

# Identification and Validation of Ferroptosis-Related Biomarkers and Therapeutic Targets in ARDS: A Bioinformatics and Experimental Study

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**Background:** Acute respiratory distress syndrome (ARDS) is a severe inflammatory lung disease with high mortality and limited effective therapies. Recent studies link ferroptosis—an iron-dependent regulated cell death—to ARDS pathogenesis. This study aimed to identify/validate ferroptosis-related diagnostic biomarkers, therapeutic targets, and fecal microbiota transplantation (FMT)'s protective role in ARDS.

**Methods:** Bioinformatic analyses of GEO datasets (GSE76293/GSE151263) included differential expression profiling, WGCNA, PPI network, and machine learning (LASSO/RF) to screen hub genes, with ROC analysis for diagnostic efficacy. An LPS-induced ARDS rat model with FMT intervention was validated via qRT-PCR, IHC, Western blot, and histological staining.

**Results:** Thirty-seven ferroptosis-linked differentially expressed genes (FDEGs) were identified, enriched in ferroptosis, mitophagy, and immune pathways. Three hub genes (MAPK8, CREB1, GPX4) showed robust diagnostic utility (LASSO AUC=0.931; RF AUC=0.993 in GSE76293) and correlated with monocytes/neutrophils/activated NK cells. LPS suppressed their mRNA/protein levels in rats, reversed by FMT.

**Conclusion:** MAPK8, CREB1, and GPX4 are potential diagnostic biomarkers and therapeutic targets for ARDS. FMT protects against ARDS by reversing these genes' downregulation and suppressing ferroptosis, providing new insights into ARDS pathogenesis and ferroptosis-targeted interventions.

**Keywords:** acute respiratory distress syndrome, ferroptosis, fecal microbiota transplantation, GPX4, MAPK8, CREB1

## Introduction

Ferroptosis, a form of regulated cell death characterized by iron-mediated lipid peroxidation, is implicated in oxidative stress and inflammatory cascades that drive tissue injury across pathological contexts such as acute respiratory distress syndrome (ARDS).<sup>1,2</sup> Within ARDS, iron-dependent mechanisms promote mortality in alveolar epithelial and endothelial cells, thereby amplifying inflammatory processes and disrupting redox balance, which collectively exacerbates pulmonary damage.<sup>1-3</sup> Experimental and clinical analyses indicate that disturbances in iron metabolism

and dysregulation of ferroptosis-associated genes serve as contributors to ARDS advancement.<sup>4,5</sup> Nonetheless, the intricate molecular cascades linking ferroptosis to immune pathways are not yet comprehensively delineated in this disease.<sup>1</sup>

Advancements in high-throughput genomic technologies enable robust bioinformatic strategies to uncover critical genes and signaling networks in multifaceted disorders like ARDS.<sup>6,7</sup> This investigation harnessed transcriptomic profiles from accessible ARDS repositories, combined with ferroptosis-related gene signatures, to screen for diagnostic biomarkers and intervention targets.<sup>6,8</sup> Through an integrative approach incorporating differential gene expression scrutiny, weighted gene co-expression network analysis (WGCNA), protein-protein interaction mapping, and machine learning-based algorithms, we identified three hub genes—MAPK8, CREB1, and GPX4—that are closely associated with ferroptosis in ARDS. Increasing evidence underscores the gut-lung axis as an essential regulatory mechanism that integrates intestinal microbiota homeostasis with pulmonary immune responses and tissue damage.<sup>9,10</sup> Disruption of gut microbiota equilibrium impairs intestinal barrier function, induces systemic inflammation, and intensifies lung injury in ARDS. In contrast, microbial diversity restoration through fecal microbiota transplantation (FMT) represents a promising therapeutic approach.<sup>10,11</sup> FMT facilitates gut-lung axis modulation by replenishing commensal bacteria that synthesize metabolites such as short-chain fatty acids; these compounds inhibit pro-inflammatory cytokine production and promote anti-inflammatory immune cell differentiation.<sup>12,13</sup> Critically, preclinical investigations have associated FMT with ferroptosis pathway regulation, where it mitigates oxidative stress—a primary driver of ferroptosis in ARDS.<sup>1,14</sup> For instance, studies indicate that FMT can counteract the suppression of ferroptosis inhibitors (eg, GPX4) and diminish lipid peroxidation in lung injury models,<sup>11,12</sup> directly supporting its relevance for exploring ferroptosis-associated biomarkers in ARDS. Consequently, this study employed FMT as an interventional strategy to evaluate the functionality of hub genes and their regulatory potential through gut-lung axis pathways.<sup>10,15</sup> To this end, we established a lipopolysaccharide (LPS)-induced ARDS rat model, in which FMT was administered to explore its modulatory effects. The expression and protective roles of these hub genes were validated in this model, complemented by structural bioinformatics analysis to characterize the functional domains of the corresponding proteins. Further experimental validation confirmed their functional importance and the modulatory outcomes.<sup>16</sup>

These results collectively underscore the involvement of ferroptosis in ARDS progression and point to potential biomarkers and therapeutic strategies aimed at alleviating ferroptotic damage.<sup>1,3</sup>

## Materials and Methods

### Data Acquisition and Processing

Gene expression datasets related to acute respiratory distress syndrome (ARDS) were retrieved from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) via the GEOquery package,<sup>17</sup> followed by preprocessing and differential expression analysis via the limma package. We downloaded the GSE76293 dataset, which contains both ARDS and normal sample data. The data were normalized with the limma package, and a linear model was constructed. Differential gene expression analysis was then performed via the Bayesian method to compare ARDS samples with normal controls. We then utilized the ggplot2 package to visualize the results of our differential gene expression analysis, creating volcano plots and heatmaps to illustrate the significance and fold changes in gene expression, as well as the expression patterns across samples. The use of GEO database data in this study did not require specific ethical approval as it involved analysis of publicly available datasets.

### Gene Set Enrichment Analysis

To investigate the biological differences between sample groups, gene set enrichment analysis (GSEA) was performed on the differentially expressed genes (DEGs). The following thresholds of significance were employed: absolute NES values > 1, adjusted  $P < 0.05$ , and FDR  $q$  values = 0.25.

## Weighted Gene Co-Expression Network Analysis (WGCNA)

We performed weighted gene coexpression network analysis (WGCNA) via the “WGCNA” package in R to construct a weighted gene coexpression network. First, we removed batch effects from the expression profiles of the sample data. Using the WGCNA package, we then constructed a correlation matrix and selected the optimal soft-thresholding power to transform the correlation matrix into an adjacency matrix, which was subsequently converted into a Topological Overlap Matrix (TOM). On the bases of the dissimilarity measure derived from TOM, average linkage hierarchical clustering was employed to group genes with similar expression patterns into modules. After the modules were identified through hierarchical clustering, we calculated module eigengenes. Finally, Pearson correlation analysis was performed to assess the relationship between phenotypes (ARDS or control samples) and each module, identifying modules significantly associated with ARDS. The genes within these modules were categorized as ARDS-related module genes. This study aimed to investigate the relationships between gene modules and ARDS and to identify key genes within the related modules.<sup>18</sup> The resulting gene dendrogram, module colors, and module-trait relationships are visualized in [Supplementary Figure S1](#).

## Identification of Ferroptosis-Related DEGs (FDEGs)

We downloaded the ferroptosis-related gene list from the GENECARD database (<https://www.genecards.org/>). We overlapped differentially expressed genes, WGCNA results, and ferroptosis genes to identify co-differentially expressed genes (co-DEGs). A Venn diagram was used to describe the details of the co-DEGs.

## Functional Enrichment Analysis

All keywords were clustered on the basis of member similarity, and the terms with the highest enrichment were selected as representatives. The “clusterProfiler” R package was utilized to perform GO and KEGG functional enrichment analyses in R, enabling the assessment of gene-related biological processes (BP), molecular functions (MF), cellular components (CC), and signaling pathways. Statistical significance was set at  $P < 0.05$ .

## Screening Hub Genes by Machine Learning

In this study, two kinds of machine learning algorithms, random forest (RF) and minimum absolute contraction and selection operator (LASSO) logistic regression, were used to screen key genes. The RF algorithm was carried out via the randomForest<sup>19</sup> package in R software (version 4.2.0). LASSO logistic regression analysis was performed using the “glmnet” package in R software (v64 4.3). The discriminative performance of the models (LASSO and RF) was quantified using the area under the receiver operating characteristic curve (AUC), where higher AUC values indicate stronger capability of the models to differentiate between ARDS patients and healthy control samples.<sup>20</sup>

## Prediction of the Structural Characteristics of Key Candidate Gene Proteins

To predict the structural characteristics of the proteins encoded by the key candidate genes *MAPK8*, *CREB1*, and *GPX4*, we utilized the online tools PredictProtein (<https://predictprotein.org/>) and ExPasy-SOPMA (<https://npsa-prabi.ibcp.fr/>) for secondary structure prediction. For tertiary structure prediction, we employed the SWISS-MODEL platform (<https://swissmodel.ExPASy.org/>).

## Gene Set Variation Analysis (GSVA)

We employed single-gene Gene Set Variation Analysis (GSVA) to elucidate the enriched KEGG pathways, utilizing the “GSVA” package to assess enrichment depicted on heatmaps. Gene sets with a P value of less than 0.05 were considered significantly enriched. The heatmap of GSVA enrichment scores for KEGG pathways is presented in [Supplementary Figure S2](#).

## Identification of Gene Clusters and Construction of Protein-Protein Interaction Network

We used the STRING database (version 11.5; [www.string-db.org](http://www.string-db.org)).<sup>21</sup> The results obtained from the STRING online database were imported into Cytoscape v3.9.1 to identify key nodes in the visual molecular interaction network. The

images downloaded from STRING were modified via Cytoscape software and important interacting genes were identified via the MCODE plug-in.<sup>22</sup> The PPI network of ferroptosis-related co-DEGs and the mRNA-TF regulatory network for FANCD2 are shown in [Supplementary Figure S3](#).

## Correlation Between Key Genes and Immune Infiltration

We utilized CIBERSORT, an analytical tool, to estimate the infiltration of immune cells on the basis of gene expression profiles. We utilized the “corrplot” R package<sup>18</sup> and employed Spearman’s rank correlation coefficient to analyze the correlation between the expression of nine diagnostic biomarkers and the content of infiltrated immune cells, with  $P < 0.05$  considered statistically significant.

## Single-Cell Data Integration and Difference Analysis

We used the Seurat software package<sup>23</sup> to comprehensively process and analyze the publicly available single-cell RNA sequencing dataset (GSE151263), achieving data integration. Subsequently, nonlinear dimensionality reduction methods, including Uniform Manifold Approximation and Projection (UMAP) and t-distributed Stochastic Neighbor Embedding (t-SNE), were employed to visualize high-dimensional data and identify potential cell populations.

## Animal Experiment Validation

### Animals and ARDS Model Establishment

Eighteen male Sprague Dawley (SD) rats, aged 8 weeks and weighing approximately 200 g each (SPF class), were purchased from Hunan Sino-Lac-Jinda Experimental Animal Co., Ltd. (6 rats per group). The animal use certificate number is SCXK (Xiang) 2019-0004. The animals were allowed a one-week acclimation period in their new environment before the start of the experiments. The animal study protocol was approved by the Scientific Research Ethics Committee of Liuzhou People’s Hospital (No. KY-2024-078), and all experimental procedures were strictly conducted in accordance with the ARRIVE guidelines. The rats were then randomly divided into three groups, each consisting of six rats: a control group, a lipopolysaccharide (LPS) group, and an LPS plus fecal microbiota transplantation (LPS+FMT) group. All rats were anesthetized with 20% urethane (intraperitoneal injection, 5 mL/kg). In the control group, 100  $\mu$ L of saline was administered via tracheal intubation. In the LPS group, 15 mg/kg LPS (O55, Sigma Aldrich, USA, Cat. No. L2880), dissolved in 100  $\mu$ L of saline (Sigma Aldrich, USA, Cat. No. S5886), was introduced via tracheal intubation to induce lung injury.<sup>21</sup> Fresh fecal samples were collected from healthy donor rats and homogenized in phosphate-buffered saline (PBS; Gibco, 14190-144) at a ratio of 1:5 (w/v). To preserve anaerobic bacteria viability, L-cysteine (Sigma-Aldrich, C7352) was added to the suspension as a reducing agent. After thorough vortexing to ensure homogeneity, the fecal suspension was sequentially filtered through a 0.8–1 mm mesh sieve to remove particulate matter. Further purification was achieved by centrifugation at 800 $\times$ g for 3 minutes to pellet insoluble solids; this low-speed protocol effectively removes debris while minimizing bacterial loss. The supernatant was stored at 4°C and utilized within 6 hours to maintain microbial viability. Acute respiratory distress syndrome (ARDS) was induced in the rats 24 hours after LPS injection. For the FMT+LPS group, the fecal microbiota from healthy donor rats was treated via oral gavage at a dose of 100 mg/kg, once daily for 7 consecutive days, starting 24 hours after LPS induction. The 100 mg/kg dosage was selected on the basis of previous studies.<sup>22</sup> Following seven days of intervention, rats were humanely euthanized via cervical dislocation under profound anesthesia, with subsequent collection of lung tissues for further analysis.

### H&E Staining and Histopathological Injury Assessment

Rat lung tissue samples were placed in 10% formalin (Chinese combination) and fixed at room temperature for 18–24 hours. After fixation, the samples were dehydrated with graded ethanol (China Joint), removed with xylene (China joint), and embedded in paraffin wax (China joint). Slices of 4–5  $\mu$ m were then sliced and placed in a slide heater at 60°C for 1 hour. The slices were dewaxed and rehydrated and then stained with hematoxylin and eosin (H&E) (Solarbio, China) following a strict H&E staining scheme. After the samples were dyed, neutral resin (Solarbio, China) was applied, and the samples were observed under a light microscope. Lung histological scoring is based on four parameters: alveolar congestion, neutrophil infiltration or aggregation in the alveolar cavity or vascular wall, hemorrhage,

and thickening and/or hyaline membrane formation of the alveolar wall. Each individual parameter is scored from 0 to 4. Calculate the total score of the four parameters. Specimens of six rats in each group were evaluated.

## Immunohistochemical (IHC) Analysis

For the immunohistochemical analysis of protein expression in lung tissue, we focused on the *MAPK8*, *CREB1* and *GPX4* proteins. Antigens were extracted from the microwave-heated sections for 10 minutes. The sections were treated with 3% H<sub>2</sub>O<sub>2</sub> (Solarbio, China) at 37°C for 20 min and then washed with PBS (Solarbio, China). To block nonspecific binding, the sections were incubated with 10% goat serum at room temperature for 30 minutes, followed by incubation with primary antibody (1:50 dilution) overnight in a humid chamber at 4°C. After the sections were washed with PBS, the enzyme labeled secondary antibody was added and the samples were incubated at 37°C for 40 minutes. After the samples were washed with PBS, color was developed with fresh DAB solution. Finally, the slices were briefly air-dried and mounted with anti-fading mounting medium, and images were captured under a microscope.

## Collection of Serum Cytokines

The concentrations of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were measured using ELISA kits according to the manufacturer's protocol. Serum was extracted from 5 mL of rat blood by centrifugation at 4000 rpm for 10 minutes at 4°C. Rat TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 levels in the serum were measured using enzyme-linked immunosorbent assay (ELISA) kits.

## RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

The levels of *MAPK8*, *CREB1*, and *GPX4* were measured via RT-qPCR. Total RNA was extracted from rat lung tissues via Trizol reagent (Thermo Fisher Scientific, USA). The RNA from each sample was then used to synthesize first-strand cDNA. The primer sequences are listed in Table 1. The cDNA was subsequently amplified, and the  $2^{-\Delta\Delta C_t}$  method was used for data analysis, with *GAPDH* used as the reference gene. The primer sequences details are provided in Table 1.

## Western Blot Assay

Protein samples isolated from lung tissue via RIPA buffer lysis underwent electrophoresis on 10% SDS-polyacrylamide gels, followed by transfer to PVDF membranes. The membrane was blocked with 5% non-fat milk at room temperature for 1 hour, then incubated with primary antibodies MAPK8 (Wuhan Sanying, 66210-1-Ig), CREB1 (ABcam Trading Co, Ltd, ab32515-10oul), and GPX4 (ABcam Trading Co., Ltd, ab125066-10oul) at a dilution of 1:1000; GAPDH antibody (ABcam Trading Co., Ltd, ab181602-10oul) at a dilution of 1:5000 at 4°C overnight. After washing with TBST three times, the membrane was incubated with HRP-conjugated secondary antibody (dilution of 1:5000) at room temperature for 1 hour. Following another round of washing, protein bands were visualized using an ECL chemiluminescence kit, and band gray values were quantified using ImageJ software. The experiment was independently repeated 3 times (n=3).

## Statistical Analysis

Statistical analyses were performed using R software (version 4.2.0). Quantitative data were expressed as “mean  $\pm$  standard deviation (mean  $\pm$  SD)”. Comparisons between two groups were conducted using the Wilcoxon rank-sum test or

**Table 1** Specific Primer Sequences for the *MAPK8*, *CREB1*, and *GPX4* Genes

| Gene Name    | Forward Primer (F)     | Reverse Primer (R)        |
|--------------|------------------------|---------------------------|
| <i>MAPK8</i> | CAGCCGTCTCCTTTAGGTGC   | CTGCTGTCTGTATCCGAGGC      |
| <i>CREB1</i> | AGCAGACAACCAGCAGAGTG   | TGCTGGCATGGATACCTGG       |
| <i>GPX4</i>  | CAACCAGTTCGGGAGGCAGGAG | CACGGCAGGTCCTTCTCTATCACCT |
| <i>GAPDH</i> | CAAGATGGTGAAGGTCGGTG   | TGCCGTGGGTAGAGTCATACTG    |

Student's *t*-test, while comparisons among multiple groups (qRT-PCR, Western blot, ELISA, and histological scoring) were performed using one-way analysis of variance (one-way ANOVA) followed by Tukey's post-hoc test. The correlation between genes and immune cell infiltration was analyzed using Spearman's rank correlation analysis.  $P < 0.05$  was considered statistically significant.

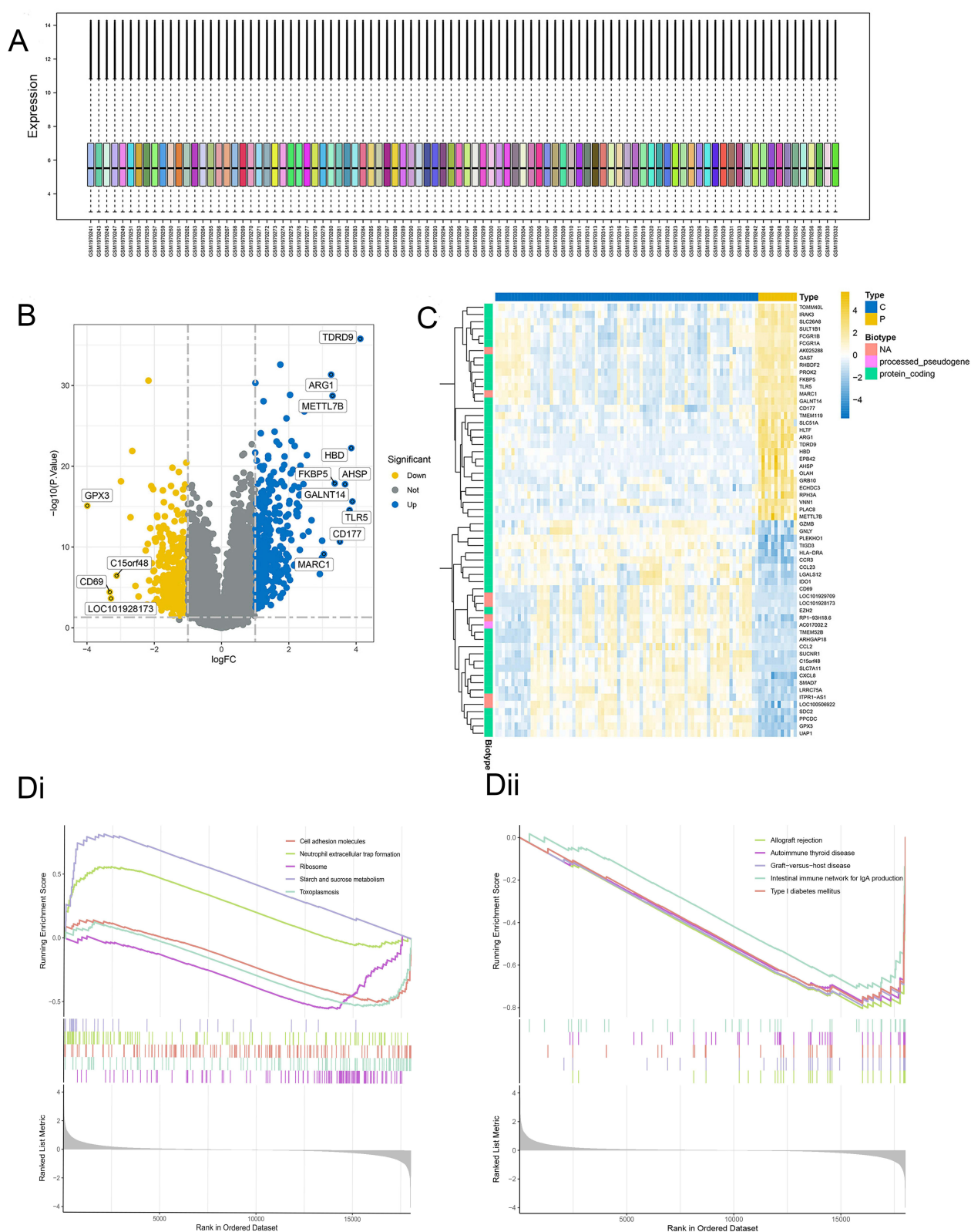
## Results

### Identification of Differentially Expressed Genes (DEGs) in ARDS and Gene Set Enrichment Analysis of DEGs

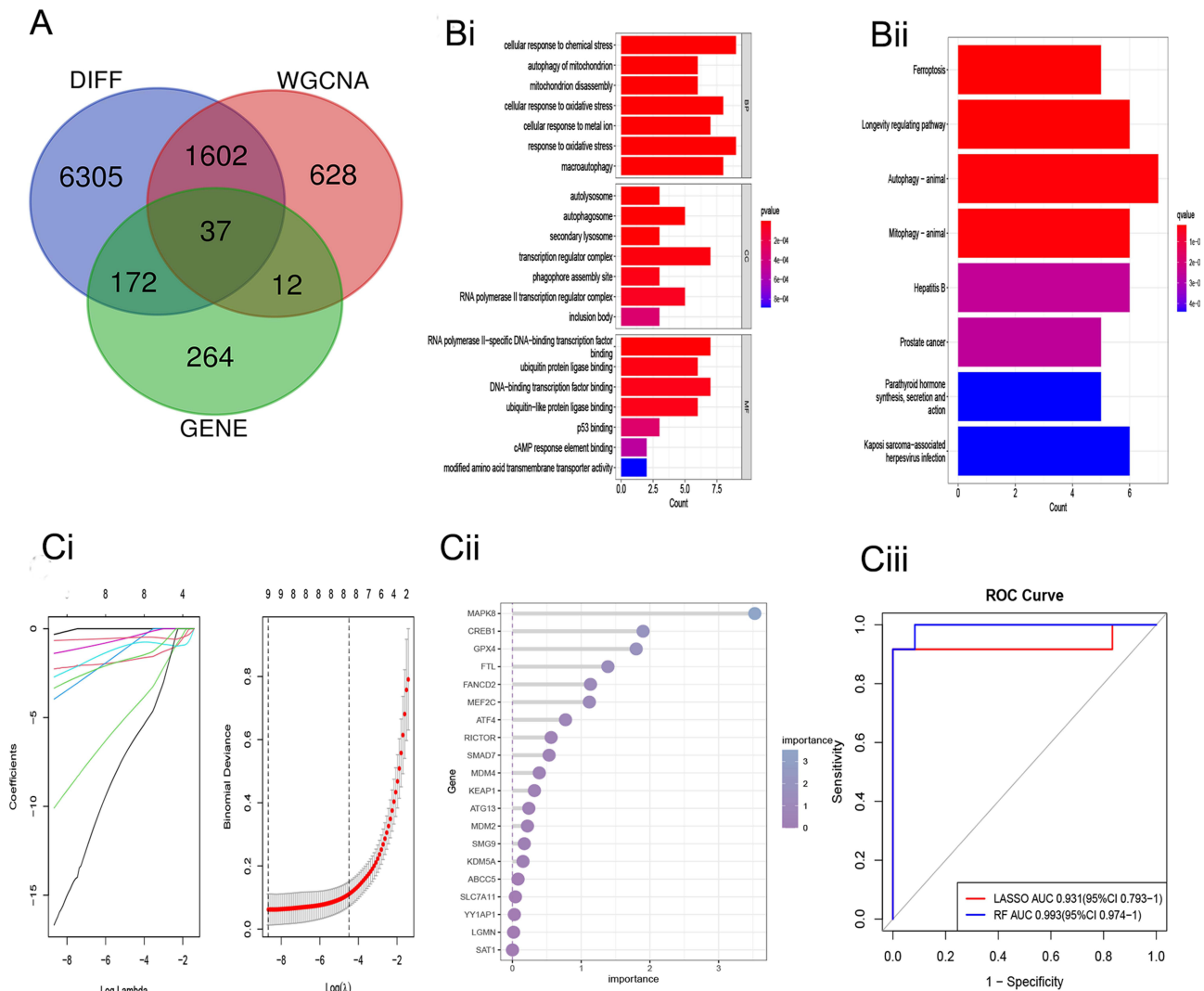
We downloaded the ARDS-related dataset from GSE76293 and performed differential expression analysis via the limma package. The raw data were initially analyzed and subsequently corrected for batch effects, followed by log normalization. Datasets from various sources often exhibit significant batch effects. Using data retrieved from the GSE76293 dataset, box plots were generated for the ARDS group and the normal group (Figure 1A). Following batch correction and log<sub>2</sub> normalization, the expression profiles across all samples showed improved consistency, enhancing the accuracy and robustness of downstream analyses. We identified a total of 633 DEGs via the Limma method with an adjusted  $P < 0.05$  and  $|\log_2FC| > 1.0$ . The volcano plot (Figure 1B) and heatmap (Figure 1C) demonstrate the differential expression patterns of these DEGs. Analysis of upregulated differentially expressed genes (DEGs) (Figure 1Di) demonstrated significant activation in various signaling pathways. Pathways associated with cell adhesion molecules were strongly enriched, potentially reflecting alveolar epithelial and endothelial damage alongside compensatory repair mechanisms. Neutrophil extracellular trap (NET) formation pathways showed marked enrichment, highlighting the critical involvement of neutrophils in inflammatory responses within acute respiratory distress syndrome (ARDS). Ribosomal function-related pathways implied disruptions in protein synthesis, while starch and sucrose metabolism pathways indicated metabolic dysregulation contributing to ARDS pathogenesis. Toxoplasmosis-associated pathways were also enriched, suggesting possible pathogen-mediated immune interactions in ARDS development. For downregulated DEGs (Figure 1Dii), gene set enrichment analysis (GSEA) identified several enriched biological processes: allograft rejection and graft-versus-host disease pathways implicated immune hyperreactivity and dysregulation; autoimmune thyroid disease pathways hinted at autoimmune involvement; the intestinal immune network for IgA production revealed a previously unexplored link to gut immunity in ARDS; and type 1 diabetes mellitus pathways indicated metabolic-autoimmune interplay in ARDS pathogenesis. GSEA significance thresholds were set as absolute normalized enrichment score (NES)  $> 1$ , adjusted  $P$ -value  $< 0.05$ , and false discovery rate (FDR)  $q$ -value = 0.25.

### Identification of Core Ferroptosis-Related Differentially Expressed Genes (FRDEGs) and Diagnostic Hub Genes

To pinpoint key ferroptosis-related core differentially expressed genes (co-DEGs), an integrative approach was employed. Genes identified as differentially expressed (DEGs) were intersected with significant modules derived from Weighted Gene Co-expression Network Analysis (WGCNA) and known ferroptosis-associated genes sourced from the GENECARD database. This overlap, visualized by a Venn diagram (Figure 2A), revealed 37 ferroptosis-related co-DEGs. Subsequent Gene Ontology (GO) enrichment analysis demonstrated that these co-DEGs were prominently associated with biological processes (BPs) such as the cellular response to chemical stress, cellular components (CCs) including transcription regulator complexes, and molecular functions (MFs) linked to transcription factor regulatory activity (Figure 2Bi). Further pathway analysis using KEGG highlighted significant enrichment in ferroptosis, mitophagy, and longevity regulating pathways (Figure 2Bii). To identify pivotal diagnostic genes (hub genes), we utilized machine learning approaches. Lasso regression analysis determined 9 candidate genes (*RICTOR*, *CREB1*, *FANCD2*, *MEF2C*, *MAPK8*, *SMAD7*, *FTL*, *MAP1LC3A*, and *IDH2*) based on coefficient profiling and the selection of an optimal tuning parameter ( $\lambda$ ) (Figure 2Ci). In parallel, the Random Forest (RF) algorithm prioritized 3 hub genes (MAPK8, CREB1, GPX4) showing importance scores exceeding 1.5 (Figure 2Cii). Diagnostic efficacy of these models was evaluated using ROC curve analysis (sensitivity vs. 1 - specificity) on the GSE76293 dataset (Figure 2Ciii). The LASSO model yielded an AUC of 0.931 (95% CI: 0.793–1), demonstrating strong performance. The RF model achieved an even higher AUC of



**Figure 1** Analysis of Differentially Expressed Genes and Enriched Functional Pathways. **(A)** Box plot illustrating expression distributions for the GSE76293 dataset following batch correction and log2 normalization, revealing enhanced consistency between ARDS and control groups; **(B)** Volcano plot depicting screening outcomes for differentially expressed genes; red circles indicate upregulated genes, while blue circles represent downregulated genes, with thresholds of  $|\log_2 FC| > 1.0$  and adjusted  $P < 0.05$ ; **(C)** Heatmap demonstrating expression profiles for all DEGs; rows correlate with genes, columns with samples, and gradient colors denote expression intensity; **(D)** Gene Set Enrichment Analysis results: The **(Di)** identifies the three most enriched pathways for upregulated genes, including cell adhesion molecules, neutrophil extracellular trap formation, and ribosome processes; the **(Dii)** panel highlights the top three pathways for downregulated genes, such as allograft rejection, autoimmune thyroid disease, and intestinal immune network for IgA production. Significance thresholds include absolute NES  $> 1$ , adjusted  $P < 0.05$ , and FDR  $q = 0.25$ .



**Figure 2** Screening of ferroptosis-related core differentially expressed genes (co-DEGs) and identification of hub diagnostic genes. **(A)**Venn diagram illustrating the intersection of DEGs (DIFF), key module genes from Weighted Gene Co-expression Network Analysis (WGCNA), and ferroptosis-related genes (GENE), leading to the identification of 37 ferroptosis-related co-DEGs; **(B)** Functional enrichment analysis: **(Bi)** Bar plot of Gene Ontology (GO) Biological Process (BP) enrichment, showcasing top enriched categories (biological process, cellular component, molecular function) with gene counts and P values color-coded to reflect significance gradients; **(Bii)** Bar plot illustrating Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, highlighting key pathways clustered by functional groups (eg, ferroptosis, disease-related) and color-scaled q values for statistical significance; **(C)** Machine learning-based hub gene identification and diagnostic validation. **(Ci)** Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis; **(Cii)** Gene importance ranking by Random Forest (RF) algorithm, with points representing genes and color indicating importance scores; **(Ciii)** Receiver Operating Characteristic (ROC) curves comparing the diagnostic performance of LASSO and RF models, with Area Under the Curve (AUC) and 95% confidence intervals (CI) provided.

0.993 (95% CI: 0.974–1.000), attributed to pronounced expression differences of the 3 hub genes between groups and specific characteristics of the GSE76293 cohort. Visually distinct curves represented each model (LASSO: solid blue line; RF: dashed red line). All analyses utilized the “pROC” package within R software (version 4.2.0).

## Weighted Gene Co-Expression Network Analysis

[Supplementary Figure S1](#) elaborates on the findings derived from Weighted Gene Co-expression Network Analysis (WGCNA) applied to ARDS transcriptomic datasets. Specifically, S1A visualizes the hierarchical clustering dendrogram of genes alongside assigned color codes for individual modules, integrating dynamic tree cutting methodology to demarcate unique co-expression modules (each color denotes a distinct module). S1B illustrates a correlation heatmap mapping module-trait associations: rows signify gene modules and columns denote phenotypic states (either control or ARDS), with Spearman correlation coefficients and color gradients reflecting association strength (P values in

**Table 2** Prediction Results for the Secondary Structure of the Key Genes and Proteins

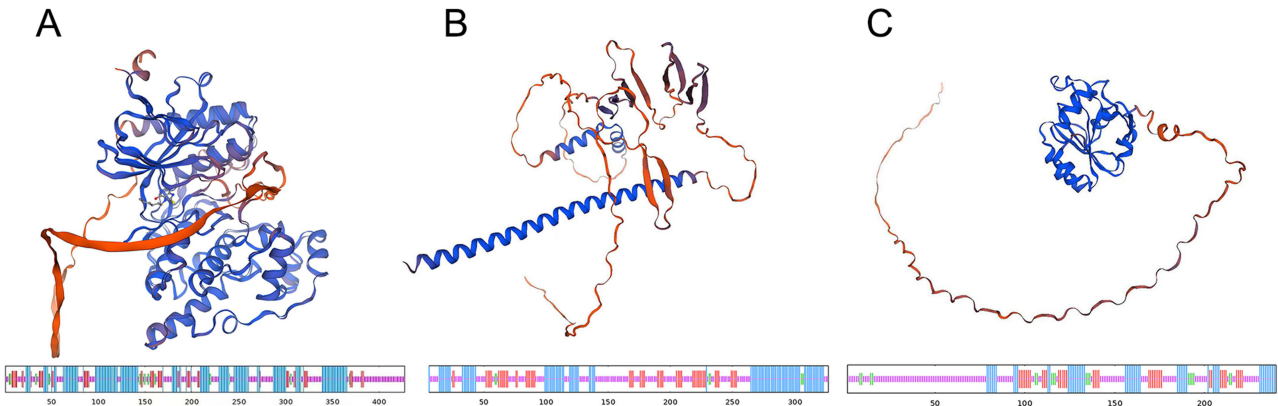
|                        | MAPK8  | CREB1  | GPX4   |
|------------------------|--------|--------|--------|
| $\alpha$ -helices (Hh) | 40.52% | 34.25% | 20.83% |
| Random coil (Cc)       | 40.98% | 43.73% | 56.25% |
| $\beta$ -turns (Tt)    | 4.92%  | 2.14%  | 7.50%  |
| Extended strands (Ee)  | 13.58% | 19.88% | 15.42% |

parentheses). This visualization accentuates modules exhibiting the most significant correlation with ARDS pathogenesis. S1C displays a clustered dendrogram post-module merging via dynamic tree cutting; the consolidation of similar modules refines the final classification, thereby optimizing reliability in identifying ARDS-related module genes. Collectively, these supplementary analyses substantiate the co-expression network’s robustness and furnish granular support for the main text’s discussion of ARDS-associated module genes.

Prediction of Secondary and Tertiary Structures of the MAPK8, CREB1, and GPX4 Proteins

The secondary structure of the three key genes was predicted via SOPMA software, and the results are presented in Figure 3A-3C and summarized in the following Table 2. Based on the structural analysis, MAPK8 exhibits 40.52%  $\alpha$ -helices and 40.98% disordered regions, with  $\beta$ -sheets comprising 4.92% and extended strands 13.58%. Its tertiary configuration (residues 50–400, Figure 3A) encompasses a canonical kinase domain interspersed with regulatory loops. CREB1 displays 34.25%  $\alpha$ -helical content and 43.73% coil structures, alongside minimal  $\beta$ -sheet representation (2.14%) and 19.88% extended strands. The tertiary architecture (residues 50–300, Figure 3B) integrates a DNA-binding domain with a transactivation domain harboring phosphorylation sites. GPX4 contains 20.83%  $\alpha$ -helices and 56.25% coil regions, with  $\beta$ -sheets accounting for 7.50% and extended strands 15.42%. Its catalytic domain (residues 50–200, Figure 3C) features essential selenocysteine motifs within the tertiary fold.

Investigation into the secondary structures of MAPK8, CREB1, and GPX4 indicates a significant prevalence of  $\alpha$ -helical regions, contributing to both ordered domains and potential disordered segments.<sup>24</sup> This composition supports a functional motif that integrates structural stability with inherent flexibility, essential for proteins engaged in cellular

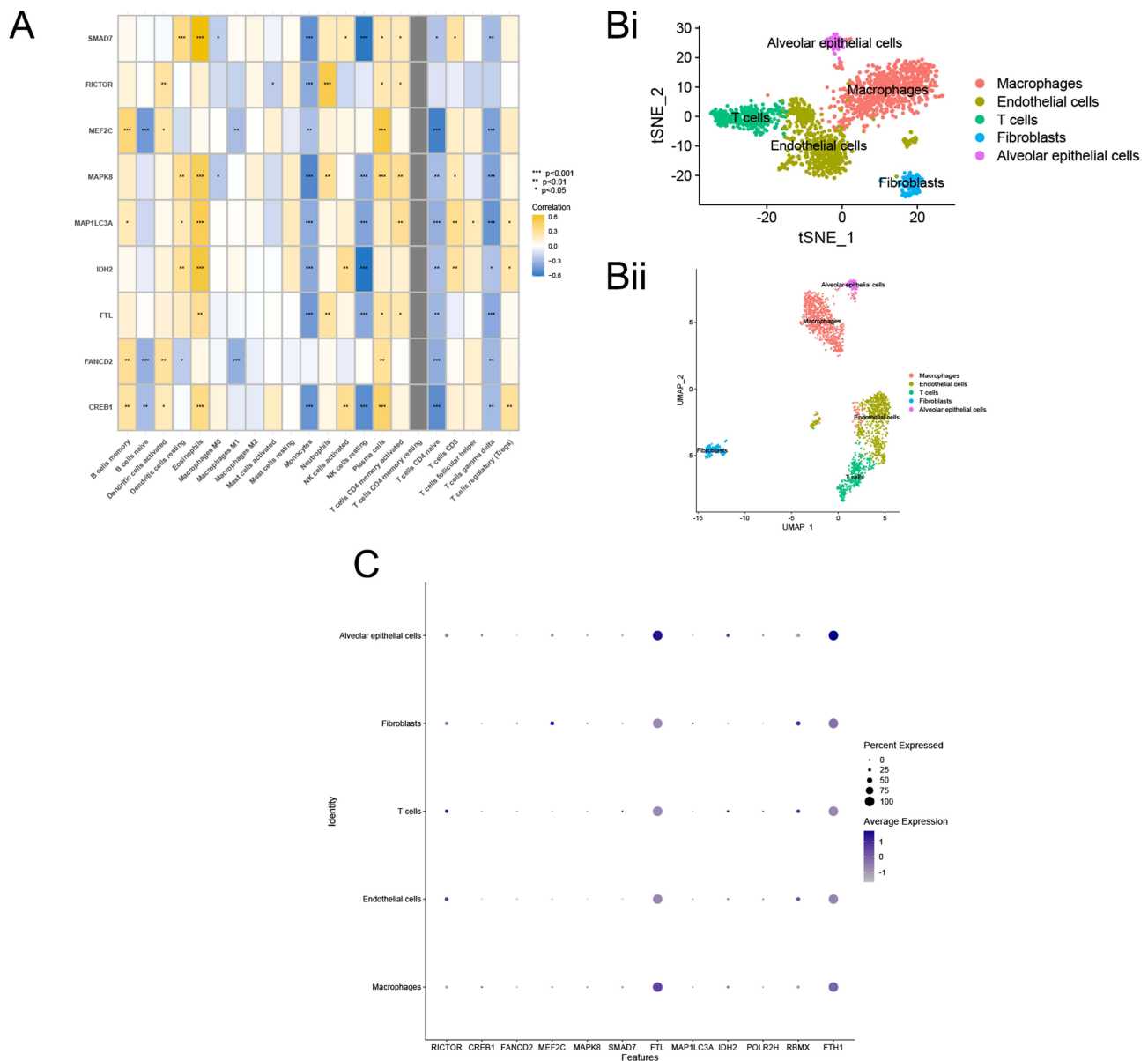


**Figure 3** Predicted structural representations of proteins derived from hub genes MAPK8, CREB1, and GPX4 . (A) Three - dimensional structure and domain architecture of MAPK8. The upper ribbon model illustrates the spatial organization of  $\alpha$ -helices (depicted in blue) along with secondary elements (rendered in Orange). In the lower section, a domain schematic identifies key functional modules, such as the kinase domain and regulatory loops, plus essential residues spanning amino acid positions 50–400; (B) Three - dimensional structure and domain architecture of CREB1. At top, the ribbon rendering emphasizes the DNA-binding domain (blue  $\alpha$ -helix) in juxtaposition to the transactivation domain (orange loops). Beneath, a detailed domain map incorporates phosphorylation sites and DNA-binding motifs located between residues 50–300; (C) Three - dimensional structure and domain architecture of GPX4. The ribbon display highlights the catalytic domain (blue-shaded) and conserved active sites. Below this, the schematic delineates domain architecture, featuring motifs incorporating selenocysteine across residues 50–200.

signaling, DNA binding events, and enzymatic regulation.<sup>25–27</sup> Such architectural properties confer the versatility needed for interaction with diverse molecular cues and functional partners.<sup>25</sup>

### Associations of Hub Genes with Immune Cell Infiltration and Expression Profiling in Single-Cell Clusters

To further investigate the immunomodulatory role of ferroptosis in ARDS pathogenesis, we integrated immune cell infiltration analysis with scRNA-seq data. Using CIBERSORT and Spearman’s rank correlation, we assessed the relationship between nine machine-learning-selected key ferroptosis regulators and the abundance of 22 distinct immune cell types. This analysis revealed significant associations between these candidate genes and specific immune populations (Figure 4A).



**Figure 4** Associations of Hub Genes with Immune Cell Infiltration and Expression Profiling in Single-Cell Clusters. **(A)** Visualization of the association matrix for hub genes and 22 immune cell types. Color gradients represent Spearman correlation coefficients (yellow: positive, blue: negative), with significance levels marked by \*For  $P < 0.05$ , \*\*For  $P < 0.01$ , \*\*\*For  $P < 0.001$ . **(B)** Single-cell clustering visualization. **(Bi)** The t-SNE projection above depicts lung tissue cells (from dataset GSE151263), color-coded by cell types—Macrophages (red), Endothelial cells (green), T cells (teal), Fibroblasts (blue), Alveolar epithelial cells (pink). **(Bii)** The UMAP display the same cell clustering with cell type labels. **(C)** Bubble diagram of hub gene expression levels across distinct cell groups. Colors encode mean expression intensities; bubble sizes denote the fraction of expressing cells per cluster.

Notably, MAPK8, CREB1, and GPX4 were inversely correlated with monocyte abundance but showed a positive correlation with neutrophil and activated NK cell levels, suggesting their potential involvement in shaping the ARDS immune landscape.

Furthermore, to validate these findings and pinpoint the cellular localization of these central biomarkers at high resolution, we analyzed the publicly accessible scRNA-seq dataset GSE151263. Both t-SNE and UMAP dimensionality reduction techniques were employed to characterize the cellular composition of the lung microenvironment. These methods consistently identified major cell populations, including macrophages, endothelial cells, T cells, fibroblasts, and alveolar epithelial cells (Figure 4Bi and Bii). Analysis of gene expression patterns across these defined cell subtypes demonstrated the widespread presence of the top ferroptosis-related candidate genes. Importantly, FTL exhibited the most ubiquitous expression profile among these core factors (Figure 4C).

## Gene Set Variation Analysis (GSVA)

[Supplementary Figure S2](#) displays the KEGG pathway enrichment heatmap based on GSVA analysis, complementing the main text's examination of core pathways. The rows correspond to key KEGG pathways divided into categories: immune-related (eg, antigen processing and presentation), metabolic (eg, glycolysis/gluconeogenesis), and disease-linked functions. Columns depict samples categorized by phenotype: control group in blue and ARDS group in orange. Color intensity indicates pathway enrichment scores (red for high, blue for low), highlighting distinct activity patterns between the two groups. Notably, immune and metabolic pathways exhibit pronounced enrichment differences, consistent with the main finding that ferroptosis-related genes participate in immune and metabolic disturbances. This supplementary visual offers a comprehensive overview of pathway variations, strengthening evidence for the functional role of DEGs in ARDS pathogenesis.

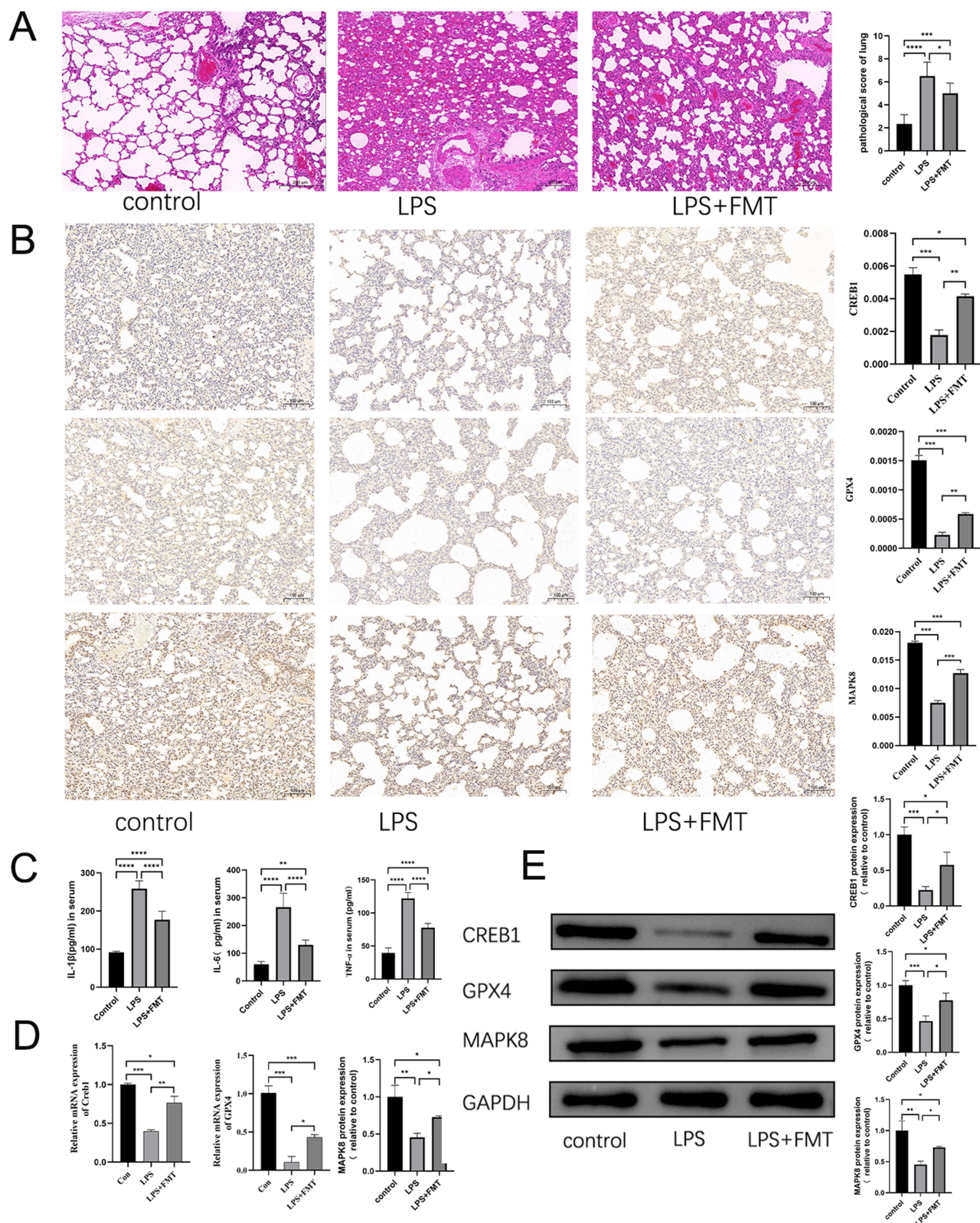
## Protein-Protein Interaction (PPI) Network and mRNA-Transcription Factor (TF) Regulatory Network

[Supplementary Figure S3](#) delineates the interaction networks for core differentially expressed genes (DEGs) linked to ferroptosis. Panel S3A displays the protein-protein interaction (PPI) network comprising 37 ferroptosis-associated DEGs, where nodes signify proteins and edges indicate associations. Core hub proteins (including MDM2, GPX4, CREB1, and MAPK8) were markedly enriched, delineating the complex functional interplay within this network pertinent to ferroptosis mechanisms. Further analysis utilizing the MCODE plug-in identified densely interconnected core modules, underscoring the pivotal role of these hubs in modulating ferroptosis and associated immune responses. Panel S3B concurrently presents the mRNA-transcription factor (TF) regulatory network centered on FANCD2 (a critical ferroptosis gene). This network employs color-coded components: FANCD2 mRNA (red node) and 16 potential regulatory TFs (eg, CTBP1, HDAC2, KLF1; blue diamond nodes). This visualization elucidates the transcriptional regulation of FANCD2, thereby complementing the main text's functional characterization of hub genes and shedding light on upstream regulatory pathways governing ferroptosis in ARDS.

## Validation of Hub Genes and Assessment of Fecal Microbiota Transplantation (FMT) Efficacy in LPS-Induced ARDS Rats

To experimentally validate our bioinformatic predictions, we established a lipopolysaccharide (LPS)-induced ARDS rat model and administered fecal microbiota transplantation (FMT) as an interventional strategy. As shown in [Figure 5](#), we subsequently systematically evaluated multiple endpoints: pathological alterations in lung tissue via H&E staining and histological scoring, concentrations of serum inflammatory factors (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ) via ELISA, and the mRNA expression (qRT-PCR) as well as protein localization (immunohistochemistry, IHC) and expression levels (Western blot) of the identified hub genes, MAPK8, CREB1, and GPX4.

Histological examination of rat lung tissues via H&E staining ([Figure 5A](#)) revealed distinct pathological changes among the three groups: the Control group exhibited intact lung tissue structure with basically normal bronchial structures at all levels, closely arranged epithelial cells, and abundant alveoli, with only occasional necrosis and shedding of epithelial cells in some bronchi and no other obvious abnormalities; in contrast, the LPS group showed moderate structural damage, characterized by vacuolation of epithelial cells in some bronchi, obstruction by desquamated epithelial



**Figure 5** Validation of hub genes and assessment of fecal microbiota transplantation (FMT) efficacy in attenuating LPS-induced acute respiratory distress syndrome (ARDS) in rats. **(A)** Lung tissue sections (H&E staining; 200× magnification) with histopathological scoring. The bar graph presents pathological scores (mean ± SD, n=6) were compared via one-way ANOVA: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . **(B)** Immunohistochemical (IHC) localization of CREB1, GPX4, and MAPK8 proteins (brown signal; 200×). Positive signal areas were quantified. Data expressed as relative positive area, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (one-way ANOVA). **(C)** Detection of serum inflammatory factors (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ) by enzyme-linked immunosorbent assay (ELISA). Data are presented as mean ± SD (n=6), \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$  (one-way ANOVA). **(D)** Relative mRNA expression of CREB1, GPX4, and MAPK8 measured by qRT-PCR. Data are mean ± SD (n=3), \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (one-way ANOVA). **(E)** Western Blot analysis of CREB1, GPX4, and MAPK8 protein expression in lung tissue, with GAPDH as a loading control. Bar graphs show relative protein expression (mean ± SD, n=3), \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (one-way ANOVA).

cells and red blood cells, reduced number of alveoli, thickening of most alveolar septa with slight collagen fiber proliferation, abundant inflammatory cell infiltration around the bronchi and in the pulmonary interstitium, and congestion of capillaries in the pulmonary interstitium; notably, the LPS+FMT group displayed milder lung tissue damage, with most bronchial structures remaining basically normal, only partial bronchial epithelial cell necrosis or shedding, a relative reduction in alveolar number, partial alveolar septal thickening with slight collagen fiber proliferation, a small amount of inflammatory cell infiltration around the bronchi, blood vessels, and in the pulmonary interstitium, and marked congestion of capillaries in the pulmonary interstitium. Histopathological evaluation was performed using a semi-quantitative scoring system assessing four key parameters: alveolar congestion, neutrophil infiltration, hemorrhage, and alveolar wall thickening/hyaline membrane formation (each parameter scored 0–4, yielding a total possible score of 0–16). Statistical analysis ( $n=6$ ; one-way ANOVA,  $P<0.001$ ) demonstrated marked variations among experimental groups. While control groups displayed minimal pathological changes, the LPS groups developed pronounced pulmonary damage ( $P<0.0001$  versus controls). Notably, FMT intervention substantially attenuated the injury severity in LPS-treated subjects, as evidenced by significantly lower composite scores in the LPS+FMT groups compared to LPS groups ( $P<0.05$ ). Detailed parameter analysis revealed FMT-mediated mitigation across all evaluated histological features, substantiating its multi-faceted protective capacity against LPS-triggered pulmonary pathology (Figure 5A).

Protein expression was assessed via immunohistochemistry (IHC) for MAPK8, CREB1, and GPX4—key regulators of ferroptosis—providing supporting evidence (Figure 5B). Representative IHC images with brown-positive signals and quantitative data on stained areas are presented in Figure 5B. Qualitatively, prominent staining for all three proteins was evident in Control group lung tissues, whereas LPS stimulation substantially attenuated immunoreactivity. FMT administration significantly restored protein expression of these ferroptosis-related regulators. Quantitative analysis verified significant reductions in positive areas for each protein in LPS versus Control groups (all  $P < 0.001$ ). Conversely, FMT intervention markedly ameliorated this LPS-induced decrease: staining areas for MAPK8, CREB1, and GPX4 in LPS +FMT group exceeded those in LPS group (all  $P < 0.01$ ), though remained slightly lower than Control group (all  $P < 0.05$ ). This demonstrates FMT's efficacy in counteracting suppression of these ferroptosis-associated proteins in LPS-induced ARDS.

The Control group exhibited minimal background levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , corresponding to a steady systemic inflammatory state that aligned with lung pathology and ferroptosis-associated protein expression (Figure 5C). By contrast, LPS challenge provoked a pronounced inflammatory reaction: concentrations of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  rose sharply in the LPS group, exceeding those observed in the Control group (all  $P < 0.0001$ ). This intense systemic inflammatory profile correlated directly with substantial pulmonary tissue injury, evidenced by alveolar damage and extensive cellular infiltration. This underscores the synergistic relationship between inflammation and ferroptosis during ARDS progression. FMT administration effectively attenuated the LPS-driven inflammatory cascade, consistent with its beneficial influence on lung histology and ferroptosis-related proteins. In the LPS+FMT cohort, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  levels were markedly lowered relative to the LPS group (all  $P < 0.0001$ ). Although cytokine concentrations stayed above Control group values (all  $P < 0.01$ ), the observed reduction affirmed FMT's capacity to inhibit systemic inflammation. This anti-inflammatory outcome coincided with mitigated lung tissue injury (Figure 5A) and renewed expression of MAPK8, CREB1, and GPX4 (Figure 5B). Together, these results demonstrate that FMT confers multi-faceted protection in ARDS, concurrently lessening inflammation, correcting ferroptosis-associated protein dysregulation, and reducing pulmonary damage.

Consistent with the bioinformatic analysis, the mRNA expression levels of MAPK8, CREB1, and GPX4 in lung tissues, as determined by qRT-PCR, were significantly downregulated in the LPS group compared to the Control group (Figure 5D). Importantly, FMT treatment effectively reversed this downregulation, restoring the expression levels of these genes towards normality. Western Blot analysis confirmed that the downregulation of MAPK8, CREB1, and GPX4 at the protein level in the LPS group was also reversed by FMT (Figure 5E).

## Discussion

Ferroptosis, an iron-dependent form of cell death characterized by lipid peroxidation, plays a key role in ARDS pathogenesis. Oxidative stress, iron dysregulation, and inflammation synergistically exacerbate lung injury. In ARDS,

iron overload disrupts metabolism, promoting lipid peroxidation and membrane damage via Fenton reaction-mediated ROS production. Critical regulators include NCOA4 and IREB2, which increase susceptibility to ferroptosis in ARDS.<sup>28,29</sup> Emerging evidence reveals a vicious cycle between ferroptosis and inflammation in ARDS pathogenesis. Characterized by neutrophil infiltration and elevated oxidative stress markers, ARDS results in feedforward amplification through crosstalk between ferroptotic pathways and inflammatory mediators such as NF- $\kappa$ B. Notably, SARS-CoV-2-induced ARDS results in the upregulation of ferroptosis-related genes, which is correlated with immune cell recruitment and tissue damage.<sup>28,30</sup> Ferroptosis exacerbates hallmark ARDS features including inflammatory amplification, endothelial dysfunction, and alveolar injury. Pathogenic triggers (bacterial/viral infections or sepsis) provoke excessive immune responses, generating ROS accumulation and glutathione (GSH) depletion that favor ferroptosis. Critical anti-ferroptotic defenses, such as GPX4 are compromised in ARDS, accelerating lung epithelial and endothelial cell death.<sup>19,28</sup> While these findings align with our observations, the precise mechanisms of ferroptosis in ARDS pathogenesis remain elusive. Elucidating these pathways may reveal novel therapeutic targets. This study utilized bioinformatics approaches to identify critical ferroptosis-related genes and pathways involved in ARDS, potentially establishing new treatment strategies.

To characterize the molecular underpinnings of ferroptosis in ARDS pathogenesis, we performed integrative bioinformatics analyses. This strategy revealed MAPK8, CREB1, and GPX4 as core ferroptosis regulators—identified through consensus across differential gene expression patterns, co-expression networks, and machine learning approaches.<sup>2,16</sup> Subsequent experimental validation in LPS-challenged rat models demonstrated significant downregulation of these hub genes—consistent with ferroptosis activation during lung injury.<sup>2,3,31</sup> Notably, this suppression was reversed by FMT,<sup>9,14</sup> reinforcing ferroptosis's pathogenic role. Our findings not only substantiate ferroptosis as a critical mechanism in ARDS<sup>4</sup> but also indicate the gut-lung axis modulates this cell death pathway through microbiota-dependent mechanisms.<sup>9,14,32</sup>

Our results strongly establish ferroptosis as a fundamental pathogenic driver in ARDS pathogenesis. The observed suppression of the critical antioxidant enzyme GPX4 in our LPS model emerges as a key element. GPX4 acts as the principal enzymatic defense against ferroptosis by reducing phospholipid hydroperoxides within cellular membranes.<sup>1,33</sup> Its significant downregulation in ARDS indicates a crucial failure in this protective mechanism, directly enabling the damaging buildup of lipid peroxides and the induction of ferroptotic cell death in critical alveolar epithelial and vascular endothelial cells.<sup>1,3,33,34</sup> This process is a direct contributor to the defining pathological features of ARDS: heightened vascular permeability, pulmonary edema formation, and inflammatory cell recruitment.<sup>1,34</sup> Furthermore, ferroptosis actively intensifies the inflammatory milieu. The rupture of ferroptotic cells releases damage-associated molecular patterns (DAMPs), thereby activating innate immune cells and establishing a self-perpetuating cycle of escalating inflammation and cellular damage.<sup>34,35</sup> Consequently, therapeutic intervention targeting the core ferroptosis pathway, particularly the GPX4 regulatory axis, represents a highly promising strategy. This approach aims to directly preserve pulmonary structure and function by interrupting this detrimental feedback loop.<sup>35</sup>

The protective role of FMT highlighted in our research points to its therapeutic value in managing ARDS through the gut-lung axis. This bidirectional network involves gut microbiota and metabolites influencing systemic lung immune and inflammatory pathways. During ARDS, dysbiosis compromises the intestinal barrier, enabling bacterial translocation that worsens systemic and pulmonary inflammation. By restoring a balanced gut microbiota, FMT strengthens barrier integrity and modulates host immune function.<sup>36–38</sup> Preclinical studies validate this, showing FMT alleviates LPS-triggered lung injury via suppression of pro-inflammatory cytokines.<sup>38</sup> Further, a reshaped gut microbiome boosts immunomodulatory metabolites, including SCFAs, which curb neutrophil-mediated inflammation and support macrophage balance. This fosters an anti-inflammatory environment, suppressing excessive lung immune activation.<sup>10,39,40</sup>

Building on our bioinformatic and experimental results, we suggest a plausible mechanism through which FMT provides protection by restoring the expression and functionality of key ferroptosis regulators identified in this study. We propose that FMT modulates the gut microbiota and enhances the gut barrier integrity, which reduces systemic inflammation and modifies immune cell signaling via the gut-lung axis. This subsequently promotes the upregulation of MAPK8, CREB1, and GPX4 in the lung, ultimately mitigating ferroptosis and lung injury.

In detail, CREB1—a major transcription factor governing genes for cell survival and inflammation resolution—may reactivate essential anti-inflammatory and cytoprotective transcriptional programs upon its recovery. The restoration of MAPK8 (JNK1), a stress-signal integrator with intrinsic structural flexibility enabling coordination of complex cellular responses, could recalibrate signaling networks away from pro-death pathways toward adaptive responses under inflammatory conditions. Crucially, GPX4 upregulation reestablishes the primary enzymatic defense by detoxifying lipid peroxides and directly inhibiting the ferroptotic cascade. The coordinated rescue of these genes (a transcriptional regulator, CREB1; a signaling kinase, MAPK8; and the terminal enzyme effector, GPX4) by FMT underscores a coherent, multi-layered protective mechanism. Although the precise gut-derived signals driving their transcriptional upregulation warrant further investigation, our findings offer strong evidence for a new gut-lung-ferroptosis axis in ARDS, positioning FMT as a potential strategy to target multiple sites in this pathogenic pathway concurrently.

Collectively, our integrated approach—spanning bioinformatics to experimental validation—uncovers a new protective mechanism in which FMT mitigates ferroptosis and lung injury in ARDS by re-establishing expression of key regulators MAPK8, CREB1, and GPX4 through the gut-lung axis.

Finally, immune infiltration analysis offers additional insights into this process, uncovering notable associations between the expression of hub genes and distinct immune cell types. The inverse link to monocytes and direct associations with neutrophils and activated natural killer cells highlight the complex connection between ferroptosis and immune regulation in ARDS. This implies that therapeutic benefits may include rebalancing these immune responses for enhanced outcomes.

Several study constraints deserve attention despite our supplementary experiments reinforcing the central findings. First, while Western blotting verified significant downregulation of the hub genes (MAPK8, CREB1, and GPX4) in LPS-induced ARDS rat lungs alongside their restoration by FMT, evaluation of other critical ferroptosis markers—ACSL4 (contributing to lipid peroxidation substrate synthesis), PTGS2 (functioning as a downstream effector of ferroptotic demise), and NCOA4 (mediating ferritinophagy to foster iron overload)—was omitted. This omission impedes a comprehensive grasp of FMT's influence on the wider ferroptosis pathway beyond the core genes. Second, the animal investigation faced two design limitations due to practical constraints: exclusion of a “Control + FMT” group precludes determining FMT's effects on uninjured pulmonary physiology; establishing this safety profile in healthy lungs is essential to verifying ARDS-specific benefits rather than nonspecific modulation. Furthermore, absence of a comparison with established ferroptosis inhibitors such as erastin or liproxstatin-1 obscures whether FMT primarily suppresses ferroptosis or acts via broader anti-inflammatory mechanisms that secondarily curtail it. Third, the sample size ( $n = 6$  per group), though aligned with routine preclinical ARDS protocols, may inadequately power the detection of subtle phenotypic or molecular alterations; expanding cohorts in future studies would enhance reliability for assessing moderate histopathological shifts or marginal changes in gene/protein expression. Fourth, the bioinformatics approach has inherent constraints. Datasets such as GSE76293 and GSE151263, while instrumental for initial gene identification, are constrained by their size and potential artifacts from transcriptomic platform variations. For instance, the Random Forest model demonstrated an Area Under the Curve (AUC) of 0.993 in the GSE76293 cohort, implying optimal diagnostic capability. Yet, this high performance could be cohort-specific rather than broadly indicative of biomarker reliability. To verify the diagnostic role of MAPK8, CREB1, and GPX4, further validation in expanded, multi-center ARDS patient populations is essential. Additionally, the LPS-induced rat model captures core ARDS traits, but interspecies variances in ferroptosis mechanisms and gut-lung interactions are poorly defined. The applicability of these biomarkers to human ARDS has not been assessed in clinical samples—such as bronchoalveolar lavage fluid, lung biopsies, or patient blood—representing a critical gap for practical implementation.

Future directions will prioritize the following investigations: (1) inclusion of an expanded array of ferroptosis biomarkers to more comprehensively assess pathway activation status; (2) extension of animal model studies to incorporate Control with FMT (Fecal Microbiota Transplantation) groups and the administration of ferroptosis antagonists; (3) confirmation of hub gene significance utilizing independent clinical cohorts; (4) elucidation of cross-species conserved mechanisms operating within the gut-lung-ferroptosis axis. These targeted investigations aim to substantiate the robustness and potential clinical translation of our data.

## Conclusions

Conclusively, this integrated approach, which merges bioinformatic analysis with experimental evidence, provides robust evidence for ferroptosis playing a central pathogenic role in ARDS. Our work pinpointed and corroborated MAPK8, CREB1, and GPX4 as pivotal diagnostic biomarkers and viable therapeutic targets. Analyses confirmed their substantial downregulation in ARDS models, an effect ameliorated by Fecal Microbiota Transplantation (FMT). The significant association of these genes with immune cell infiltration within affected tissues further emphasizes their functional importance in the disease microenvironment. These outcomes substantially enhance the understanding of ARDS pathogenesis and identify FMT, alongside interventions targeting ferroptosis inhibition, as particularly promising treatment avenues. Importantly, the study suggests a link connecting intestinal microbial transfer (FMT), pulmonary pathophysiology, and ferroptosis regulation, presenting a novel gut-pulmonary-ferroptotic axis worthy of deeper exploration and potential therapeutic exploitation.

## Declaration of AI Use

Throughout the article preparation process, DeepSeek was utilized to enhance readability and refine language expression. The author(s) subsequently undertook comprehensive review and revision of all content, ensuring accuracy and coherence. The author(s) assume full responsibility for the final published material.

## Data Sharing Statement

The datasets generated and/or analyzed during the current study are available in the GEO repository under accession numbers GSE76293 and GSE151263. Additional data and materials supporting the findings of this study are available from the corresponding author upon reasonable request.

## Ethics Approval

The animal study was approved by the Scientific Research Ethics Committee of Liuzhou People's Hospital (Approval No. KY-2024-078). All procedures were conducted in accordance with the ARRIVE guidelines.

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## Author Contributions

Biying Dong: Conceptualization, Methodology, Software, Validation, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Supervision, Project Administration. Bing Zhong: Conceptualization, Methodology, Software, Validation, Writing - Review & Editing, Visualization. Jing Zuo: Software, Validation, Investigation, Data Curation, Funding Acquisition, Writing - Review & Editing.

Longxiong Liao: Validation, Investigation, Data Curation, Methodology, Formal Analysis, Writing - Review & Editing. Weitong Zeng: Conceptualization, Methodology, Software, Validation, Data Curation, Writing - Review & Editing. Minjun Xiong: Software, Validation, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Data Curation. Yi Wei: Supervision, Project Administration, Investigation, Data Curation, Writing - Review & Editing. Dongwei Zhang: Writing - Original Draft, Project Administration, Methodology, Investigation, Validation.

Xianming Fan: Resources, Data Curation, Software, Supervision, Funding Acquisition, Writing - Review & 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 author(s) report no conflicts of interest in this work.

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