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ORIGINAL RESEARCH

Identification of a Four-Biomarker Panel for the Diagnosis of Tuberculous Pleural Effusion Using Olink Proteomics

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Background: Tuberculous pleural effusion (TPE) results from an inflammatory response triggered by tuberculosis infection. If not promptly diagnosed and treated, it can lead to severe pulmonary dysfunction and the risk of infection spread. However, TPE diagnosis remains a significant challenge. This study employs Olink proteomics to explore novel methods for diagnosing TPE.

Methods: In this study, we collected 20 cases each of TPE, malignant pleural effusion (MPE), and parapneumonic pleural effusion (PPE) from patients at the First Affiliated Hospital of Xinxiang Medical University in Xinxiang, China, between January and April 2024. Using Olink proteomics, we quantified 92 inflammation-related proteins in pleural effusions across these three patient groups. Differentially expressed proteins were identified, followed by enrichment and pathway analyses to explore potential underlying mechanisms. An independent validation cohort, consisting of 36 TPE samples and 29 non-tuberculous pleural effusion samples collected between April and July 2024, was used to validate the diagnostic performance of selected biomarkers by enzyme-linked immunosorbent assay (ELISA). Diagnostic accuracy was evaluated using logistic regression and receiver operating characteristic (ROC) curve analysis.

Results: A total of 92 inflammation-related proteins were identified in this study. Differential analysis identified 43 proteins with distinct expression levels between TPE and MPE, and 33 between TPE and PPE. Of these, 25 proteins were uniquely expressed in TPE. ELISA validation confirmed the expression of four key inflammatory proteins: IFN-γ, CXCL9, TNF-β and PD-L1. The combined area under the curve (AUC) for these markers was 0.963, with a sensitivity of 0.944 and specificity of 1, surpassing the sensitivity and specificity of individual or other biomarker combinations.

Conclusion: This study identified a diagnostic method using a combination of four biomarkers: IFN-γ, CXCL9, TNF-β, and PD-L1, which can aid in the diagnosis of TPE.

Keywords: tuberculous pleural effusion, Olink technology, inflammation-related biomarkers

Background

In 2023, the estimated number of incident tuberculosis (TB) cases worldwide was 10.80 million, representing a slight increase compared with 10.70 million cases in 2022 and exceeding the estimates for 2021 (10.40 million) and 2020 (10.10 million). Despite this, the overall pace of decline in TB incidence remains far below the milestone targets set by the End TB Strategy. Accordingly, TB was responsible for approximately 1.25 million deaths worldwide in 2023, once again becoming the leading cause of death from a single infectious disease.[1] While tuberculosis primarily affects the lungs, it can also involve the pleura, lymph nodes, bones, and other organs. Tuberculous pleural effusion (TPE) is one of the most common extra pulmonary manifestations of tuberculosis, with its incidence varying by region.[2,3] In areas with a high prevalence of tuberculosis, the

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incidence can reach up to 30%.[4] TPE develops due to an inflammatory response triggered by tuberculosis infection, leading to fluid accumulation in the pleural cavity. This not only exacerbates patient discomfort but also poses risks of severe complications such as respiratory failure and infection dissemination.[5] Therefore, timely and accurate diagnosis of TPE is crucial for improving patient outcomes.

Currently, traditional bacteriological, immunological, molecular biology and pathological methods for diagnosing TPE still have significant limitations. The detection rate of Mycobacterium tuberculosis (MTB) in pleural fluid using conventional smear microscopy is less than 10%,[6] and culturing pleural fluid for MTB can take several weeks, delaying treatment, and the culture yield is less than 30%.[6] Immunological tests, such as the tuberculin skin test (TST) based on the intradermal injection of purified protein derivative (PPD), offer advantages including ease of use and low cost. However, the antigens contained in the PPD compound may cross-react with several species of mycobacteria, potentially leading to low specificity in detecting Mycobacterium tuberculosis infection. The paucibacillary nature of pleural effusion is a key factor contributing to the low sensitivity of molecular biological tests.[7] Although a novel cartridge-based nucleic acid amplification test (Gene Xpert) has improved diagnostic rates for paucibacillary clinical specimens, its performance in pleural effusion is suboptimal. The overall sensitivity for pleural fluid is 50.9%.[8] Isolation and/or culture of MTB from pleural effusion or pleural biopsy samples, or histopathological confirmation of granulomas from pleural biopsy, can provide definitive diagnosis of TPE, offering higher sensitivity (93–100%) and accuracy.[9] However, it is undeniable that medical thoracoscopy, while effective, is an invasive and costly diagnostic procedure with a complication rate of 2–6%.[10,11] Furthermore, some patients with advanced underlying conditions or elderly patients may be unable to tolerate this procedure.[12]

Several biomarkers are currently recommended to improve the diagnosis of tuberculous pleural effusion (TPE). Among them, adenosine deaminase (ADA) and interferon-γ (IFN-γ) are commonly used biomarkers for evaluating TPE. A review study indicated that the sensitivity and specificity of ADA are 0.88 and 0.91, respectively, while the sensitivity and specificity of IFN-γ are 0.91 and 0.96, respectively.[13] These findings highlight the high diagnostic value of ADA and IFN-γ in TPE diagnosis, offering non-invasive and rapid detection methods that provide convenient diagnostic support for clinicians. However, it is important to acknowledge that there are still some limitations associated with these biomarkers. ADA is not specific to tuberculous pleuritis, as other infectious pleural diseases can also lead to elevated ADA levels.[14] While IFN-γ is highly correlated with tuberculosis, its levels can be influenced by the patient's immune status. In immunocompromised patients, the potential for false-negative results is higher.[15] Olink proteomics is based on the Proximity Extension Assay (PEA) technology, which successfully combines antibody-based immunoassays with the strengths of quantitative real-time PCR (qPCR) and next-generation sequencing (NGS). This approach enables a multiplexed and highly specific method, capable of simultaneously quantifying up to 3072 protein biomarkers. PEA is particularly well-suited for large-scale precision proteomics studies due to its high sensitivity, speed, high-throughput capacity, and specificity at the multiplex level. Olink proteomics technology has been widely applied in the research of cardiovascular diseases, cancers, and neurological disorders.[16–18]

In summary, traditional diagnostic methods for tuberculous pleural effusion (TPE) face significant challenges, highlighting the urgent need for biomarkers with high specificity and sensitivity. In this study, Olink proteomics was employed to identify differences in inflammation-related protein expression among patients with TPE, malignant pleural effusion (MPE), and parapneumonic pleural effusion (PPE). These differentially expressed proteins may serve as potential biomarkers and, if successfully translated from the discovery platform into ELISA-based assays or multi-marker diagnostic panels for clinical application, could aid in the rapid and minimally invasive diagnosis of TPE, thereby improving its diagnosis and clinical management.

Methods

Sample Collection

The study was approved by the Ethics Committee of the First Affiliated Hospital of Xinxiang Medical University (Approval No. EC-023-433) and conducted in accordance with its guidelines. Pleural effusion samples from 60 patients at the First Affiliated Hospital of Xinxiang Medical University were collected for the measurement of differential proteins using the Olink platform, which was part of the discovery phase of the study. This included 20 cases of TPE, 20 cases of MPE, and 20 cases of PPE. The discovery set samples were collected between January and April 2024. Given the results of the power analysis, no additional

samples were required. We selected TPE, MPE, and PPE groups based on their clinical relevance, as TPE is the primary focus of this study and MPE and PPE serve as the control groups to assess the specificity of the identified biomarkers. Subsequently, we collected an additional 36 cases of tuberculous pleural effusion as the experimental group, along with 29 cases of malignant and parapneumonic pleural effusions as the control group, for ELISA validation experiments. The validation set samples were collected between April and July 2024. Patient information is presented in Table 1. The complete workflow of this study can be found in Figure 1. We conducted case screening based on the following criteria:

Table 1 Information of TPE, MPE, and PPE Patient Samples Used for Olink Analysis

Discovery Cohort				
Index	**TPE (n=20)**	**Non-TPE (n=40)**		**P value**
		MPE (n=20)	**PPE (n=20)**	
Gender				0.002
Male	19	9	15	
Female	1	11	5	
Age (Mean ± SD)	43.60±17.04	69.95±10.42	63.95±12.18	<0.001
BMI (Mean ± SD)	20.89±3.19	21.47±2.67	24.62±5.01	0.009
Sample Collection Time (Mean ± SD)	52.95±26.97	68.2±20.38	75.20±24.12	0.019
Smoking History				0.125
Smoker	8	7	13	
Non-Smoker	12	13	7	
Comorbidity with Diabetes				0.074
Yes	4	2	8	
No	16	18	12	
Comorbidity with Hypertension				0.035
Yes	4	12	9	
No	16	8	11	
Comorbidity with Cardiovascular Disease				0.418
Yes	1	3	1	
No	19	17	19	
Validation cohort				
Index	**TPE (n=36)**	**Non-TPE (n=29)**		**P value**
Gender				0.774
Male	26	20		
Female	10	9		
Age (Mean ± SD)	52.00±14.95	66.41±14.84		0.790
BMI (Mean ± SD)	24.35±3.55	23.29±3.88		0.512
Sample Collection Time (Mean ± SD)	97.00±44.09	125.72±57.58		0.323
Smoking History				0.476
Smoker	13	13		
Non-Smoker	23	16		
Comorbidity with Diabetes				0.321
Yes	4	6		
No	32	23		
Comorbidity with Hypertension				0.532
Yes	11	11		
No	25	18		
Comorbidity with Cardiovascular Disease				0.227
Yes	2	5		
No	34	24		

Abbreviations: TPE, Tuberculous Pleural Effusion; Non-TPE, Non-tuberculous Pleural Effusion; MPE, Malignant Pleural Effusion; PPE, Parapneumonic Pleural Effusion; BMI, body mass index.

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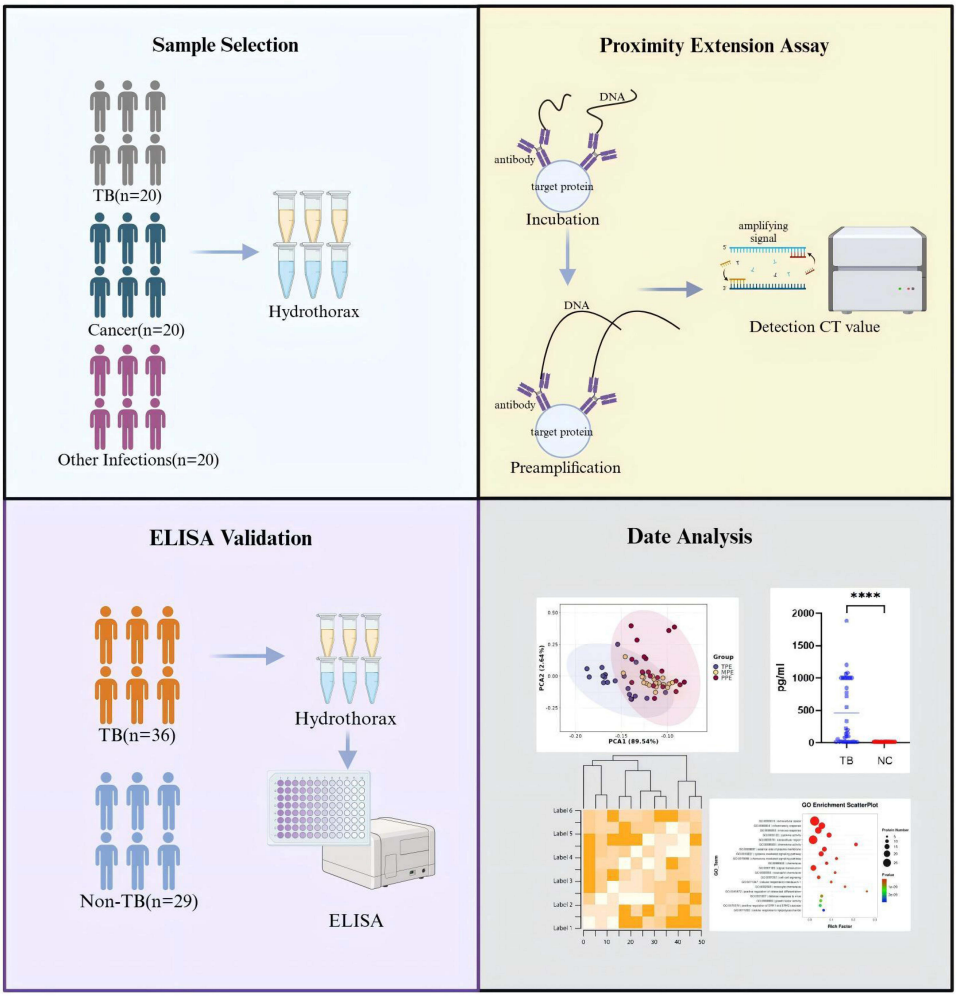

Figure 1 Flowchart of Sample Collection, Detection, and Analysis in This Study.

Tuberculous Pleural Effusion (TPE) Group

Patients were selected based on the presence of tuberculosis-related symptoms, such as cough or sputum production lasting for ≥ 2 weeks, hemoptysis, or other clinical signs suggestive of pulmonary tuberculosis. Diagnostic criteria included chest imaging and pleural fluid or pleural biopsy demonstrating tuberculous lesions, or chest imaging combined with pleural fluid bacteriological examination meeting microbiological criteria, confirming exudative pleural effusion.

Malignant Pleural Effusion (MPE) Group

Patients with symptoms of chronic malignancy, such as weight loss and fatigue, were included. Chest imaging revealed mass shadows, and histological or cytological examination confirmed malignant tumors. Tumor diagnoses were made according to the Chinese Society of Clinical Oncology (CSCO) guidelines for common malignancies (2020 edition) and the American Joint Committee on Cancer (AJCC) 8th edition TNM staging system. Patients were pathologically confirmed to be in stages I–IV, with exudative pleural effusion present. Individuals with latent or active tuberculosis were excluded from this group.

Parapneumonic Pleural Effusion (PPE) Group

Patients exhibiting clinical signs of infection, including chest pain, cough, sputum production, fever, and respiratory distress, were selected. Bacteriological examination of pleural fluid was positive, with evidence of inflammatory cell infiltration, and other causes of exudative pleural effusion were excluded.

Olink Proteomics

We applied Olink high-throughput protein analysis using the Target 96 Inflammation panel to qualitatively and quantitatively analyze 92 inflammation-related proteins in the three patient groups. Olink employs the Proximity Extension Assay (PEA) technology, where a pair of antibodies, equipped with specific nucleotide sequence probes, binds to the target protein. When the probes are in close proximity, complementary 5 bp overhangs pair and are extended by a DNA polymerase to form a double-stranded DNA template, which is then detected using qPCR or NGS. The detection is based on the specific nucleotide sequence signal, which reflects the amount of the target protein. This method requires only 1 μL of sample and is highly sensitive. For the analysis of these protein expression values, the NPX (Normalized Protein Expression) values were treated as quantitative variables. These values were used to assess the differential expression between groups, and all data were normalized and log2-transformed to ensure comparability across samples. To ensure analytical and sample quality, four internal controls were added per sample. Quality control involved assessing internal plate control deviation (<0.2 NPX) and sample deviation from median (<0.3 NPX), only data meeting these criteria were included.

Bioinformatics Analysis

The data exported from the Signature software was reformatted and supplemented with protein annotations from databases such as UniProt (https://www.uniprot.org/), Gene Ontology (https://geneontology.org/), and KEGG (https://www.kegg.jp/), resulting in a comprehensive protein information table. Gene Ontology (GO)functional enrichment analysis involved mapping all significantly differentially expressed proteins to the various terms in the Gene Ontology database. The number of proteins associated with each term was calculated, and hypergeometric testing was applied to identify GO terms that were significantly enriched in the differentially expressed proteins compared to the entire proteome background. A P-value of ≤ 0.05 was considered to indicate significant enrichment. KEGG is a major public database related to pathways, and pathway enrichment analysis was performed using KEGG pathways. Hypergeometric testing was employed to identify pathways significantly enriched among the differentially expressed proteins compared to the background proteome. Protein interaction analysis for identified proteins was conducted using the StringDB (https://string-db.org/). If the corresponding species was available in the database, the sequences of the species were directly extracted; if not, sequences from closely related species were retrieved. The sequences of differentially expressed proteins were then aligned with the extracted sequences using Basic Local Alignment Search Tool (BLAST), and the corresponding interaction information was obtained to construct a network diagram.

ELISA Validation

In this study, 65 cases were used for ELISA validation., including 36 cases in the TPE group and 29 cases in the non-TPE control group for ELISA validation. Five inflammatory factors were validated: IFN-γ (EHC102g, Xinbosun Technology Co, Ltd, China), CXCL9 (EHC114, Xinbosun Technology Co, Ltd, China), TNF-β (EHC039b, Xinbosun Technology Co, Ltd, China), PD-L1 (EHC071, Xinbosun Technology Co, Ltd, China), and CD6 (MM-62195H1, Enzyme Immunoassay Co, Ltd, China). The procedures and analyses were conducted according to the manufacturer's protocols.

Statistical Analysis

Data analysis for the discovery cohort was conducted using R software (version 4.1.3). For the validation cohort, data analysis and visualization were performed using IBM SPSS Statistics (version 26.0) and GraphPad Prism (version 10.1.2). $P < 0.05$ was considered statistically significant.

Results

Comprehensive Visualization of Protein Expression

Heatmap (Figure 2A) presents the expression levels of 92 inflammation-related proteins across different samples. The clustering of protein expression patterns among the samples is prominent, reflecting distinct classifications of inflammatory states. Based on these expression patterns, the samples are clearly divided into three groups, representing

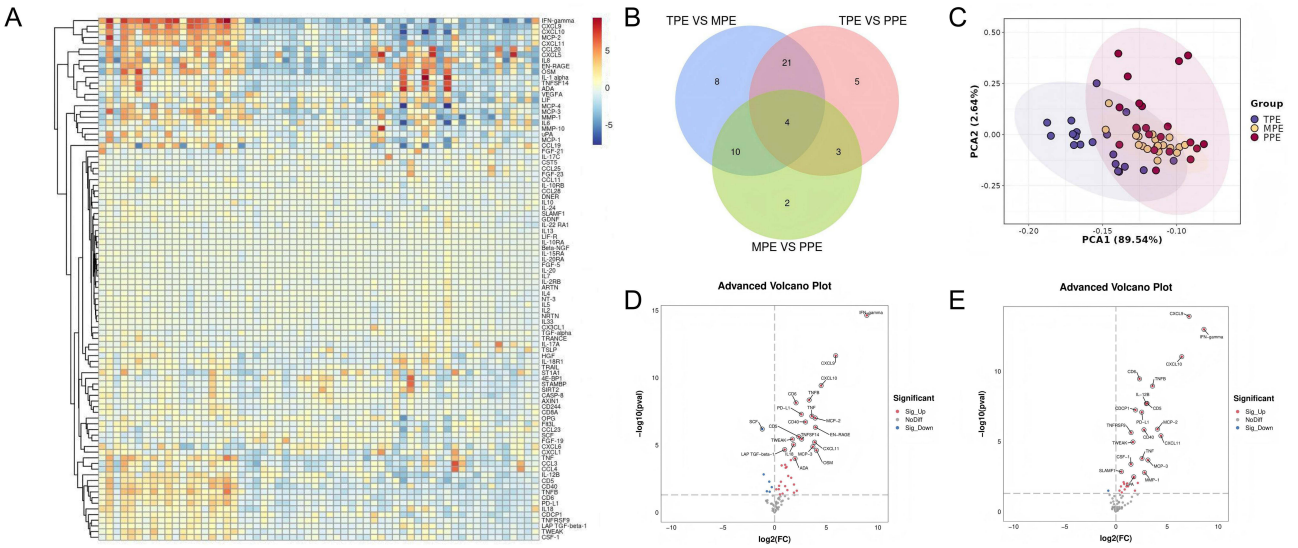

Figure 2 Differentially expressed inflammation-related biomarkers among TPE, MPE, and PPE groups. (**A**) Heatmap of 92 inflammation-related proteins. (**B**) Venn diagram illustrating the overlap and specificity of differentially expressed proteins among TPE, MPE, and PPE groups. (**C**) PCA plot displaying the distribution of TPE, MPE, and PPE samples along the first two principal components (PCA1 and PCA2). (**D**) Volcano plot showing differentially expressed inflammatory biomarkers between TPE and MPE. (**E**) Volcano plot visualizing the differential expression of inflammation-related biomarkers between TPE and PPE samples.

different inflammatory conditions. This grouping is consistent with clinical diagnoses, further confirming the differential protein expression profiles. The Venn diagram (Figure 2B) illustrates the overlap and specificity of gene expression among the three sample groups. A total of 53 genes are commonly expressed across all three groups. In the TPE group, 21 proteins are uniquely expressed, indicating their possible association with specific inflammatory responses or biological processes in this group. We used principal component analysis (PCA) (Figure 2C) to depict the distinct protein expression levels among the three sample types. After normalization, PCA was performed using a mean-centered approach, scaled by the standard deviation of each variable. PCA demonstrate clear separation between TPE, MPE, and PPE samples. The first principal component explains 89.54% of the total variance, while the second accounts for 2.64% of the variance. Differential protein analysis was conducted between the TPE and PPE, as well as the TPE and MPE groups. The volcano plot reveals that, compared to the MPE group, 37 proteins were up regulated and 6 were down regulated in the TPE group (Figure 2D). Similarly, compared to the PPE group, 32 proteins were up regulated and 1 was down regulated in the TPE group (Figure 2E).

Differential Analysis of Inflammation-Related Proteins

To investigate tuberculosis-specific inflammatory biomarkers, we established two comparison groups: TPE vs. MPE and TPE vs. PPE. In the TPE vs. MPE group, 43 inflammation-related proteins were differentially expressed, Figure 3A shows the top 20 differential proteins. In the TPE vs. PPE group,33 inflammation-related proteins were differentially expressed, Figure 3B shows the top 20 differential proteins. A total of 25 biomarkers were specifically differentially expressed in TPE compared to both non-TPE control groups, including IFN-γ, CXCL9, CXCL10, TNF-β, CD6, PD-L1, TNF, MCP-2, CD40, CD5, TWEAK, CXCL11, MCP-3, LAP TGF-beta-1, CSF-1, CD244, SLAMF1, TNFRSF9, IL-12B, CCL25, MMP-1, IL-10RB, IL6, and IL8. We hypothesize that these 25 inflammatory factors play a critical role in tuberculosis infection.

Functional Enrichment Analysis of Differential Proteins

In the comparison between TPE and MPE, GO enrichment analysis (Figure 4A) revealed significant enrichment of TPE in terms related to inflammatory response (GO:0006954), extracellular space (GO:0005615), and cytokine activity (GO:0005125), indicating a more active immune and inflammatory response in TPE. Furthermore, KEGG enrichment (Figure 4C) showed that TPE was significantly enriched in the TNF signaling pathway (hsa04668), JAK-STAT signaling

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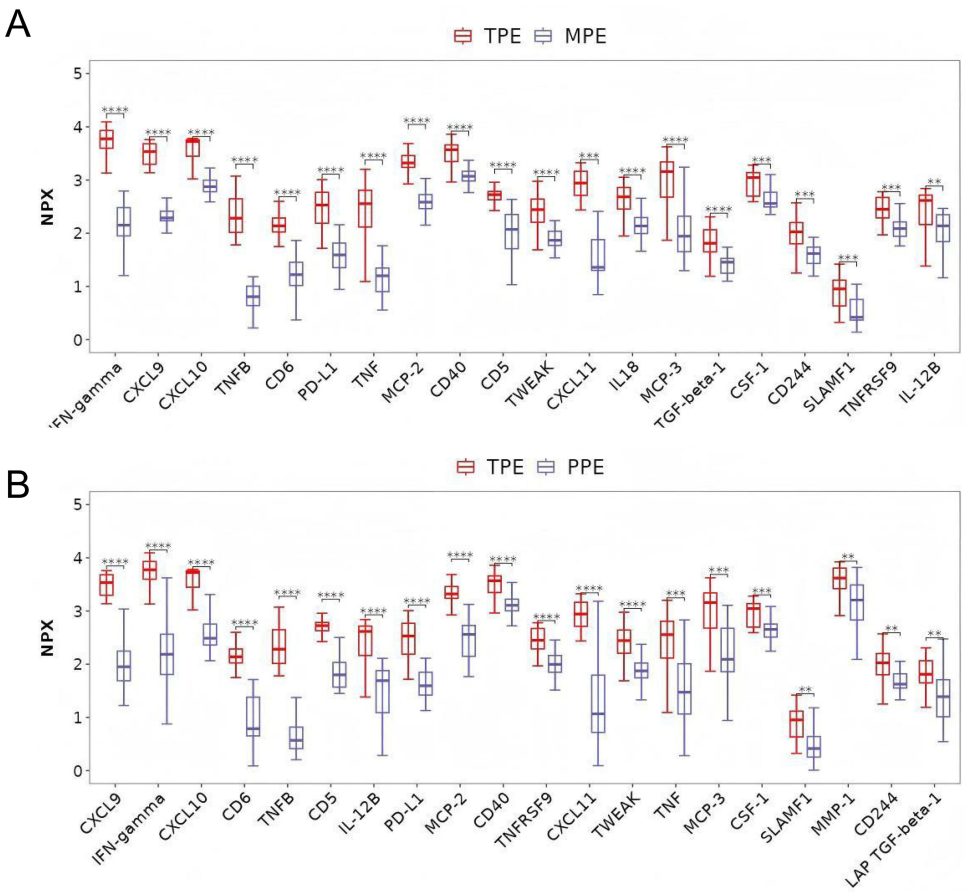

Figure 3 Comparative analysis of inflammation-related protein components in TPE, MPE, and PPE pleural effusions. (**A**) Box plot illustrating the relative expression levels (NPX) of various inflammatory biomarkers between TPE and MPE (**p < 0.01, ***p < 0.001, ****p < 0.0001). (**B**) Box plot illustrating the relative expression levels (NPX) of various inflammatory biomarkers between TPE and PPE (**p < 0.01, ***p < 0.001, ****p < 0.0001).

pathway (hsa04630), and apoptosis pathway (hsa04210), which are closely related to immune regulation and cell death processes in tuberculosis. These enriched pathways suggest a stronger immune response and cellular damage in tuberculous pleural effusion, highlighting the specificity of its pathological process.

In the comparison between TPE and PPE, Figure 4B shows that TPE also demonstrated significant enrichment in GO terms such as inflammatory response, cytokine activity, and chemokine activity (GO:0008009). Additionally, KEGG pathway analysis (Figure 4D) revealed significant enrichment of the NF-kappa B signaling pathway (hsa04064) and the JAK-STAT signaling pathway, indicating greater activity of these immune signaling pathways in tuberculous pleural effusion. This suggests that TPE exhibits a more distinct regulatory pattern in chronic inflammation and immune modulation.

Protein-Protein Interaction

The analysis of the two network diagrams revealed that TNF and IL6 occupy central positions in both comparison groups, indicating that they play a pivotal role as key signaling molecules in immune regulation in patients with TPE. Additionally, the network for TPE vs. MPE (Figure 5A) is more complex, with a higher number of protein interaction nodes and greater network density, particularly in inflammation-related signaling pathways such as the TNF signaling pathway. This suggests that immune and inflammatory responses are more prominent in TPE compared to MPE, supporting the dominant role of chronic inflammation and immune responses in the pathology of tuberculous pleural effusion.

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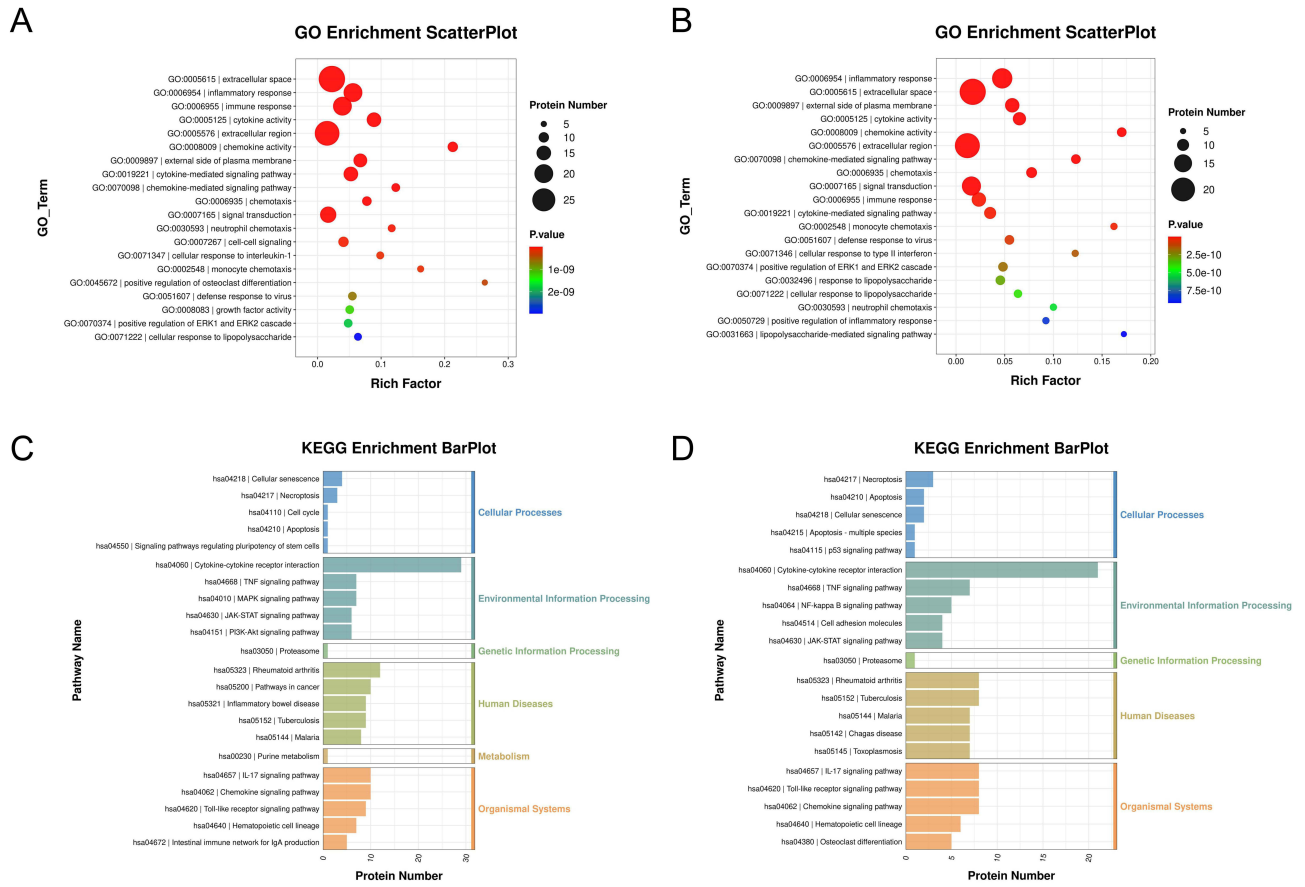

Figure 4 Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differentially expressed inflammation-related proteins. (**A**) Enrichment of differentially expressed proteins in TPE compared to MPE samples. (**B**) Enrichment of differentially expressed proteins in TPE compared to PPE samples. (**C**) KEGG enrichment bar plot of differentially expressed proteins between TPE and MPE. (**D**) KEGG enrichment bar plot of differentially expressed proteins between TPE and PPE.

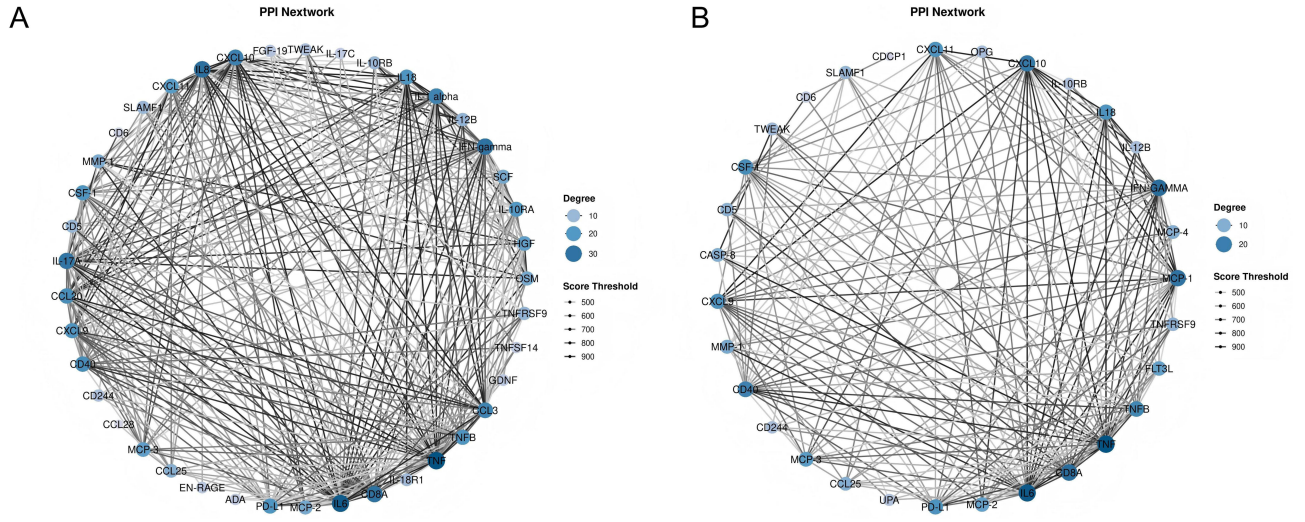

Figure 5 Protein-Protein Interaction (PPI) networks of differentially expressed proteins in TPE, MPE, and PPE samples. Each node in the figure represents a protein, with the color intensity indicating the degree of the protein in the network (the number of connections with other proteins), and the size of the node representing the relative interaction strength. (**A**) PPI network of differentially expressed proteins between TPE and MPE. (**B**) PPI network of differentially expressed proteins between TPE and PPE.

In contrast, the network diagram for TPE vs. PPE (Figure 5B) presents a relatively simpler structure, but still highlights the central roles of key inflammatory factors such as TNF, IL6, and IFNG. This indicates that while both groups involve inflammatory responses, certain regulatory mechanisms in TPE may be more specific, particularly in the key regulatory

functions of the TNF signaling pathway. Additionally, the significant enrichment of CXCL family chemokines in TPE compared to PPE suggests that chemokine activity may play a crucial role in the pathology of TPE, further supporting the significance of chemokine-related terms identified in the GO enrichment analysis.

ELISA Validation of Selected Biomarkers

We selected five inflammatory factors—IFN-γ, CXCL9, TNFB, PD-L1, and CD6—for ELISA validation. As shown in Figure 6A, the concentrations of IFN-γ and CXCL9 were significantly higher in the TB group compared to the non-TPE control

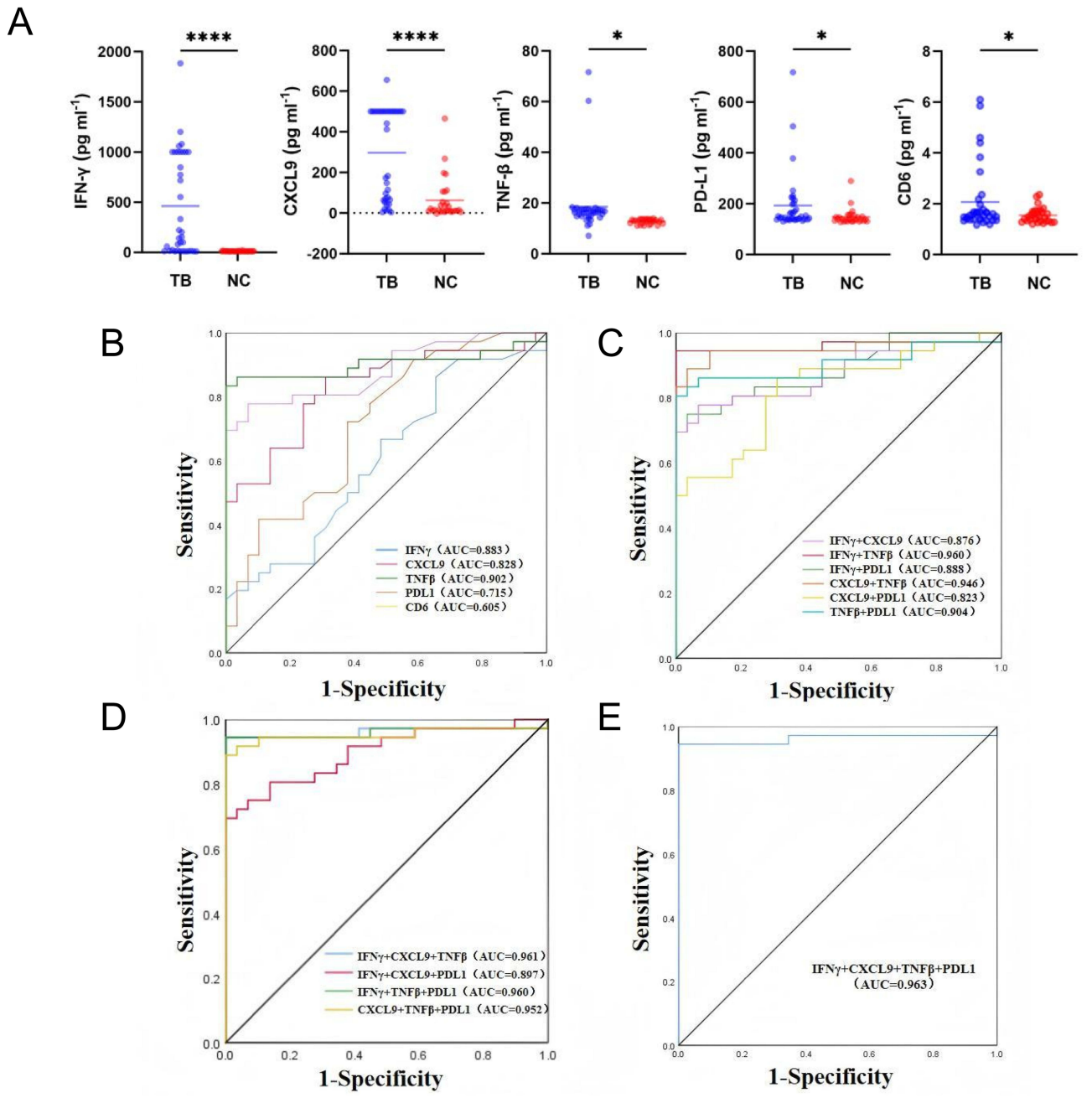

Figure 6 ELISA validation of five inflammation-related proteins in pleural effusions from 65 patients. (**A**) Concentrations (pg/mL) of IFN-γ, CXCL9, TNF-β, CD6, and PD-L1 in the pleural effusions of TPE (n=36) and non-TPE (n=29) groups (*p < 0.05, ****p < 0.0001). (**B**) Receiver operating characteristic (ROC) curves for IFN-γ, CXCL9, TNF-β, CD6, and PD-L1. (**C**) ROC curves for paired combinations of IFN-γ, CXCL9, TNF-β, and PD-L1. (**D**) ROC curves for three-factor random combinations of IFN-γ, CXCL9, TNF-β, and PD-L1. (**E**) ROC curve for the combined diagnostic performance of IFN-γ, CXCL9, TNF-β, and PD-L1.

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group, indicating their critical roles in tuberculosis infection. The concentrations of TNF-β and PD-L1 were also slightly higher in the TB group but remained statistically significant. These results are consistent with the findings from the Olink proteomics analysis. Moreover, the ROC curve analysis (Figure 6B) demonstrated that the AUC for IFN-γ was 0.883 (P<0.001, 0.801–0.964), for CXCL9 it was 0.828 (P<0.001, 0.728–0.928), for TNF-β it was 0.902 (P<0.001, 0.816–0.988), for PD-L1 it was 0.715 (P=0.003, 0.588–0.841), and for CD6 it was 0.605 (P=0.071, 0.467–0.744). Based on these results, we selected IFN-γ, CXCL9, TNF-β, and PD-L1 for further analysis. Using logistic regression, we created two-factor (Figure 6C), three-factor (Figure 6D), and four-factor combinations (Figure 6E) using logistic regression to fit a combined ROC curve. The results showed that the four-factor combination yielded the highest AUC, at 0.963, with a sensitivity and specificity of 0.944 and 1, respectively, demonstrating a clear advantage over single-factor or other combinations.

Discussion

Tuberculous pleurisy is a highly detrimental disease that, if not promptly diagnosed and treated, can lead to severe pulmonary dysfunction, including the development of pulmonary fibrosis or respiratory failure. Additionally, the high transmissibility of tuberculosis poses a significant public health threat, making early diagnosis and treatment crucial. Tuberculous pleurisy is caused by the infection of the pleura by Mycobacterium tuberculosis. However, in the pleural effusion immune environment, the proliferation of Mycobacterium tuberculosis is inhibited by immune cells and cytokines, and the oxygen and nutritional conditions of the pleural effusion are also unfavorable for bacterial growth, resulting in a low bacterial load in the pleural effusion. This characteristic makes traditional diagnostic methods for tuberculous pleural effusion, such as culture and microscopy, often unreliable for detecting the pathogen, which may lead to false-negative results or delayed diagnosis. However, when Mycobacterium tuberculosis infects the pleura, the immune response is activated, with macrophages as the first responder immune cells, along with other immune cells, releasing a variety of cytokines that activate T cells and initiate adaptive immunity. From the progression of the disease, inflammatory biomarkers can reflect the immune response to tuberculosis infection, making them valuable as potential diagnostic tools and providing strong support for early diagnosis. ADA,[19] IFN-γ,[20] IL27[7] and soluble interceptor (sIL-2R)[21] are among the most extensive diagnostic biomarkers for TPE. However, to date, no reliable and stable biomarkers have been identified to accurately confirm TPE, and in areas with a high prevalence of tuberculosis, the diagnostic accuracy of a single biomarker is generally lower than expected.[22]

In this study, 24 differentially expressed inflammatory proteins were identified as potential biomarkers to distinguish TPE from non-TPE patients. Through differential expression analysis and enrichment analysis, we ultimately selected five inflammatory factors—IFN-γ, CXCL9, TNF-β, PD-L1, and CD6—for ELISA validation. IFN-γ, a cytokine secreted by T cells and natural killer cells, plays a critical role in combating tuberculosis infection. Direct detection of IFN-γ in TPE using non-stimulated methods has demonstrated high sensitivity (89%) and specificity (97%), potentially surpassing ADA as a diagnostic marker.[6,23] IFN-γ concentrations exceeding 140 pg/mL offer a sensitivity of 94%, while concentrations over 240 pg/mL yield a sensitivity of 95%.[24,25] Recent studies suggest that combining both methods provides 100% specificity.[26] CXCL10 and CXCL9, members of the chemokine family, are key regulators of cell migration and inflammatory responses, functioning via the CXCR3 signaling pathway.[27,28] Studies have shown that CXCL9 is significantly upregulated in patients with MTB infection, particularly in those with active tuberculosis. CXCL10 and CXCL9 have been explored as auxiliary biomarkers for distinguishing between active and latent tuberculosis infections,[29,30] though their diagnostic accuracy may decrease with age.[31] In this study, both IFN-γ and CXCL9 demonstrated good diagnostic performance. TNF-β, a pro-inflammatory cytokine, plays a key role in the TNF signaling pathway, which involves apoptosis mediated by the caspase family, TRAF-mediated activation of the transcription factor NF-κB, and JNK protein kinase, all of which are crucial in combating tuberculosis infection.[32] Studies in zebrafish have revealed a marked increase in TNF-β expression during latent and chronic infection stages, suggesting its important role in controlling latent MTB infection.[33] While TNF-β shares some functional similarities with TNF-α, which has been extensively studied in the diagnosis and pathophysiology of TPE, its lower specificity limits its potential as an independent diagnostic marker for TPE.[34,35] CD6 is a 105–130 kDa type I transmembrane glycoprotein,[36] expressed on most T cells and a subset of B cells and natural killer (NK) cells.[37,38] By interacting with ligands such as ALCAM (activated leukocyte cell adhesion molecule), CD6 promotes the adhesion and migration of immune cells, which is critical for immune cell aggregation in tuberculous pleurisy.[39] Recent studies have reported altered CD6 expression in cancer and autoimmune diseases;[40,41] however, its role in TPE diagnosis remains limited. Although our ELISA validation did

not demonstrate diagnostic value for CD6, the Olink proteomics results showed an opposite trend, which may be attributed to sample size or the detection limit of the ELISA assay. Further studies are warranted to explore the potential diagnostic value of CD6. Programmed death-ligand 1 (PD-L1), a key molecule in the immunosuppressive pathway, is overexpressed in tuberculosis infections, potentially leading to antigen-specific T-cell dysfunction (T-cell exhaustion), which limits the host's ability to clear MTB.[42] This immune evasion mechanism contributes to the persistence of MTB infection.[43] Studies have found that PD-L1 expression is significantly elevated in neutrophils and monocytes/macrophages in patients with active tuberculosis, and a reduction in PD-L1 expression is closely associated with clinical improvement.[44] In vitro experiments show that blocking the PD-1/PD-L1 pathway enhances macrophage phagocytosis and intracellular killing of MTB, indicating that PD-L1 may serve as a potential diagnostic marker for TPE. Due to the low AUC and non-significant P-value (>0.05) for CD6 in ELISA validation, we excluded it from further analysis. Instead, we performed a combined analysis of the remaining four inflammatory factors, finding that the combined diagnostic sensitivity and specificity were 0.944 and 1, respectively. This suggests that the combined diagnostic biomarkers identified in this study may offer a diagnostic advantage for TPE.

Enrichment analysis of 92 inflammation-related proteins revealed significant pathway differences between TPE, MPE, and PPE. TPE showed enhanced enrichment in immune response, cell death, and tuberculosis-specific pathways. Cytokine-cytokine receptor interaction, TNF signaling pathway, MAPK signaling pathway, JAK-STAT signaling pathway, and PI3K-Akt signaling pathway: These pathways are closely associated with immune responses, inflammation, and cell signaling, and may play a crucial role in identifying TPE-specific biomarkers. The cytokine-cytokine receptor interaction pathway is a crucial signaling pathway in the immune system, involving interactions between cytokines and their receptors. In tuberculous pleural effusion (TPE), tuberculosis infection triggers a strong immune response, leading to the release of inflammatory cytokines such as IFN-γ, TNF-α, and IL-6. These cytokines bind to their respective receptors, regulating the activity of immune cells, recruiting additional immune cells to the site of infection, and assisting in the clearance of the pathogen.[45] TNF plays a key role in the immune response against Mycobacterium tuberculosis (MTB). During the immune response, TNF performs several critical functions: inducing apoptosis in infected macrophages, activating macrophages and promoting the formation of reactive oxygen and nitrogen species, dendritic cell maturation, subsequent activation of T cells, secretion of interferon-gamma (IFN-γ), stimulation of T lymphocyte and monocyte chemotaxis, and granuloma formation.[46] The MAPK signaling pathway is vital for immune cell proliferation, differentiation, and survival. In tuberculosis infection, the MAPK pathway regulates immune cell responses by activating molecules such as ERK, JNK, and p38, playing a significant role in the inflammatory process.[47] The activation of the MAPK pathway helps the immune system recognize and eliminate MTB, thus playing a key role in the immune response in TPE. The JAK-STAT pathway, by mediating the signaling of cytokines (especially IFN-γ), regulates the activity of immune cells.[48] STAT1, as a key transcription factor in this pathway, along with its associated molecules, can serve as potential biomarkers for TPE.[49] The activation of the PI3K-Akt signaling pathway is closely related to immune responses, cytokine secretion, and immune cell survival. By detecting Akt and its phosphorylated forms, the activation state of this pathway in TPE can be assessed, which may help distinguish TPE from other types of pleural effusion.[50,51] A comparison of protein interaction networks between tuberculous pleural effusion and malignant or inflammatory pleural effusions highlighted the central roles of TNF, IL6, IFNG, and chemokines in tuberculous pleural effusion. The enhanced interaction between IL17A and IFNG suggests a specific Th1/Th17 immune response.[52] These findings provide critical insights into the molecular mechanisms of tuberculous pleural effusion and reveal potential diagnostic and therapeutic targets. Future studies could further validate the functional roles of these differentially enriched pathways in TPE, integrating multi-omics data (eg., proteomics, metabolomics) for a more in-depth mechanistic understanding. The key proteins in these enriched pathways hold promise as specific biomarkers for TPE, offering potential targets for early diagnosis and targeted therapy. Additionally, exploring how these biomarkers can be applied in clinical detection and treatment will provide new perspectives and directions for precision medicine in tuberculosis.

Limitations

We must also acknowledge that this study has certain limitations. The relatively small sample size might limit how well the findings apply to larger populations. Additionally, by focusing on specific inflammatory proteins through Olink proteomics,

other potentially relevant biomarkers may have been missed. The study also lacks validation in more complex clinical environments, which may affect the reliability of the novel biomarker panel. Another limitation of this study is the relatively high detection limit of the ELISA kits used, which may restrict the validation of proteins present at very low concentrations. As a result, additional methods with lower detection limits, such as mass spectrometry or ultrasensitive assays, may be needed to fully assess the diagnostic potential of low-abundance differential proteins.

Conclusion

This study identified an inflammatory biomarker panel comprising IFN-γ, CXCL9, TNF-β, and PD-L1 that demonstrated high diagnostic performance for distinguishing tuberculous pleural effusion from other types of pleural effusion. This biomarker combination has the potential to improve diagnostic accuracy and reliability and may contribute to the development of more effective diagnostic strategies for TPE. However, given the modest sample size and single-center design, these findings should be considered preliminary, and further validation in larger, multicenter cohorts is required before clinical implementation.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Research Ethics and Consent to Participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University (No.EC-023-433). Informed consent was obtained from all individual participants included in the study. Additionally, this study adheres to the RECORD (Reporting of studies Conducted using Observational Routinely-collected health Data) guidelines.

Author Contributions

Xiaolin Xing; Conceptualization, Formal analysis, Methodology, Writing-Original draft. Can Guo; Resources, Methodology, Writing-Original draft. Guizeng Zhao; Resources, Methodology, Writing-Original draft; Duanduan Xie; Validation, Formal analysis, Writing-Review and editing. Linbo Zhang; Resources, Formal analysis, Writing-Review and editing. Ke Shi; Data curation, Writing-Review and editing. Zhiqiang Zhang; Conceptualization, Writing-Review and editing. Yu Pang; Conceptualization, Funding acquisition, Methodology, Writing-Review and editing. Junwei Cui; Conceptualization, Funding acquisition, Methodology, Writing-Review and editing. All authors gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors state no conflict of interest.

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