticoagulant. The specimen will be sent to laboratory within 2 hours and centrifuged at 1200g for 10 minutes. The supernatants of the specimen will then be transferred to 10 Eppendorf tubes (550 μl tube) and immediately frozen at –80°C for later use. A serum sample will be collected from the same patient within 24 hours before or after the pleural fluid collection to be frozen at –80 °C if available. A case report form will be used to record demographic and clinical details of the subjects, such as age, sex, side of PE (left-, right- or two-sided effusion), smoking history, conventional laboratory tests and microbiological findings.

All subjects will be enrolled by a pulmonologist (L Yan). The subjects will be not consecutively enrolled because they may: (i) refuse to sign the informed consent; (ii) be admitted at weekend when Dr. Yan is not on duty; (iii) not be admitted to the Department of Respiratory and Critical Care Medicine of our institution.

The laboratory technician who determines the concentration of investigated markers will be blinded to clinical presentations of the subjects.

Sample size estimation

As this is not a hypothesis-driven research and no new target disease or marker is proposed, we did not estimate the sample size before subjects' enrollment. The study execute time will last from September 2018 to July 2021. It is estimated that 200 to 300 subjects will be enrolled.

Final diagnosis

This is an observational study that will not affect the further management of the enrolled subjects. The clinicians will decide the further diagnostic, treatment and management independent of this study. The etiologies of PE are diverse and the diagnostic criteria for the major diseases are listed in **Table 1**. The final diagnosis will be made by two researchers independently (L Yan and ZD Hu) and the results of investigated markers will not be known by them when making diagnosis.

Table 1 Diagnostic criteria for major diseases related to PE

Etiology	Diagnostic criteria
Tuberculous	Identification of *Mycobacterium tuberculosis* in the sputum, pleural fluid, or pleural biopsy specimens [20], either by microscopy or cultures. In some cases with adequate clinical context, the diagnosis can be made with presence of granuloma in the parietal pleura, good response to anti-tuberculosis treatment, elevated level of pleural fluid adenosine deaminase or positive nucleic acid amplification tests (NAATs) [20–22].

Congestive heart failure	Typical clinical picture of CHF including the Framingham score, medical history and physical examination, the response to diuretic therapy, typical CHF features on chest X-ray, the echocardiographic evidence of left ventricular systolic dysfunction [15,23–25].
Malignant diseases	Identification of cancer cells in pleural fluid, sputum or bronchoalveolar lavage fluid by cytological examination, ultrasound or thoracoscopy-guided pleural biopsy [5,24].
Parapneumonic effusion	Typical clinical and radiological evidences of pneumonia, or a positive bacterial culture from pleural fluid, or good response to antibiotic therapy [5,24].
Pulmonary embolism	Computed tomographic pulmonary angiography (CTPA) [26].

Given that approximately 30% of PEs have more than one etiology and the most common secondary cause is CHF [27,28]. Therefore, all patients will be evaluated for concomitant presence of CHF. For some subjects, the differential diagnosis can change during their admission only the diagnosis at the time of specimen collection will be used. We also realize that a confirmed diagnosis is not possible in all cases with PE at the time of discharge. This can be due to the fact that some subjects might refuse to receive further diagnostic invasive approaches such as thoracoscopy. The number of these subjects will be recorded and reported. The patients without final diagnosis will be excluded from the final analysis or will be considered as control in the data analysis.

Markers will be investigated in SIMPLE study

In addition to routinely measured markers (e.g. ADA, NT-proBNP), several novel markers will be studied (e.g. soluble Fas ligand, presepsin and pentraxin-3 and interleukin 27). The serum and fluid markers intended to be investigated are listed in Table 2. It should be noted that the decision of whether or not to investigate these markers is greatly determined by the prevalence of target disease as well as statistical power. Novel markers which are not listed in Table 2 may also be studied if they showed high accuracy in identifying the etiologies of PE. In addition, the accuracy of Light criteria in differentiating exudate from transudate will also be studied.

Table 2 Markers will be investigated in SIMPLE study

Target disease	Markers
Congestive heart failure	Mid-regional pro-atrial natriuretic peptide (MR-proANP)
Tuberculous pleurisy	Soluble Fas ligand; Interleukin 27 (IL-27); C-X- C motif chemokine receptor 3 (CXCR3) ligands
Malignant diseases	Soluble B7-H4; Human epididymis 4 (HE4); Cancer ratio; Dickkopf-1 (DDK1)
Parapneumonic effusion	Presepsin; Pentraxin-3

Patient and public involvement

Figure 1 is a flowchart depicting the study procedure. Subjects who will meet the inclusion and exclusion criteria will be invited to participate in this study. An informed consent will be signed before their participation. All subjects will not be involved in the recruitment and conduct of the study.

Routine serum and pleural fluid analysis will be ordered for these subjects. Pleural fluid specimens will be obtained for routine laboratory analysis, including cell count and differentiation, tumor markers, biochemistry, bacterial culture, Gram staining, cytology and nucleic acid amplification tests (NAAT). These laboratory tests will be ordered by the attending clinicians independent of this study. Approximately 5-10 milliliters of pleural fluid specimen will be collected simultaneously for research aims. All fluid and serum specimens will be collected at the time of admission before final diagnosis. The time period between the pleural fluid specimen collection and final diagnosis will be usually within one weeks, except for some subjects that need follow-up and therapy response to make diagnosis. Imaging (CT, MRI, CTPA), thoracoscopy and bronchoscopy will be ordered if necessary. The results of markers will be disseminated to the subjects via email or telephone upon request after the conclusion of the study.

Dissemination and ethics

The results of SIMPLE will be submitted to international scientific peer-reviewed journals or conferences in laboratory medicine or respiratory medicine, thoracic diseases. This study has been approved by the Ethic Committee of the Affiliated Hospital of Inner Mongolia Medical University (NO: 2018011).

Patient and public involvement statement

All subjects in SIMPLE study will not directly involved in the study design, recruitment and study conduction. There is no public involvement.

Statistical analysis

Normal distribution of continuous data will be tested by Kolmogorov-Smirnov test. For the data with normal distribution, independent t-test or one-way ANOVA will be used for comparison; Otherwise, Mann-Whitney or Kruskal-Wallis tests will be used. Chi-square test will be used to compare categorized data. Receiver operating characteristics (ROC) curve analysis will be used to evaluate the diagnostic accuracy of the investigated markers. Area under ROC curve (AUC) will be used to estimate the overall diagnostic accuracy of a markers. AUCs of markers will be compared by the approach proposed by Delong et al [29]. The optimal threshold will be determined with the method proposed by Pepe et al. [30] or maximum Youden index. When evaluating the diagnostic accuracy of a marker for a given disease, the subjects with this disease will be categorized into a disease group, regardless of whether other etiologies co-occur. Multivariable logistic regression model, net reclassification index (NRI) and integrated discriminatory index (IDI) will be used to evaluate whether a given marker provides added diagnostic information [31,32] beyond conventional diagnostic tools and clinical details (e.g. side information, age, sex, smoking history). Decision tree approach and decision curve analysis (DCA) [33] will be created to evaluate the preferred diagnostic strategy. All analyses will be performed with SPSS 18.0 (IBM Corporation, Chicago, United States), Sigmaplot 12.0 (Systat Software, Inc., San Jose, CA), Graphpad Prism 6.0 (GraphPad Software, La Jolla, CA) and R (http://www.r-project.org).

Discussion

Multiple new diagnostic markers have been identified in the evaluation of PE with the advancement in omics approach and basic research. Therefore, it is valuable to evaluate the diagnostic accuracy of these markers rigorously. Although several studies have been performed on this topic [12,34,35], the result of these studies need to be validated. This is because that the diagnostic accuracy of a given marker may be affected by the disease spectrum of a study cohort

[36]. Besides, majority of previous published studies evaluated only the diagnostic accuracy of single marker, and did not compare it with other promising markers. Furthermore, whether multi-marker strategy can improve the diagnostic accuracy remains largely unknown.

Compared with previous studies, SIMPLE study has some strength. First, this is a registered, prospective, double-blind study. Therefore, the results of this study are more reliable. Second, majority of the previous studies did not consider the subjects with multiple etiologies of PE and this issue will be considered by SIMPLE study. Third, only limited studies have investigated whether a novel marker could provide added diagnostic information beyond traditional markers. In SIMPLE study, we will investigate this issue with IDI and NRI, although they have some shortcomings [37–40].

Multiple potential serum and fluid markers will be investigated in the SIMPLE study. Previous studies have indicated that presepsin is a useful diagnostic marker for bacterial infection [41,42]; nevertheless, it remains unknown whether presepsin in serum or pleural fluid is useful for the diagnosis of parapneumonic effusion. Serum mid-regional pro-atrial natriuretic peptide (MR-proANP) has been reported to have high diagnostic accuracy for CHF [43]. However, only one study has investigated the diagnostic accuracy of MR-proANP in PE caused by CHF [23], and the results of this study need to be validated. In addition, some novel markers will also be studied, such as soluble Fas ligand [44] and interleukin 27 [12] for tuberculous pleurisy, soluble B7-H4 [45] and human epididymis 4 (HE4) [46] for malignant effusion. The results of SIMPLE study will be reported in accordance with the Standards for Reporting of Diagnostic Accuracy Studies (STARD) guideline [47]. For researches with multivariable prediction model, the report will comply with Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) statement [48].

SIMPLE study has some limitations. First, this is a single center study and representativeness of the study cohort is a limitation. Second, because it is not ethical to let all subjects receiving all diagnostic tools once a diagnosis has been made, partial verification bias can not be avoided. Indeed, establishing one diagnosis does exclude other etiologies. Third, the prognostic value of markers will not be evaluated in this study.

Taken together, SIMPLE study is a prospective, double-blind diagnostic study aims to investigate the diagnostic accuracy of serum and pleural fluid markers. Although it has some

limitations, we believe that this study will provide a new insight into the PE etiological field.

Authors' contributions

Z.D. Hu conceived and designed the study; L Zhang, P.H. Ouyang and P Li provided administrative support; L Zhang, P.H. Ouyang, P Li, L Yan and Y.Q. Han drafted the manuscript. Z.D. Hu critically revised the manuscript; all authors approved the final version of the manuscript.

Competing interests

None declared.

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Figure legend

Figure 1. Flowchart of study procedure.

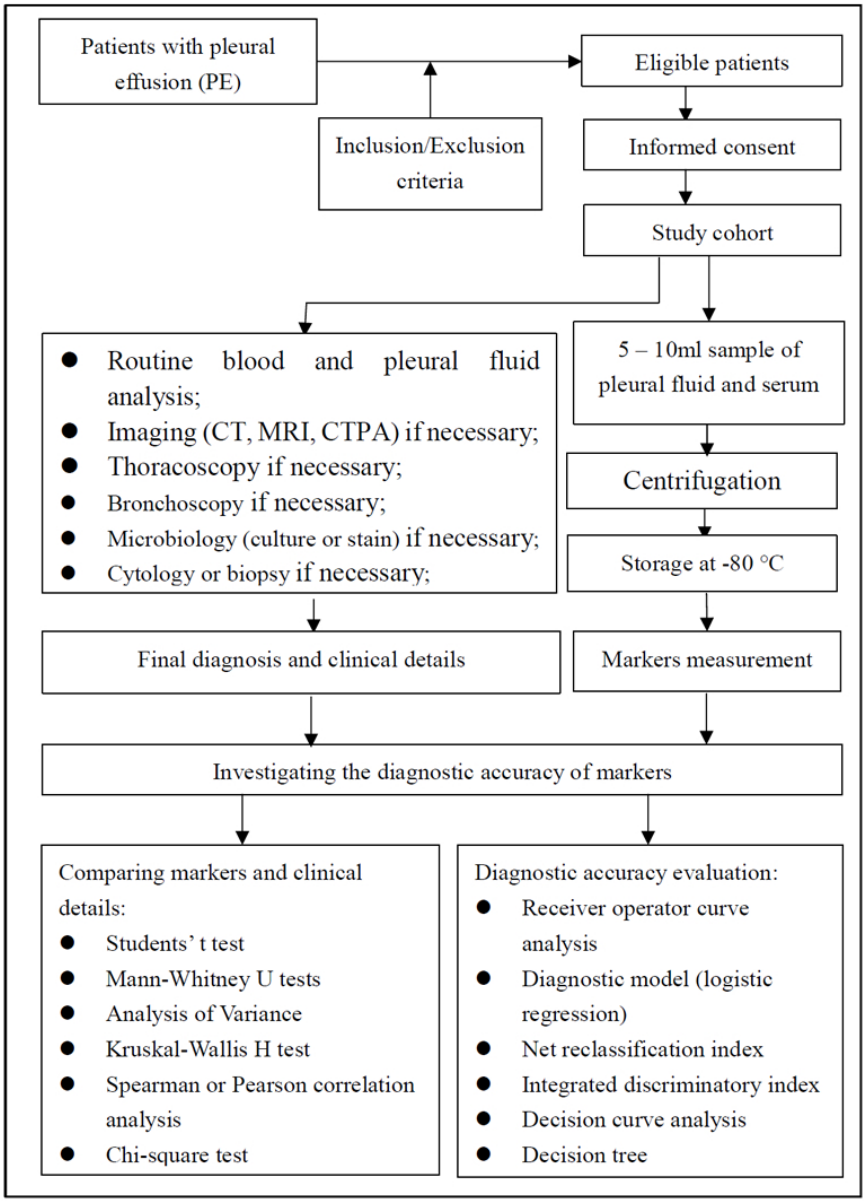

Figure 1. Flowchart of study procedure.

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