

# Bioinformatics Analysis and Identification of Ferroptosis-Related Gene TIMP1 as a Potential Biomarker of Asthma

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**Background:** Asthma is a chronic inflammatory airway disease characterized by multiple pathophysiological mechanisms. Ferroptosis is a novel form of programmed cell death. Studies have shown that ferroptosis may play a crucial role in the pathogenesis of asthma. However, ferroptosis-related genes have not yet been identified in the airway epithelial cells of patients with asthma. This study aims to identify ferroptosis-related genes in asthmatic airway epithelial cells, providing potential therapeutic targets for management of asthma.

**Methods:** We obtained the asthma dataset GSE63142 from the GEO database and the ferroptosis genes from the FerrDb database. Key co-expression modules and core genes were identified through Weighted Gene Co-expression Network Analysis (WGCNA), followed by functional enrichment analyses on the core genes. By overlapping the core genes with ferroptosis-related genes, we identified a hub gene, Tissue Inhibitor of Metalloproteinase 1 (TIMP1). It was validated using an independent dataset GSE43696 and clinical samples. Gene Set Enrichment Analysis (GSEA) was performed to further explore its biological relevance.

**Results:** WGCNA analysis yielded 23 core genes. Functional enrichment analysis revealed that these genes were significantly enriched in metabolic processes and ferroptosis. TIMP1 was identified as the sole hub gene. The expression of TIMP1 was significantly elevated in the SA groups compared to both the MMA groups ( $P < 0.05$ ) and normal controls ( $P < 0.0001$ ). The AUC values for diagnosing MMA and SA were 0.713 and 0.865, respectively. GSEA results indicated that TIMP1 was primarily enriched in the HIF-1 signaling pathway. TIMP1 mRNA expression levels were significantly higher in asthmatic groups compared to non-asthmatic control groups ( $P < 0.05$ ), while TIMP1 protein expression levels were markedly higher in asthmatic groups ( $P < 0.001$ ).

**Conclusion:** Our findings suggest that TIMP1 is associated with ferroptosis in asthma and may serve as a novel biomarker or therapeutic target.

**Keywords:** asthma, airway epithelial cells, ferroptosis, WGCNA, GSEA

## Introduction

Bronchial asthma is a heterogeneous disease with complex pathological mechanisms, characterized by recurrent episodes of wheezing, coughing, and dyspnea due to chronic airway inflammation and remodeling.<sup>1</sup> It has become one of the most common chronic lung diseases worldwide, affecting about 350 million people.<sup>2</sup> According to statistics, about 10% of adults and 2.5% of children will progress to severe asthma, which is poorly controlled by conventional treatment, and is associated with reduced quality of life, increased risk of exacerbation, and increased medical burden.<sup>3</sup> Damage and apoptosis of airway epithelial cells compromise epithelial integrity, serving as a key initiating factor in asthma-related pathological changes.<sup>4</sup> Following injury, airway epithelial cells release transforming growth factor beta (TGF- $\beta$ ), vascular endothelial growth factor (VEGF), and other mediators, which promote inflammatory response and airway remodeling.<sup>5</sup> Therefore, protecting airway epithelial cells to maintain integrity represents an effective strategy for asthma treatment.

Ferroptosis is a new type of cell death and is usually accompanied by a large amount of iron accumulation and lipid peroxidation during the cell death process.<sup>6</sup> Recent studies have shown that ferroptosis plays an important role in various diseases, including cancer, neurodegenerative diseases, autoimmune diseases, ischemia-reperfusion and other pathological processes.<sup>7,8</sup> The pathophysiology of ferroptosis involves inhibition of system Xc- leading to glutathione depletion, loss of GPX4 activity through inhibition/degradation/inactivation, depletion of reduced CoQ10, and iron-dependent lipid peroxidation caused by peroxides or accumulation of polyunsaturated fatty acids (PUFAs).<sup>9</sup> These mechanisms lead to irreversible oxidative damage of the cell membrane, mitochondrial dysfunction, and inflammatory responses, ultimately resulting in cell death.

Asthma and ferroptosis are closely associated. Ma et al were the first to discover ferroptosis in type II alveolar epithelial cells induced by birch pollen allergen Bet v1 in asthmatic mice, and use of ferroptosis inhibitors was found to alleviate Bet v1- induced asthma.<sup>10</sup> A study has shown that lipid peroxidation and reactive oxygen species (ROS) levels are elevated in house dust mite induced asthma, suggesting a potential role for ferroptosis in asthma.<sup>11</sup> Ferroptosis triggers the degradation of E-cadherin in airway epithelial cells, resulting in compromised epithelial barrier function and exacerbated inflammatory responses, which collectively contribute to asthma pathogenesis.<sup>12</sup>

Weighted Gene Coexpression Network Analysis (WGCNA) is a systematic bioinformatics approach designed to analyze correlation patterns among genes across microarray datasets. It can cluster genes with similar expression patterns and analyze the correlation between gene modules and specific traits or phenotypes, identifying modules most strongly associated with phenotypes and core genes.<sup>13</sup> This method has been widely used to identify candidate biomarkers or therapeutic targets. A key advantage of WGCNA is its ability to utilize information from all genes rather than being limited to differentially expressed genes, thereby offering a more comprehensive understanding of gene expression patterns.

However, the genes associated with ferroptosis in airway epithelial cells have yet to be identified. Therefore, this study was designed with three primary aims: (1) to identify ferroptosis-related genes in asthmatic airway epithelial cells via bioinformatics analysis, (2) to assess the diagnostic potential of the hub gene as a novel biomarker for asthma, and (3) to experimentally validate the expression of the hub gene in clinical asthmatic samples using Quantitative real-time PCR (qRT-PCR) and Western blotting. Our findings suggest that TIMP1 is associated with ferroptosis in asthma and may serve as a novel biomarker or therapeutic target.

## Materials and Methods

### Collection of Asthma and Ferroptosis-Related Genes

To explore the relationship between asthma and ferroptosis at the genetic level, we used asthma transcriptome data from the Gene Expression Omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>) database. We analyzed the GSE63142 dataset (n=155) and selected a subset of 27 normal controls and 56 severe asthmatic (SA) patients for comparative analysis to identify differentially expressed genes associated with severe asthma. Severe asthma is defined as asthma that remains uncontrolled despite adherence to maximally optimized high-dose ICS-LABA treatment and management of contributory factors, or that worsens when high-dose treatment is decreased.<sup>14</sup> The GSE63142 microarray dataset was downloaded from R software's "GEOquery" package. Ferroptosis-related genes (FRGs) were obtained from the FerrDb database (<http://www.zhounan.org/ferrdb>), including driver, marker, suppressor, unclassified genes. After removing duplicate genes, 565 FRGs were finally obtained for subsequent analysis.

### WGCNA

Modules of highly correlated genes were identified using WGCNA, and candidate biomarkers were subsequently screened. In this study, WGCNA was performed using the R package "WGCNA" to identify modules most relevant to SA patients. Specifically, we first calculated the variance of genes in the GSE63142 dataset and ranked the genes based on their variance. The top 25% of genes with the highest variance were selected for further analysis. To ensure suitability for WGCNA, the "goodSamplesGenes" function and a sample network approach were used to evaluate the 4892 selected genes. An appropriate soft threshold was determined to ensure the constructed network adhered to scale-free network standards. A TOM matrix gene clustering tree was generated based on the power value. A weighted coexpression network

model was established to partition the gene expression matrix into related modules. The module most strongly correlated with SA was selected for further analysis. Module membership (MM), defined as the correlation coefficient between a gene and the characteristic gene of a given module, quantifies the degree to which a gene belongs to that module. Genes meeting the criteria ( $|GS| \geq 0.2$  and  $|MM| \geq 0.8$ ) exhibited the strongest correlation with SA.

## Gene Enrichment Analysis

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were used to investigate biological activities and pathways. To understand the functional characteristics of the core genes of SA, we executed GO and KEGG enrichment analyses using the online tool DAVID (<https://david.ncifcrf.gov>), and the results were visualized using the “ggplot2” package.

## Hub Gene Related to Ferroptosis and Asthma

Through the “VennDiagram” package of R language, Common hub gene of ferroptosis and asthma was obtained by taking the intersection of the core genes of WGCNA and FRGs.

## Validation and Assessment of Hub Gene in an External Dataset

To reduce the possibility of false positives, we validated the hub gene using another asthma dataset from the GEO database. We selected GSE43696 for validation, which includes 20 normal controls, 50 mild-moderate asthmatic (MMA) patients and 38 SA patients. Using the R “pROC” package, draw ROC curves and evaluate the diagnostic efficacy of the hub gene based on the area under the curve (AUC). With  $AUC > 0.7$  as the screening criterion, the larger the AUC, the higher the diagnostic accuracy of the gene.

## Gene Set Enrichment Analysis (GSEA)

In order to further clarify the molecular mechanism of the hub gene of ferroptosis in asthma, asthma samples were divided into two subgroups with high and low expression according to the median expression of hub gene, and GSEA analysis was performed using R “clusterProfiler” package. The gene sets  $|NES| > 1$ ,  $P\_val < 0.05$  and  $FDR < 0.25$  were selected as the significantly enriched gene sets.

## Collection of Clinical Samples

To further verify the hub gene validated above, We enrolled 14 patients with pulmonary nodules from the Department of Thoracic Surgery at Henan Provincial People’s Hospital, categorized into two groups: (1) lung cancer patients with comorbid asthma, and (2) lung cancer patients without asthma. The inclusion criteria for all participants were: (i) preoperative diagnosis of lung cancer confirmed by pathological puncture or strong suspicion on high-resolution CT, and (ii) planned segmental bronchial resection. Asthma diagnosis required patients to be  $\geq 18$  years old and meet the Global Strategy for Asthma Management and Prevention 2024 guidelines.<sup>15</sup> Whereas non-asthmatic groups were confirmed by negative bronchial provocation tests and no history of chronic airway diseases. Exclusion criteria included postoperative pathological confirmation of distant metastasis; chronic obstructive pulmonary disease (COPD), severe pulmonary bullae, pneumothorax, pleural effusion, pulmonary fibrosis, or severe bronchiectasis; Long-term use of corticosteroids or immunosuppressants; other malignancies; severe organ dysfunction. We collected paracancerous bronchial tissue (more than 5cm from the tumor margin) samples from these patients, including 7 non-asthmatic patients and 7 asthmatic patients. The samples were obtained within 30 minutes post-surgery and stored in a  $-80^{\circ}\text{C}$  freezer. Non-asthmatic patients and asthmatic patients were matched for age and sex. This study was approved by the Ethics Committee of Henan Provincial People’s Hospital (No. 2024–077-01), and all subjects signed informed consent.

## Quantitative Real-Time PCR (qRT-PCR)

The bronchial tissues were cut and ground into powder. The total RNA was extracted by the Trizol method (Solarbio, Beijing, China). Then the RNA was reversely transcribed into cDNA using a reverse transcription kit (ABclonal, Wuhan, China), and PCR reaction was performed on a fluorescent quantitative PCR instrument (ABI, USA). The primers were synthesized by

Sangon Biotech (Shanghai, China) and are listed as follows (5'-3'):TIMP1-F,TACACACCTGGATTCCCTTCCT.TIMP1-R, CTGGGCCCCAACTACAACAT.  $\beta$ -actin-F, CCCATCTATGAGGGTTACGC. $\beta$ -actin-R, TTTAATGTCACGCACGATTC. The relative expression of TIMP1 mRNA was calculated using the  $2^{-\Delta\Delta CT}$  method with  $\beta$ -actin as an endogenous control. The experiment was carried out 3 times.

## Western Blot

The expression levels of TIMP1-related protein in bronchial tissues were detected by Western blot. Bronchial tissues of each group were homogenized in RIPA lysate containing a protease inhibitor, centrifuged at 4°C at 3000 r/min for 10 min, and the supernatant was collected. A BCA protein assay kit was used to determine the protein concentration. Protein samples of 40  $\mu$ g were separated by SDS-PAGE gel electrophoresis and then transferred to polyvinylidene fluoride membrane. After blocking 5% skim milk at room temperature for 2h, the membrane was incubated at 4°C overnight with the primary antibodies TIMP1 (1:1000, Proteintech, Wuhan, China) and GAPDH (1:2000, Diabio, Hangzhou, China), respectively. The next day, after the film was thoroughly washed, goat anti-rabbit IgG coupled with 1:5000 horseradish peroxidase was incubated at room temperature for 2h. Finally, ECL reagent was added for development. The gray values of protein bands were analyzed by Image J software. GAPDH was used as the internal parameter for calculation: relative expression of target protein = protein gray level/GAPDH gray level. The experiment was carried out 3 times.

## Statistical Analysis

Bioinformatics analysis was performed using R software (v4.3.2). GraphPad Prism version 10.4 was used for statistical analysis. An independent sample *t*-test was used to analyze the relative expression of the TIMP1 gene in non-asthmatic control groups and asthmatic groups, The Variance analysis was used to compare the differences between different groups in the GSE43696 dataset. The diagnostic efficacy of the TIMP1 gene for asthma was analyzed using ROC curve.  $P < 0.05$  was considered statistically significant.

## Results

### WGCNA

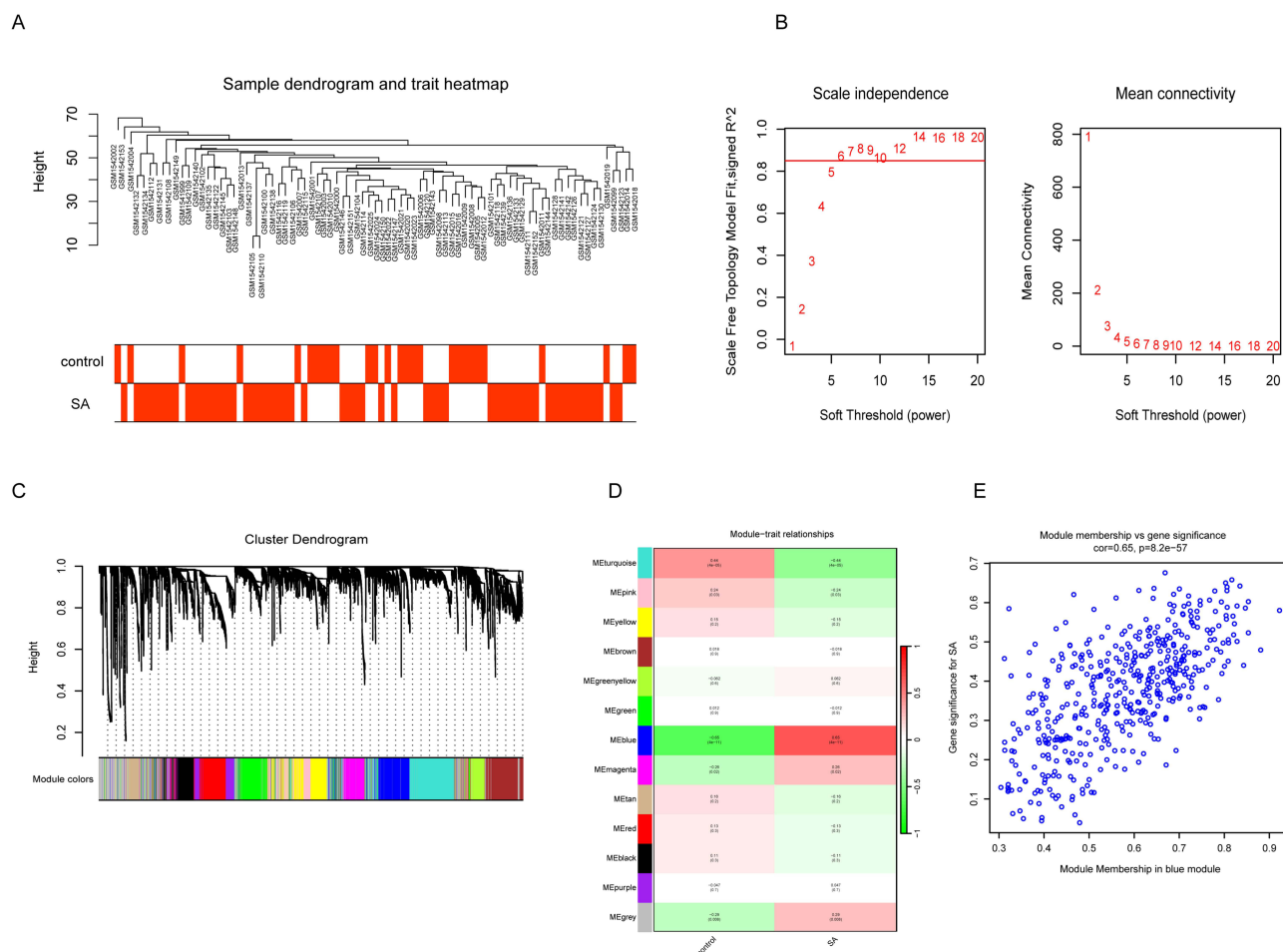
Hierarchical clustering was performed to determine the sample size, and outliers were removed with a cutting height of 70 and a minimum module size of 10 (Figure 1A). In this study, A soft-thresholding power of 6 (R-square = 0.85) was used to convert the adjacency matrix into the topological overlap matrix (TOM). This choice of parameters resulted in a network that closely resembles a scale-free network (Figure 1B). Using the selected power value, the weighted correlation coefficients were used to construct the TOM matrix for gene clustering (Figure 1C). The weighted gene co-expression network model partitioned the gene expression matrix into 13 co-expression modules, with the blue module showing the highest correlation to SA ( $r = 0.65$ ,  $P = 4e-11$ ) (Figure 1D). The genes in the blue module exhibited a strong association with SA ( $cor = 0.65$ ,  $P = 8.2e-57$ ). Core genes in the blue module were selected based on the criteria of  $|MM| \geq 0.8$  and  $|GS| \geq 0.2$ , yielding 23 core genes: FUT5, HPN, GALNT5, COMTD1, C20orf46, S100A14, DNAJC1, PYCR1, SDCBP2, CEACAM5, GALE, C7orf26, CSH1, GSN, CCBL1, VSIG2, FUT3, PRKAR2B, DHX35, TIMP1, SLC24A3, LRRC31, and LOC100132287 (Figure 1E).

### Function and Pathway Enrichment Analyses

Next, we performed GO and KEGG analyses on the core genes of the blue module to elucidate the biological functions and pathways. The GO analysis revealed significant enrichment in processes such as xenobiotic metabolic process, glutathione metabolic process, and glutathione transferase activity (Figure 2A). The KEGG analysis showed enrichment in pathways including Metabolic pathways, Drug metabolism - cytochrome P450, and Ferroptosis (Figure 2B).

### TIMP1 is the Only Ferroptosis-Related Gene of Asthma

Using R “VennDiagram” package, the intersection of the core genes and FRGs genes was analyzed. A Venn plot revealed that TIMP1 is the only one ferroptosis-related gene (Figure 3).



**Figure 1** WGCNA results. (A) Sample-Trait Clustering Chart. (B) WGCNA with 0.85 and 6 as adjacency cutoff value and soft threshold, respectively. (C) Formation of 13 co-expression gene modules via clustering. (D) Heatmap showing association between each module and SA. (E) Correlation analysis of genes and phenotype within blue module.

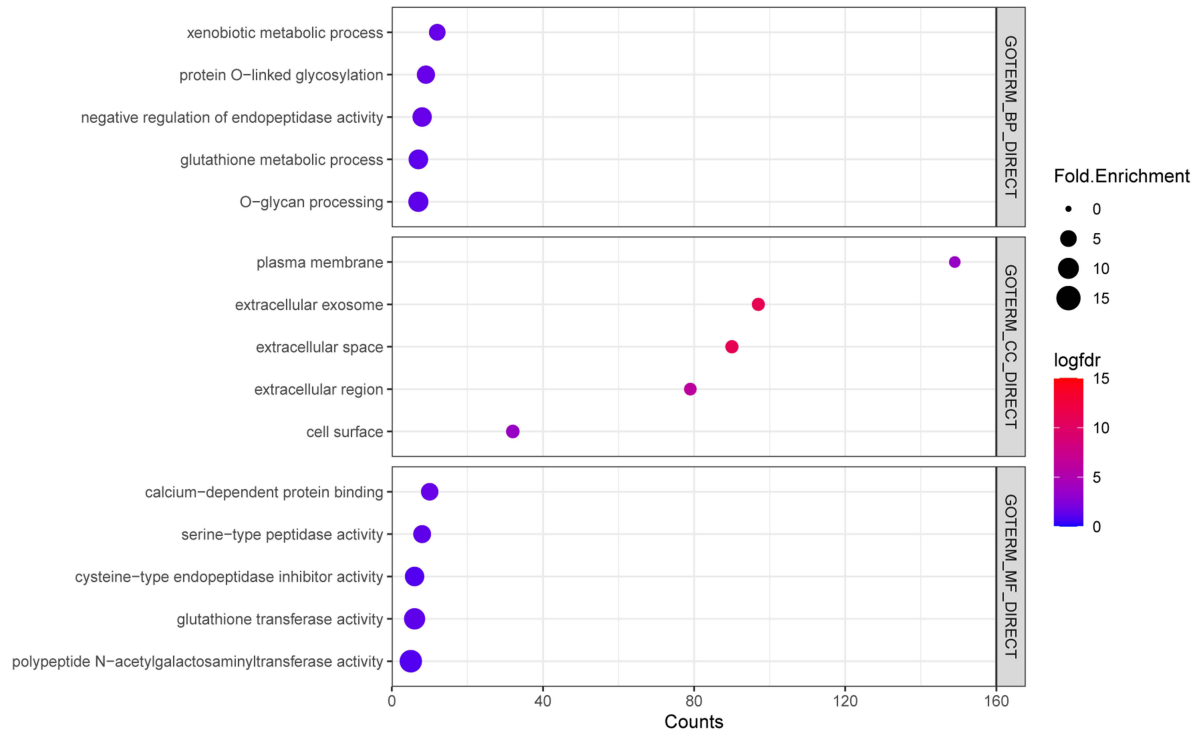
## Validation of Hub Gene in an External Dataset

Validation using the GSE43696 dataset revealed that, compared to the control groups, the gene expression of TIMP1 was elevated in the asthma groups ( $P < 0.05$ ), irrespective of asthma severity. Notably, the expression of TIMP1 was significantly elevated in the SA groups compared to both the MMA groups ( $P < 0.05$ ) and normal controls ( $P < 0.0001$ ) (Figure 4A and B). We utilized the expression level of TIMP1 as a predictor to construct ROC curves and calculate AUC values. TIMP1 demonstrated good predictive efficacy for asthma (AUC=0.769; 95% CI:0.653–0.886; Cutoff:10.568; Sensitivity:0.591; Specificity:0.9). Among the asthmatic groups, SA showed greater diagnostic value (AUC=0.865; 95% CI:0.774–0.956; Cutoff:10.486; Sensitivity:0.757; Specificity:0.865) compared to MMA (AUC=0.713; 95% CI:0.577–0.849; Cutoff:10.568; Sensitivity:0.48; Specificity:0.9) (Figure 4C–E).

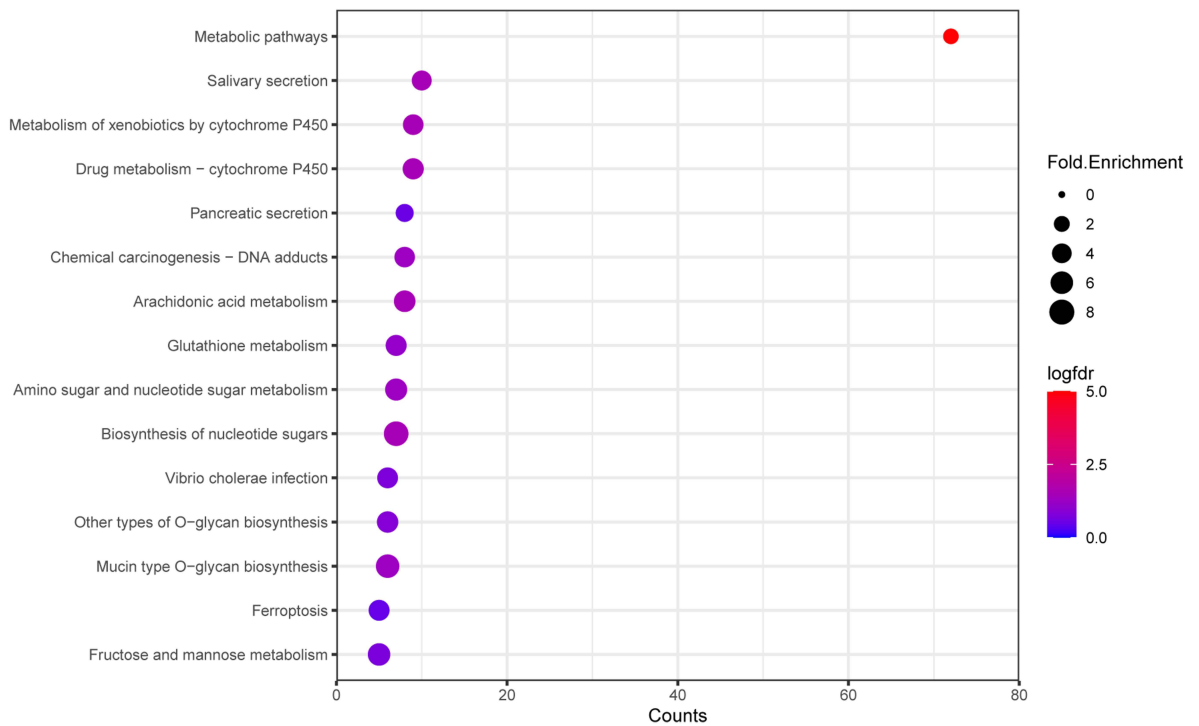
## GSEA

In the asthma samples, we divided the patients into high and low expression groups based on the median expression level of the TIMP1 gene. A heatmap was used to display the top 30 genes in both groups (Figure 5A). Subsequently, we analyzed all genes in both groups, and the results highlighted the HIF-1 signaling pathway, which is associated with the gene set containing TIMP1 (Figure 5B and Table 1).

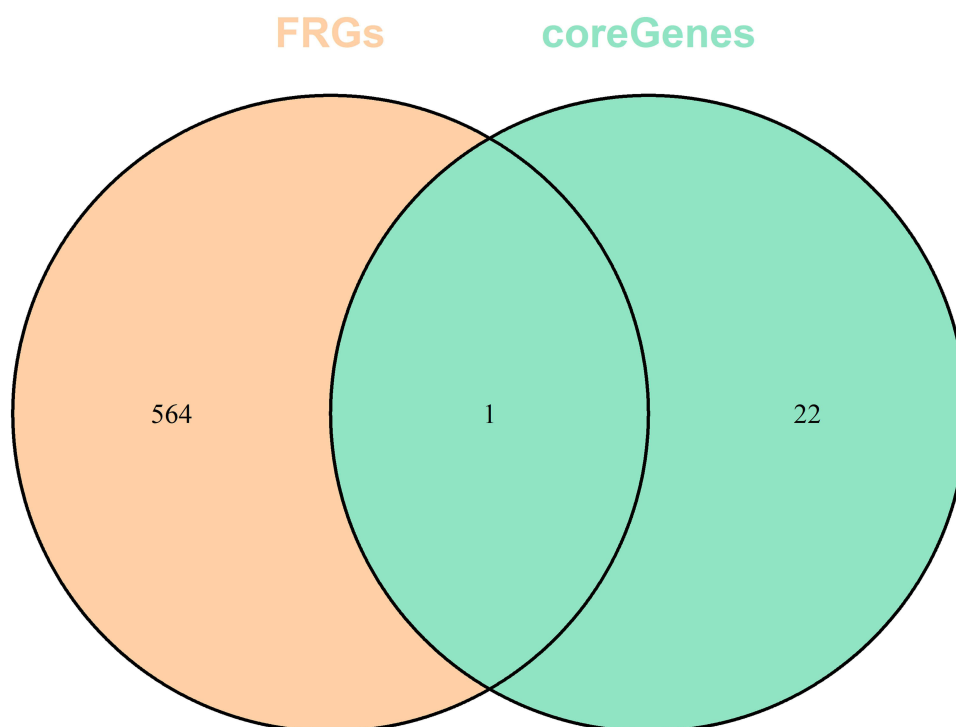
A



B



**Figure 2** Enrichment functional analyses of the 23 core genes. **(A)** Bubble plot of GO enrichment analysis of the 23 core genes. **(B)** Bubble plot of KEGG enrichment analysis of the 23 core genes.



**Figure 3** Common gene representation through a Venn diagram. One gene from among Five hundred and sixty-five FRGs genes and twenty-three core genes was found to be common gene.

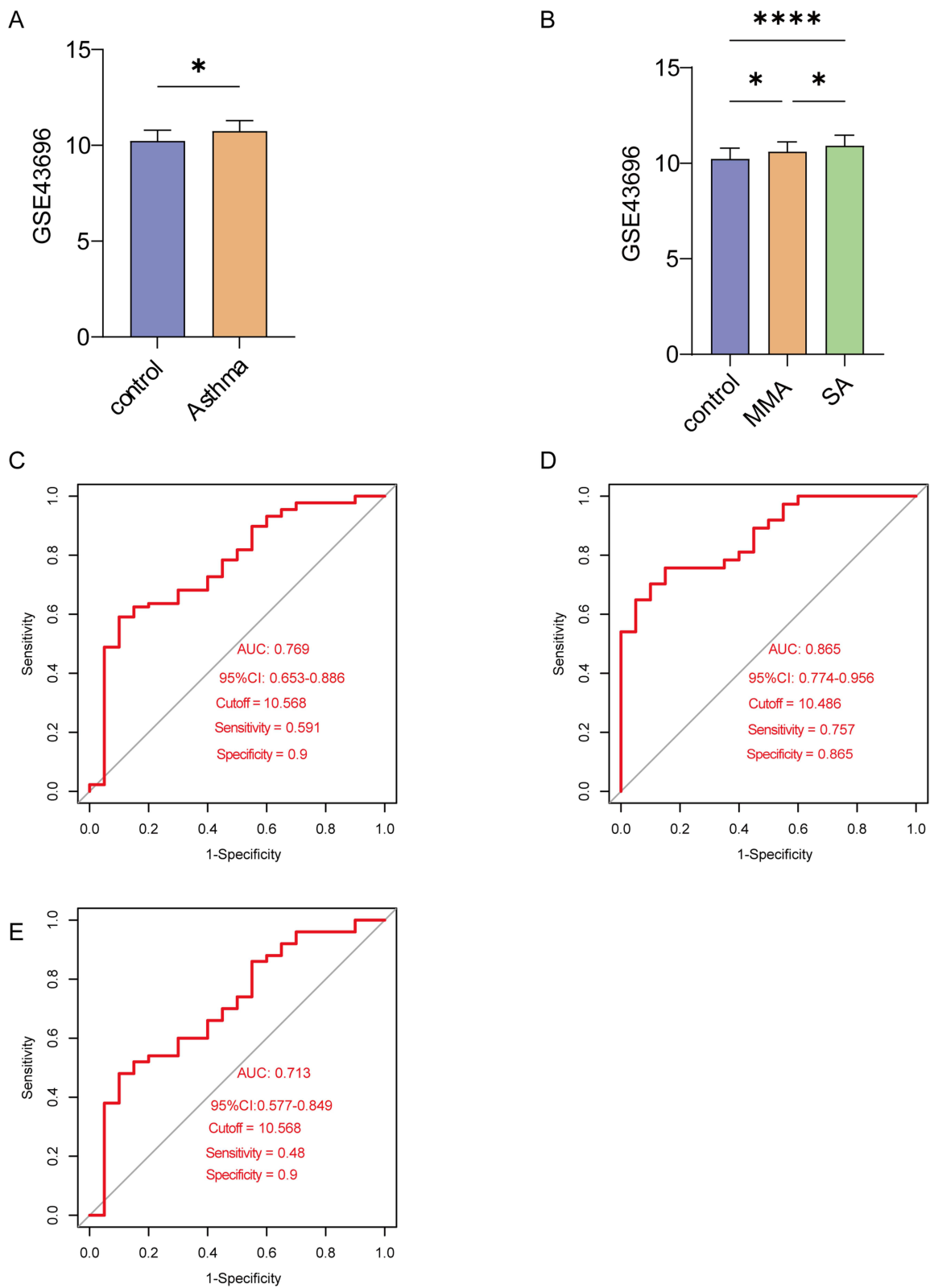
## Verification of Bronchial Tissue TIMP1 Levels in Human Samples

To verify whether TIMP1 is a hub gene involved in ferroptosis associated with asthma, we validated TIMP1 with bronchial tissue samples. The protein encoded by TIMP1 is a secreted glycoprotein, which belongs to the family of metalloproteinase inhibitors, and is widely present in various tissues and body fluids. We measured the levels of TIMP1 in the bronchial tissues of non-asthmatic patients and asthmatic patients. The results indicated that the mRNA levels of TIMP1 in the asthmatic groups were higher than those in the non-asthmatic control groups ( $P < 0.05$ ), and the protein expression levels of TIMP1 in the asthmatic groups were also elevated compared to the non-asthmatic control groups ( $P < 0.001$ ), as shown in (Figure 6A and B).

## Discussion

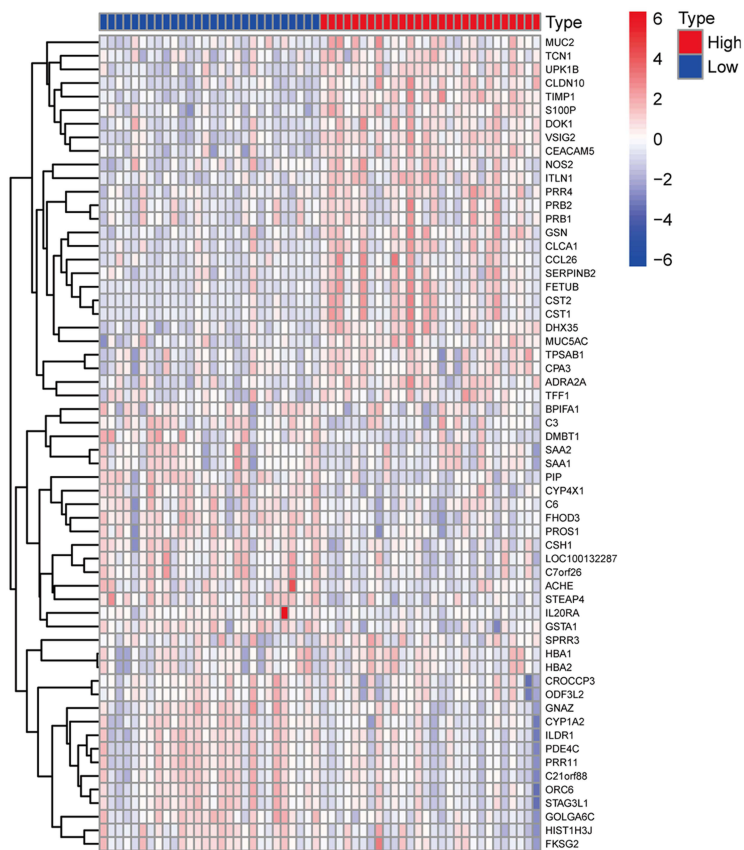
Ferroptosis, a newly discovered mode of cell death, is gradually recognized by the scientific community for its relationship with asthma. However, informatics analysis has not yet clearly defined the genetic-level connections. Inhaled corticosteroids (ICS) have been the cornerstone of asthma treatment, significantly reducing morbidity and mortality.<sup>16</sup> Nevertheless, prolonged use of ICS may lead to risks such as osteoporotic fractures, pneumonia, diabetes, and obesity.<sup>17</sup> Despite a range of preventive and therapeutic measures, there are still a few asthma patients who cannot be well controlled. Therefore, identifying biomarkers associated with ferroptosis in asthma is significant, providing new evidence for asthma treatment and early prediction.

This study identified a total of 23 core genes related to asthma through WGCNA. GO functional enrichment analysis indicated that these core genes were mainly involved in biological processes such as xenobiotic metabolic process, glutathione metabolic process, and glutathione transferase activity. The xenobiotic metabolic process refers to the metabolic process by which organisms process foreign substances, such as toxic chemicals and environmental pollutants.<sup>18–20</sup> Studies have shown that long-term exposure to dust, air pollution, and toxic chemicals increases the risk of developing asthma.<sup>18,19,21</sup> Toluene diisocyanate (TDI), one of the primary allergens responsible for occupational asthma, can induce Th2 and Th17 responses and is often accompanied by airway infiltration of neutrophils and

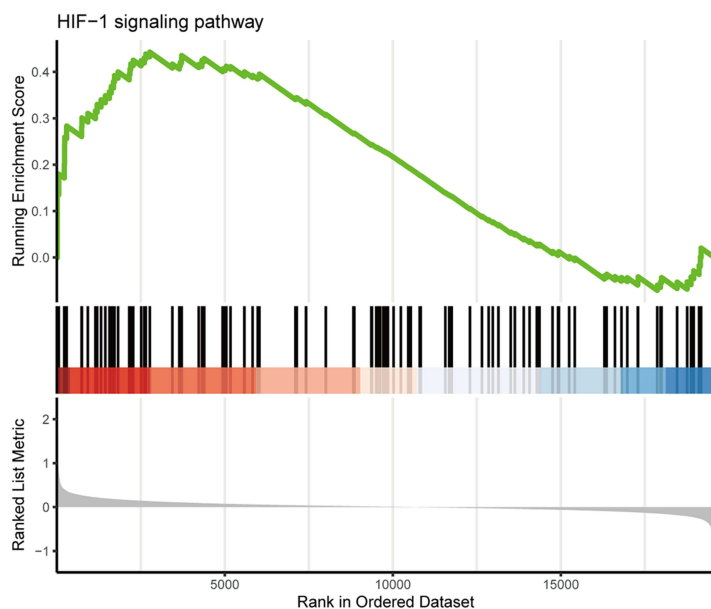


**Figure 4** Prognostic value of TIMP1 in asthma. **(A and B)** TIMP1 highly expressed in asthma and expressed higher in SA compared with in MMA. **(C–E)** ROC curves of TIMP1 in asthma, SA, and MMA in GSE43696 asthma dataset. \* $P < 0.05$ ; \*\*\*\* $P < 0.0001$ .

A



B



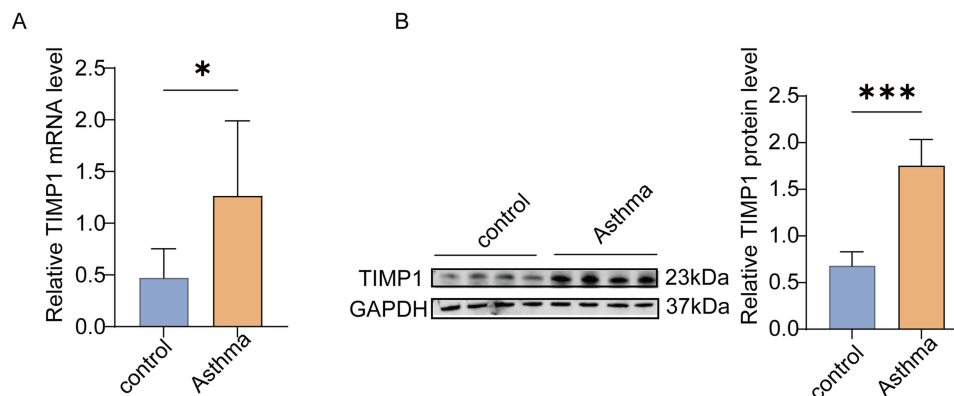
**Figure 5** Gene Set Enrichment Analysis of TIMP1 in asthma. **(A)** A heatmap was used to visualize the top 30 genes in the two groups. **(B)** The HIF-1 signaling pathway was significantly enriched in the high-expression group.

**Table 1** Gene Sets Enriched in the HIF-1 Signaling Pathway

Description	NES	p value	FDR	Core Enrichment
HIF-1 signaling pathway	1.621	0.002	0.013	NOS2/TIMP1/LDHC/EIF4E2/PFKP/HK2/EIF4EBP1/PRKCB/GAPDH/MAP2K2/HK1/LDHA/CDKN1A/ALDOA/CAMK2G/LTBR/CYBB/MKNK2/RELA/PGK1/NFKB1/HMOX1/RPS6KB2/TLR4/CAMK2B/EGLN2/MAPK3

eosinophils.<sup>22</sup> Moreover, a study has confirmed that a decrease in glutathione levels can aggravate the severity of type 2 inflammation and asthma.<sup>23</sup> Exposure to air pollution or tobacco smoke, especially those related to glutathione transferase activity polymorphisms, can affect prenatal and postnatal lung development, increase the risk of developing asthma in young adulthood, and increase the risk of decreased lung function during adolescence.<sup>24</sup> In our study, multiple terms of GO enrichment analysis were related to metabolic processes and enzyme activities, which further demonstrated the significant roles of both in the progression of asthma. KEGG pathway enrichment analysis revealed that these core genes are significantly associated with Metabolic pathways, Drug metabolism - cytochrome P450 and Ferroptosis. Jiang utilized an LC-MS metabolomics approach to analyze and compare the urine of asthmatic children and healthy controls. The results revealed that dysregulation of  $\alpha$ -linolenic acid metabolism plays a critical role in the development of childhood asthma and may serve as a novel therapeutic target for asthma.<sup>25</sup>  $\alpha$ -Linolenic acid is a polyunsaturated fatty acid belonging to the n-3 fatty acid family, known for its anti-inflammatory and lipid-regulating effects.<sup>26,27</sup> Interestingly, studies have found that dietary supplementation of n-3 polyunsaturated fatty acids promotes lipid peroxidation in cancer cells within an acidic tumor microenvironment, inducing ferroptosis in cancer cells and thereby exerting anti-tumor effects.<sup>28</sup> Cytochrome P450 is a superfamily composed of a group of heme-thiolate proteins. It is not only related to the metabolism of endogenous substances but also participates in the metabolism of exogenous substances. CYP3A is a subfamily of CYP450 and the body's most abundant metabolic enzyme. CYP3A5, as a member of the CYP3A family, has its gene polymorphism associated with the aggravation of adult asthma patients' conditions. Research indicates that patients carrying the CYP3A5\*1 allele have a significantly higher rate of disease worsening. Additionally, individuals with this allele exhibit a reduced response to corticosteroid treatment.<sup>29</sup> Notably, cytochrome P450 is involved in the phospholipid peroxidation reaction of ferroptosis.<sup>30</sup> Consequently, the KEGG pathway analysis findings suggest that these core genes may play a role in directly or indirectly regulating ferroptosis, which could potentially worsen the clinical manifestations of asthma.

We then performed an intersection analysis between the core genes and FRGs genes. TIMP1 was identified as a hub gene and further validated using an external dataset and human samples. These results suggest that TIMP1 is the only ferroptosis-related gene and may be associated with ferroptosis in asthmatic patients. In order to further investigate the molecular mechanism of TIMP1 regulating ferroptosis, GSEA analysis was performed in this study. The results indicated



**Figure 6** Verification of bronchial tissue TIMP1 levels in human samples. **(A)** TIMP1 mRNA expression was elevated in asthma patients. **(B)** The expression levels of TIMP1 was detected by Western blot and the relative intensity was quantified by image J software. \* $P < 0.05$ ; \*\*\* $P < 0.001$ .

a significant association between TIMP1 and the HIF-1 signaling pathway. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor widely present in the body under hypoxic conditions, playing a crucial role in maintaining internal homeostasis. It is significant biologically in various physiological and pathophysiological processes such as cancer, anemia, cerebral and cardiac ischemia, inflammatory responses, and embryonic development. Recently, researchers have discovered that the HIF family is simultaneously activated when the expression of TIMP1 in periosteal osteoblasts is significantly upregulated.<sup>31–33</sup> Subsequently, it has been confirmed that HIF1 $\alpha$  can directly bind to the promoter and enhancer regions of the TIMP1 gene, leading to increased production of TIMP1 by periosteal osteoblasts.<sup>34</sup>

Original naming of proteins is based mostly on one function only, and TIMP-1 was initially named for its ability to inhibit matrix metalloproteinases, with its primary function being the regulation of extracellular matrix degradation.<sup>35</sup> However, as research has advanced, elevated levels of TIMP-1 were found to be associated with a poor prognosis in a multitude of inflammatory diseases, including coronavirus disease 2019 and sepsis, suggesting a proinflammatory feature of TIMP-1 beyond metalloproteinase inhibition.<sup>36,37</sup> Our findings indicate that TIMP1 may be associated with ferroptosis in asthma. Shi et al found that silencing TIMP1 markedly reduced the iron ion level (Fe<sup>2+</sup>), and the content of Reactive oxygen species (ROS) and Malondialdehyde (MDA) expression.<sup>38</sup> Importantly, ROS plays a vital role in airway epithelial remodeling and hyper-responsiveness, which are mechanistically connected to ferroptosis.<sup>39</sup>

## Limitations of the Study

The study has several limitations, which must be acknowledged. Firstly, limited availability of clinical samples and use of paracancerous lung tissues may introduce confounding effects due to cancer-associated gene expression, potentially reducing statistical power and limiting the generalizability of our findings. To further validate our findings and address potential confounding effects, we will expand our cohort to include additional patient groups, such as individuals with severe asthma and normal controls, in subsequent studies. Additionally, although TIMP1 has been validated in clinical samples, its functional mechanisms in asthma remain poorly understood. Therefore, additional functional studies are essential to clarify the role of TIMP1 in asthma pathogenesis.

## Conclusion

In summary, Our findings reveal a novel association between TIMP1 and ferroptosis in asthma pathogenesis, providing new mechanistic insights. To elucidate the functional role of TIMP1 in regulating airway epithelial cell ferroptosis, we will combine in vitro and in vivo approaches. In human bronchial epithelial cells, TIMP1 will be modulated (siRNA/overexpression) followed by erastin-induced ferroptosis, with assessment of lipid peroxidation, mitochondrial ultrastructure (TEM), and ferroptosis markers (GPX4/ACSL4/SLC7A11). In TIMP1-knockout mice challenged with ovalbumin or HDM, we will measure MDA and Fe<sup>2+</sup> in airway tissues. These comprehensive analyses will clarify whether TIMP1 regulates ferroptosis primarily through iron metabolism or lipid metabolism pathways.

## Abbreviations

TIMP1, Tissue Inhibitor of Metalloproteinase 1; MMA, mild-moderate asthma; SA, severe asthma; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; WGCNA, Weighted gene Coexpression network analysis; GSEA, Gene Set Enrichment Analysis.

## Data Sharing Statement

We provide details of the materials and methods in our manuscript.

## Ethical Approval and Consent to Participate

This study was performed in accordance with the Helsinki declaration. All patients obtained informed consent and were approved by the Ethics Committee of Henan Provincial People's Hospital (No. 2024-077-01).

## Acknowledgments

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## Disclosure

The authors declare that they have no conflicts of interest in this work.

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