

Unveiling Ventilator-Associated Pneumonia: S100A8 as a Promising Biomarker Through Integrated RNA-Seq Analysis

Qixing Wang^{1,*}, Jiayong Dong^{2,*}, Xin Jing¹, Yanling Long¹, Hongyu Jia¹, Yingchuan Li¹, Junjie Wang^{1,*}

¹Department of Critical Care Medicine, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, 200072, People's Republic of China; ²Department of Liver Surgery and Organ Transplantation, Changzheng Hospital, Naval Medical University, Shanghai, 200003, People's Republic of China

*These authors contributed equally to this work

Correspondence: Junjie Wang; Yingchuan Li, Email megenehappy@126.com; yingchuan_li@tongji.edu.cn

Background and Objectives: Ventilator-associated pneumonia (VAP) was a common and severe complication of invasive mechanical ventilation. The traditional VAP diagnostic model relied on laboratory microbiological cultures. However, VAP had unclear pathogenesis, and its accurate identification was difficult due to the varying levels of pathogen detection in different laboratories. There was an urgent need for new diagnostic biomarkers for VAP.

Methods: The transcriptome of VAP patients was analyzed and computed using bioinformatics techniques in this study. The screen identified differentially expressed genes (DEGs) from the chemokine CC family, the S-100 family, and the α -defensin family, which are highly associated with immune-related antimicrobial functions. Single-cell landscape data revealed an increase in MTRNR2L12+ cells and a decrease in naïve CD4+ T cells, ciliated cells, and CD8+ T cells in VAP patients, indicating a significant change in the homeostatic profile in patients. Moreover, this paper explored differential gene expression at the single-cell level.

Results: The study analyzed 46 VAP samples and 48 normal samples to explore VAP pathogenesis and identify potential biomarkers. Both bulk RNA seq and scRNA-seq analysis revealed that S100A8 was highly expressed in the VAP group. This phenomenon was caused by the cellular level differential expression of B cells. In contrast, the reduced FN1 and HLA-DRB5 expressions in the VAP group may be influenced by the expression of T cells, macrophages, and ciliated cells. Western blot experiments detected S100A8 expression in the patient samples.

Conclusion: In this study, we combine bulk RNA-seq and scRNA-seq analyses to screen and validate the potential of S100A8, a gene with consistent expression, as a biomarker, providing a new perspective for VAP diagnosis.

Keywords: ventilator-associated pneumonia, biomarker, s100A8, single cell, prediction

Introduction

Ventilator-associated pneumonia (VAP) is a lung infection developed in patients receiving mechanical ventilation support through an endotracheal or tracheostomy tube.¹ VAP is a severe complication that can cause prolonged hospitalization, increased healthcare costs, and even death.² The high morbidity and mortality rates, especially in critically ill patients, make VAP dangerous.³ VAP causes severe respiratory distress⁴, sepsis,⁵ and multiple organ failure⁶. VAP patients require longer mechanical ventilation duration, prolonged hospital stays, and increased healthcare costs.

Furthermore, VAP may increase the risk of antibiotic resistance,⁷ leading to difficult-to-treat infections and worse outcomes for future patients. VAP is one of the most common healthcare-associated infections in critically ill patients. Many studies were conducted to identify the risk factors,^{8,9} pathogenesis¹⁰, prevention¹¹, and treatment strategies¹² of VAP. These studies led to the development of guidelines and protocols to reduce the incidence of VAP, including

implementing strict hand hygiene practices, reducing the duration of mechanical ventilation, and using oral care protocols to prevent oral colonization by pathogenic bacteria.

With the advancement of the post-genomic era and single-cell histology technologies, research on utilizing omics to detect VAP-related biomarkers became increasingly abundant. Biomarker research was an important area of investigation to aid in the early diagnosis and treatment of the condition.^{13–15} Biomarkers are measurable indicators of a biological state, and in the case of VAP, they can help identify the presence of lung infection. One commonly studied biomarker for VAP is procalcitonin (PCT),¹⁶ a precursor of calcitonin hormone. PCT levels are often elevated in patients with bacterial infections, including VAP. Other biomarkers studied for VAP include interleukin-1 β (IL-1 β), interleukin-8 (IL-8),¹⁷ and tumor necrosis factor- α (TNF- α),¹⁸ inflammatory cytokines produced in response to infection. Numerous studies have confirmed the potential of omics techniques for identifying new biomarkers. This article explores the pathogenesis and potential VAP marker through bulk RNA-seq and single-cell RNA-seq (scRNA-seq).

A novel pipeline for VAP-biomarker identification was developed in this study. Initially, 289 DEGs were filtered out via DESeq2. KEGG and GO functional enrichment revealed that the DEGs were closely associated with immune regulation functions such as viral protein interaction, defense response to viruses, chemokine activity, and antimicrobial humoral response. Disease and immunity analysis revealed that the chemokine CC family, S-100 family, and alpha-defensin family played a bridge-like role in the VAP phenomenon. Furthermore, single-cell landscape data revealed that MTRNR2L12+ cells increased in the VAP group, and naïve CD4+ T cells, ciliated cells, and CD8+ T cells decreased in VAP patients.

Moreover, DEGs at the single-cell level were investigated. We discovered that S100A8 was highly expressed using both bulk RNA seq and scRNA-seq in the VAP group, with the differential expression of B cells leading to this phenomenon. The reduced FN1 and HLA-DRB5 expression may be influenced by the expression of T cells, macrophages, and ciliated cells. Western blot experiments confirmed S100A8 expression in the patient samples.

Methods

Datasets

The original data (GSE168017 and GSE168018) were obtained from the gene expression omnibus (GEO, <https://www.ncbi.nlm.nih.gov/geo/>). The GSE168017 series is a bulk RNA-seq dataset, and the 94 RNA-seq samples can be downloaded for further analysis. The VAP group had 46 samples, while the non-VAP group had 48 samples. The GSE168018 series is a scRNA-seq dataset with data for eight non-VAP and five VAP patients.

Differentially Expressed Genes Screening and Identification

DESeq2¹⁹ was used for differential gene expression analysis, and the gene counts were considered metadata. R packages of org.Hs.eg.db were used to convert ensemble ID values to gene symbols. The DEGs selection threshold parameters used here consisted of two parts: $|\log_2(\text{Fold-change})| \geq 1$ and adjusted p-value < 0.05 .

Enrichment Analysis

Additional biological functional analysis of the aforementioned DEGs was performed using R package clusterProfiler²⁰ and enrichplot. In the KEGG database analysis, the enrichment parameter was set as organism = “has”, while the other parameters remained unchanged. Furthermore, in the analysis of the Gene Ontology (GO) Resource database, this paper enriched the three ontologies of biological process (BP), molecular function (MF), and cell component (CC) at the same time, with the enrichment parameter is set to pvalueCutoff = 0.05, pAdjustMethod = “BH”.

Disease Association Analysis

DisGeNet database (<https://www.disgenet.org/>)²¹ was used for VAP, and 56 disease-related genes were obtained. The relationship between DEGs and the disease gene list was analyzed using a Venn diagram.

Metascape Analysis

Metascape website (<https://metascape.org/>)²² was used to perform functional clustering analysis. A total of 289 DEGs were uploaded to the website using the default submission parameters.

Immune Factor Analysis

The immune factor analysis background data were exported from the ImmPort database (<https://www.immport.org/shared/genelists>).^{23,24} The genelists.txt file included 2483 items.

Single Cell Analysis

The R package Seurat²⁵ was used for single-cell data pre-processing, data integration, normalization, highly variable features finding, value scale, PCA, UMAP, Marker labeling, and other processes.

Cell Type Identification

CD14 and LYZ for CD14+ Monocytes; CSF3R and CXCR4 for CXCR4+ Neutrophils; CSF3R and FCGR3B for FCGR3B+ Neutrophils; MTRNR2L12 for MTRNR2L12+ Cells; CCR7 and IL7R for Naive CD4+ T Cell; CD68 and EREG for EREG+ Macrophages; CD68 and DNAJB1 for DNAJB1+ Macrophages; TFF3 and SCGB1A1 for Ciliated Cells, CD8A for CD8+ T Cells; MS4A1 for B Cells; IRF4 and STMN1 for STMN1+ T Cells.

Western Blot Analysis

This study was carried out following the guidelines of the Helsinki Declaration (World Medical Association Declaration of Helsinki). The ethical approval was obtained from Shanghai tenth people's hospital Ethics Board (ethical approval code: 22k204), and written informed consent was obtained from all participants prior to the commencement of the study. Endotracheal aspirates were collected through an endotracheal tube using a sterile suction catheter, 0.1 g of endotracheal aspirate was weighed, and 1 mL RIPA lysis buffer was added. The tissue was further ground using an electric homogenizer ([Supplementary 3](#)). After complete grinding, the tissues were lysed with lysis solution at 4°C for 30 min, followed by centrifugation ($12,000 \times g$ at 4°C for 10 min), and the supernatant was obtained. Total protein was quantified using BCA assays, and the remainder was frozen at -80°C until use. All samples were subjected to SDS-PAGE electrophoresis and transferred to PVDF membranes. The membrane was blocked with 5% skim milk powder in TBST solution. Primary antibodies were incubated overnight, and the secondary antibodies were incubated for one hour. The ECL method was used for Color development. The Gelpro32 software was used for the gray value analysis of the target band.

Results

Profile of Gene Expression and Gene Function in VAP

The experimental group in this study included 46 VAP samples, and the control group consisted of 48 normal samples to investigate the pathogenesis of VAP and screen for suitable biomarkers. The age of the VAP group ranged from 22 to 72 years, with an average age of 52.14 ± 14.30 years. The patient's ages in the non-VAP group ranged from 31 to 79, with an average age of 53.70 ± 14.02 years. There was no significant difference in age ($p = 0.8365$), gender ($p = 0.4089$), race ($p = 0.2440$), and ethnicity ($p = 0.4089$) between the two groups (Student's *t*-test).

There were 289 DEGs meeting the threshold of $|\log_2\text{FC}| \geq 1$ and adjusted $p\text{-value} < 0.05$. Most genes (223 genes) were highly expressed in the VAP group, while the remaining genes (66 genes) had a lower expression. The volcano diagram for DEGs screening is shown in [Figure 1A](#). The upregulated DEGs were colored red, and downregulated DEGs were colored dark blue. The KEGG enrichment analysis showed that these DEGs were primarily associated with Morphine addiction, Viral protein interaction with cytokine and cytokine receptors, GABAergic synapse, Hematopoietic cell lineage, and Ovarian steroidogenesis signaling pathways ([Figure 1B](#)). The enrichment results of the GO database revealed that these DEGs were primarily composed of cellular components such as haptoglobin-hemoglobin complex, collagen-containing extracellular matrix, blood microparticle, endocytic vesicle lumen, and other

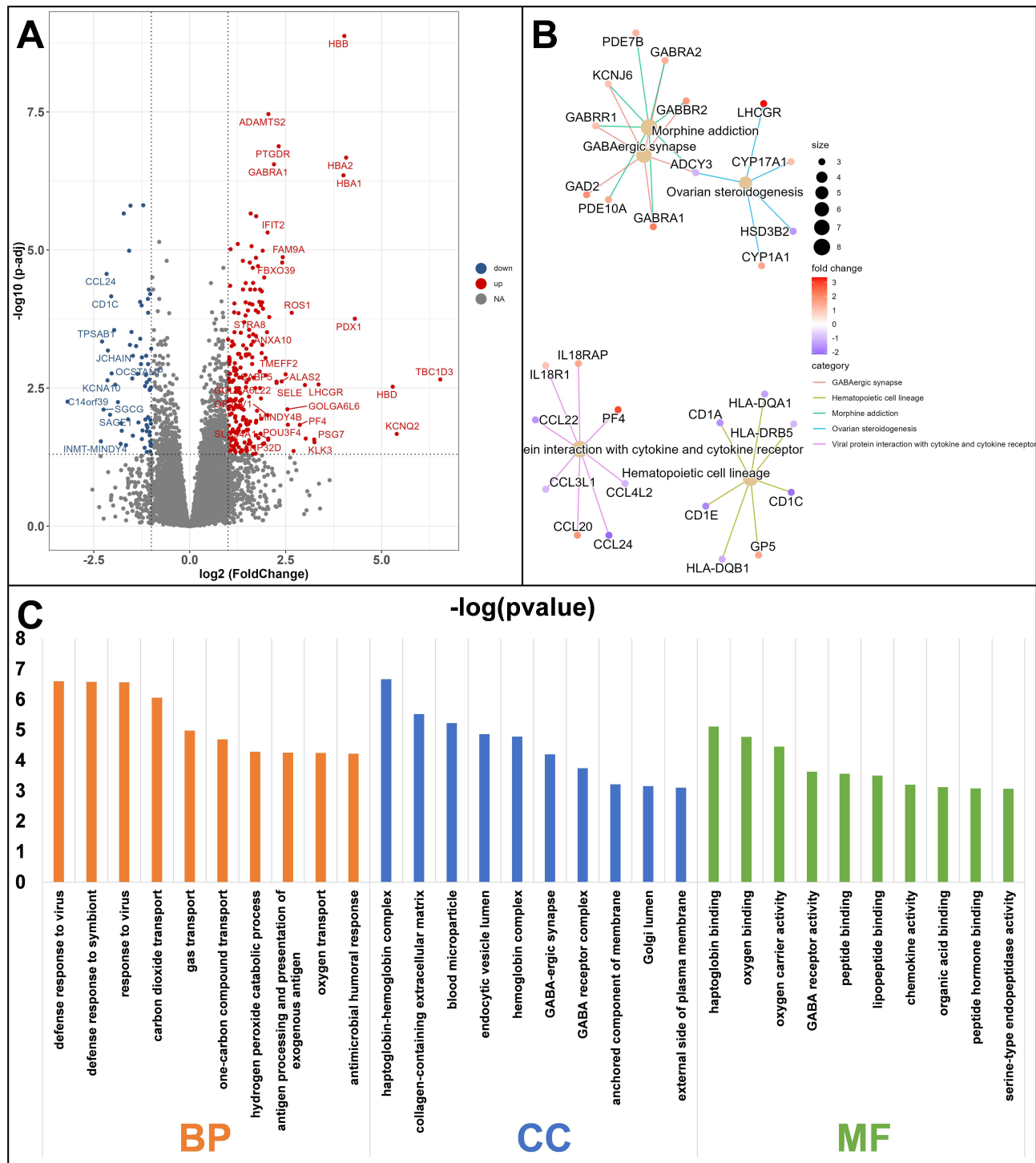


Figure 1 (A) The DEGs in VAP and non-VAP groups; red color indicates differentially upregulated genes, blue indicates differentially down-regulated genes, and gray indicates insignificantly differential genes. The horizontal axis represents the ploidy difference, and the vertical axis represents the negative logarithm of the p-value with a base of 10. (B) The KEGG enrichment results for DEGs. The size of the dot represents the number of genes in the pathway. The relationship between signaling pathways and genes is represented by different color lines. (C) The GO enrichment results for DEGs. BP = Biological Process; CC = Cell Component; MF = Molecular Function.

components. They were involved in haptoglobin binding, oxygen binding, peptide binding, lipopeptide binding, chemokine activity, serine-type endopeptidase activity, and other molecular binding effects. Moreover, these DEGs further influence numerous biological processes, such as defense response to viruses and symbionts, carbon dioxide transport, and antimicrobial humoral response (Figure 1C).

Disease and Immunity Analysis

The DEGs were compared with the DisGeNet database, a discovery platform containing one of the largest publicly available collections of genes and variants associated with human diseases, to further investigate the association between DEGs and diseases.^{21,26,27} The database included 56 VAP-related genes, and the analysis showed that only S100A8 was among the DEGs (Figure 2A). We then used Metascape²² to perform a functional clustering analysis of differential genes, with each term (including KEGG, GO, Reactome, Wiki Pathways, and Canonical Pathways) represented by a circle node, its size was proportional to the number of input genes that fall under that term, and its color represents its cluster identity. Terms with a similarity score > 0.3 were linked by an edge. Furthermore, the Metascape enrichment results showed that NABA matrisome associated response to viruses, inflammatory response, response to bacteria, scavenging of heme from plasma, and other immune functions might be related to DEGs (Figure 2B). This article analyzed the annotation of DEGs in the ImmPort database to explore the immune mechanism in diseases further. The immune-related DEGs (IR-DEGs) and their corresponding immune functions are shown in Figure 2C. The most important role of IR-DEGs is their antibacterial activities.

Profile of Cell Populations in VAP

The resolution of bulk-RNAseq cannot explain whether the disease was caused by changes in cell populations; therefore, this article also analyzed VAP-related single-cell RNA-seq data (GSE168018) from the same batch. This dataset included single-cell sequencing samples of airway aspirates from eight non-VAP patients and five VAP patients. Data integration and standardization follow the Seurat guidelines. Figure 3A shows the data integration results of most individuals, and Figure 3B shows the integration comparison results of VAP and non-VAP groups. When the resolution was set to 0.7, a preliminary cell population of 17 categories (cluster 0–16) was obtained (Figure 3C), and the representative markers of each group are shown in Figure 3D.

Identification and Analysis of Cell Types

Based on the literature and existing knowledge, 11 cell types were identified in this study. The marker for each cell type could be found in the methods part (including CD14⁺ Monocytes, CXCR4⁺ Neutrophils, FCGR3B⁺ Neutrophils, MTRNR2L12⁺ Cells, Naive CD4⁺ T Cell, EREG⁺ Macrophages, DNAJB1⁺ Macrophages, Ciliated Cells, CD8⁺ T Cells, B Cells, and STMN1⁺ T Cells). Figure 4A shows the labelled-cell types of the non-VAP (Ctrl) and VAP groups. Figure 4B shows the percentages of cell types by Samples (left) and group (right).

Moreover, scRNA-seq revealed significant individual differences. We identified a cell type, MTRNR2L12⁺ cells, with high expression of mitochondrial-related genes in this study. Figure 4B shows that the percentage change of this type of cell was significantly affected by the VAP05 sample. Similarly, the proportion of ciliated cells of Ctrl01 in the non-VAP group was also significantly higher than in other individuals. MTRNR2L12⁺ cells in the VAP group increased at the group level compared to the non-VAP group, while naive CD4⁺ T cells, ciliated cells, and CD8⁺ T cells decreased.

Consistency Check for S100A8

The expression levels of the top-10 highly expressed upregulated DEGs (Supplementary Figure 1) and down-regulated DEGs (Supplementary Figure 2) were evaluated separately to investigate DEGs' expression in bulk-RNAseq at the cellular level. The expression of the aforementioned upregulated and down-regulated DEGs was inconsistent with the bulk-RNAseq results. Only differences in S100A8 expression were found in B cells in the upregulated DEGs group, while differences in FN1 and HLA-DRB5 expression were found in T cells, macrophages, and ciliated cells in the down-regulated DEGs group (Figure 5A). We then performed a Western blot to test S100A8 expression to determine whether they were good markers for VAP disease in different situations (Figure 5B and C). The results showed that S100A8 protein expression was significantly higher in the VAP group than in the non-VAP group.

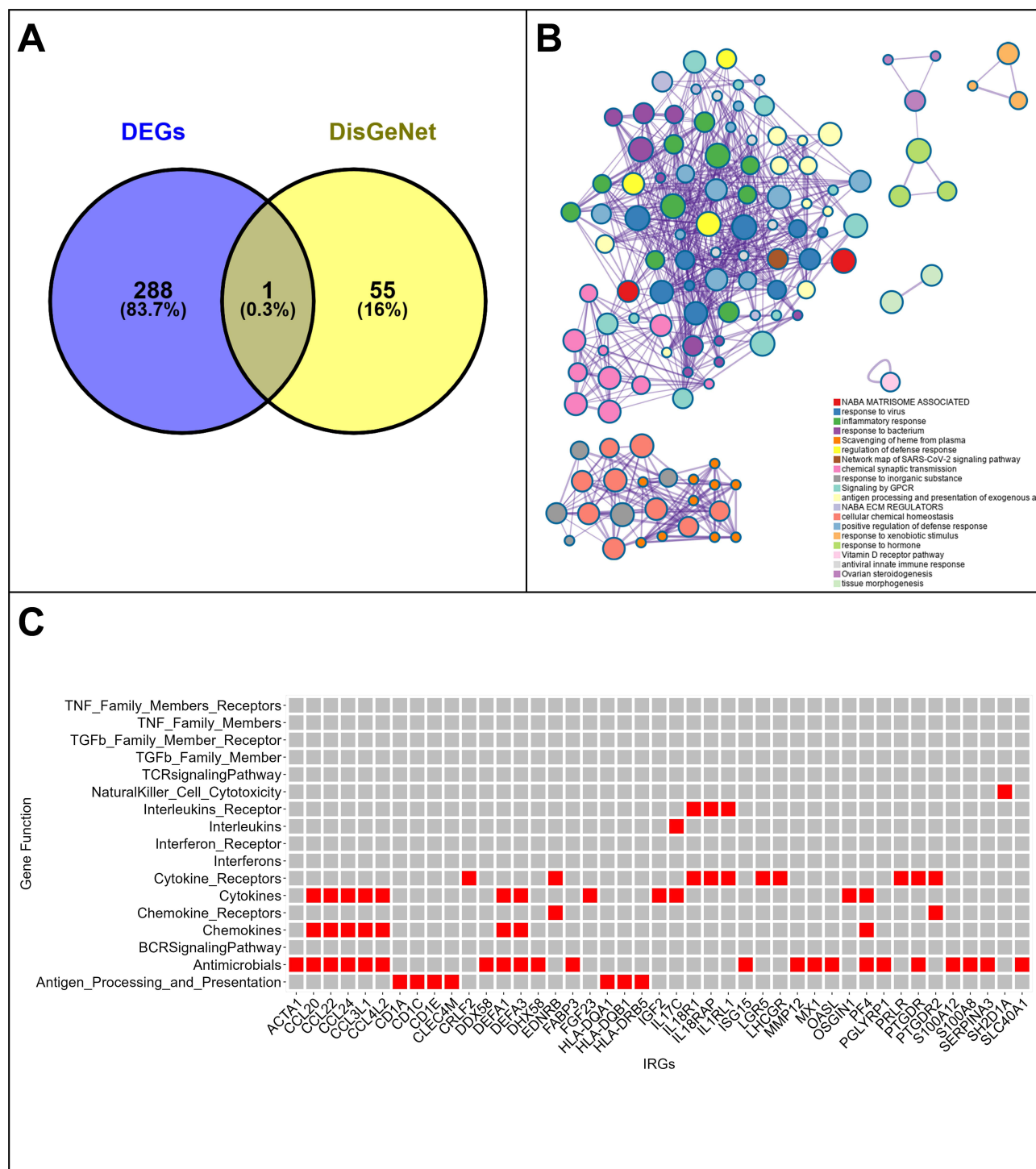


Figure 2 (A) Venn diagram representing the intersection interval of DEGs and DisGeNet. (B) The enrichment results of Metascape for DEGs. The dot size was proportional to the number of input genes falling under that term, and its color represents its cluster identity. Terms with a similarity score > 0.3 were linked by an edge. (C) Annotation of immune-related activities for DEGs. The horizontal axis represents immune-related genes (IRGs) in DEGs, while the vertical axis represents the function of each IRG.

Discussion

Ventilator-associated pneumonia is a common nosocomial pneumonia in invasive mechanically ventilated patients,²⁸ leading to prolonged mechanical ventilation, higher mortality, and severe illness. The pathogenesis and diagnosis criteria for VAP are currently unclear.^{29,30} Positive lower respiratory tract specimen results are critical VAP indicators based on

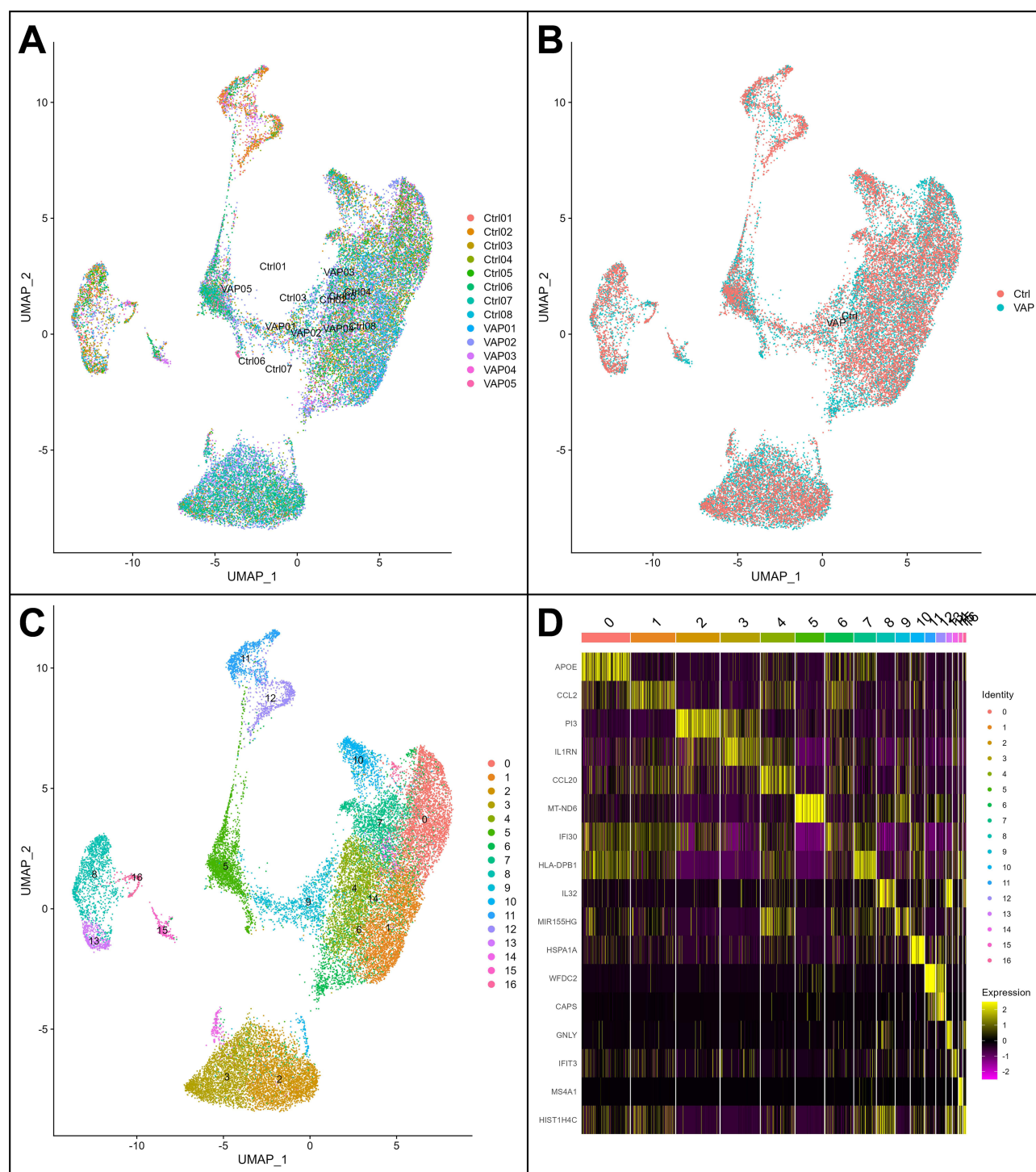


Figure 3 (A) The UMAP dimensionality reduction chart differentiated by sample type. (B) UMAP dimensionality reduction chart differentiated by group type. (C) Cluster results of cells at resolution = 0.7 (17 types of cell clusters were obtained initially); (D) Cell marker provided by Seurat for distinguishing different cell clusters (Top-1).

the existing diagnostic criteria. The above diagnostic criteria may result in a false negative, especially if antibiotics were used before sampling. It usually takes several days to obtain microbial culture results, affecting the clinical decision-making to use antibiotics.

We used bioinformatics methods to analyze the disease status of VAP patients in this study. The biomarkers screened in this study belong to the response process based on the sequencing data that are mainly derived from the antimicrobial

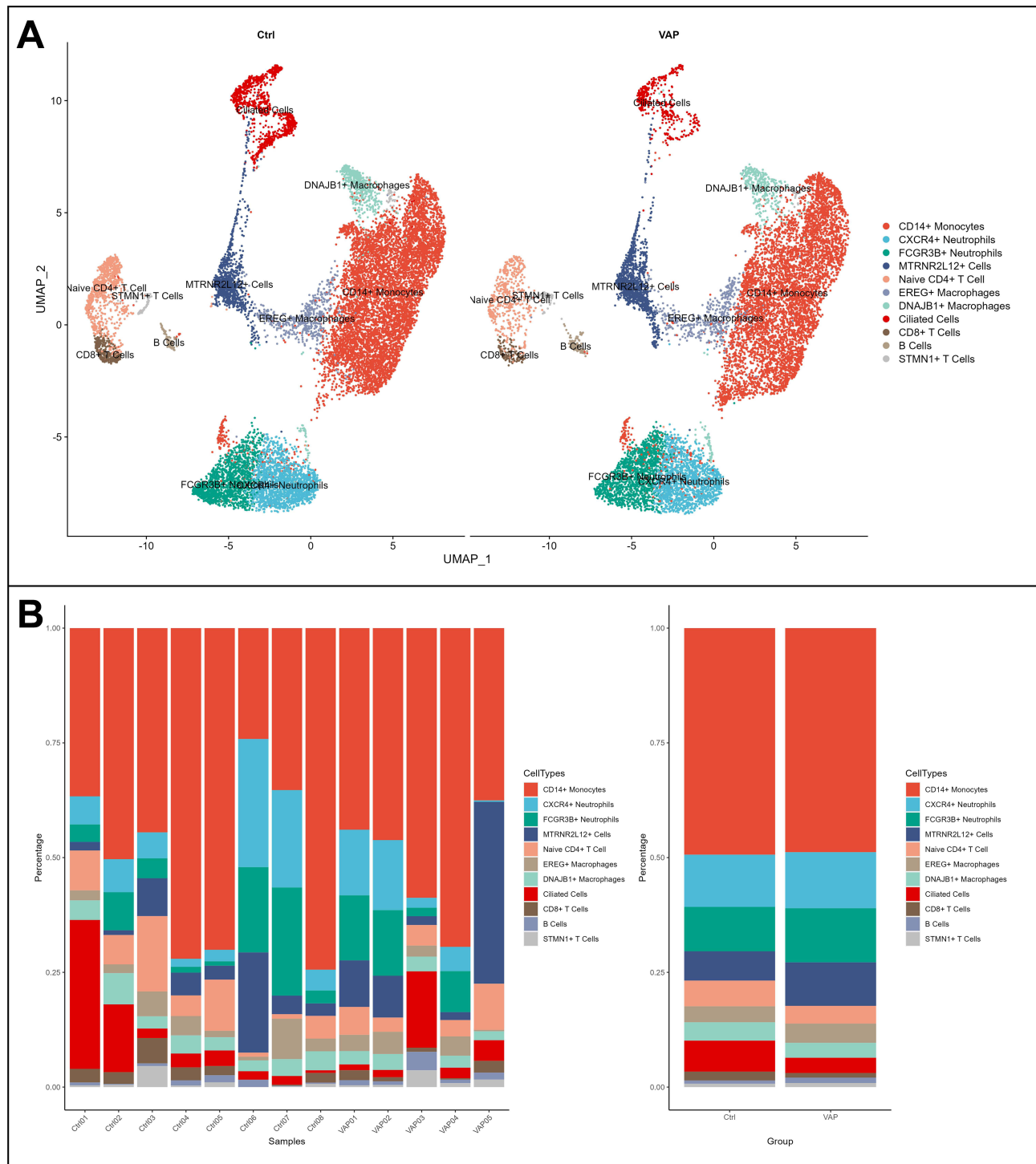


Figure 4 (A) Schematic diagram of cell types in VAP and non-VAP groups (Ctrl). The cells on the graph are in the following order: CD14+ Monocytes, CXCR4+ Neutrophils, FCGR3B+ Neutrophils, MTRNR2L12+ Cells, Naive CD4+ T Cells, EREG+ Macrophages, DNAJB1+ Macrophages, Ciliated Cells, CD8+ T Cells, B Cells, and STMN1+ T Cells. (B) Cell abundance in different samples (left) and groups (right).

mechanism of innate immunity. The accuracy and stability of sequencing-based biomarkers could be easier to control than traditional culture-based methods.

The single-cell analysis results demonstrated that the proportion of innate immune-related cells (monocytes, neutrophils, and macrophages) was relatively high in both patient groups, which may be related to the fact that all patients

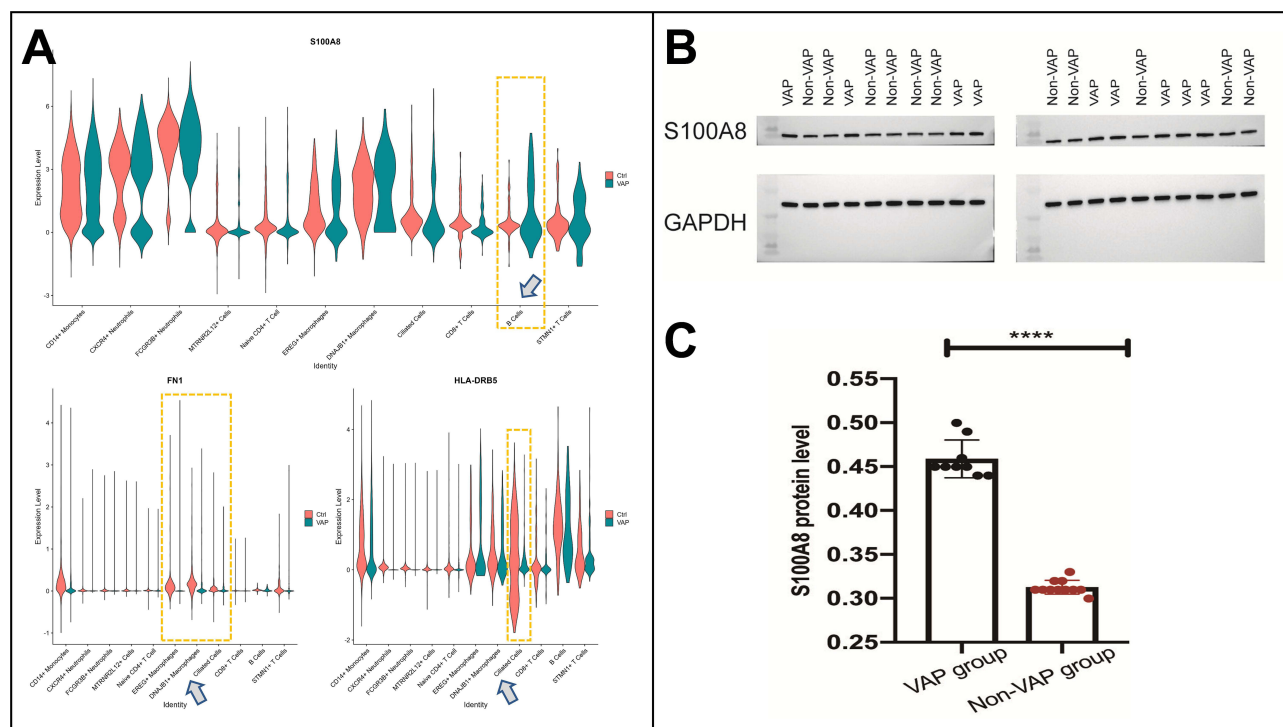


Figure 5 (A) DEGs expression in bulk RNA-seq at the single cell level. Three genes with consistent expression trends were screened for display: S100A8, FN1, and HLA-DRB5. The horizontal axis represents the different cell types, and the vertical axis represents the average expression level of the gene in the current cell. The red color indicates the non-VAP group (Ctrl), and the dark green color indicates the VAP group. The direction of the arrows indicates the distribution of specific gene expression changes in different cell types. Upregulated DEGs group: Differences in S100A8 expression were found only in B cells. Downregulated DEGs group: Differences in FN1 and HLA-DRB5 expression were mainly found in T cells, macrophages, and ciliated cells. **(B and C)** West blot showed that the protein expression level of S100A8 in the VAP group was significantly higher than the level in the non-VAP group. **** represents p-values less than 0.05, specifically $p = 0.0047$.

were infected with COVID-19. However, we discovered that B and T cells were relatively low. B and T lymphocytes are the major components of the adaptive immune response,³¹ which usually takes weeks or months after the infection is established.³² After analyzing the data, we hypothesized that the low levels of B and T cells might be related to the short disease courses. A Literature review into innate immunity showed that neutrophils are involved in various lung diseases.^{33,34} Although neutrophils are relatively nonspecific in different diseases, they are susceptible to pneumonia in critically ill patients and patients with non-neutropenic immunosuppression.^{35,36}

They primarily maintain the inflammatory environment in the current study by releasing antimicrobial proteins (Supplementary Figure 1) such as S100A8,^{37–39} S100A12,⁴⁰ and ISG15.^{41,42} On the other hand, DEGs from VAP patients were significantly enriched in gas transport-related pathways, which included high expression of HBA1, HBA2, HBB, and HBD and low expression of CA2. The upregulated genes are hemoglobin-related, and previous studies have shown that HBD can improve the antibacterial properties of neutrophils by inducing the formation of neutrophil extracellular traps (NETs).^{43,44}

Combined with the existing literature review, it can be concluded that S100A8 was not only differentially expressed in the transcriptome, but its proteome was abnormally highly expressed in VAP patients.³⁸ On this basis, a Western blot experiment detecting protein expression in VAP patients was performed to determine whether S100A8 could be a good marker for VAP disease in different situations. The results showed that the S100A8 protein expression level in the VAP group was significantly higher than in the non-VAP group.

We searched some biomarkers in the clinical OMIM database to evaluate the feasibility of the selected genes as VAP markers. Several studies have demonstrated that S100A8 could be a surrogate marker of lung inflammation during tuberculosis.⁴⁵ The antimicrobial peptide S100A8/A9 produced by airway epithelial cells is also a potent and direct regulator of macrophage phenotype and function.³⁹ Furthermore, it has been shown that S100 proteins such as S100A8 and S100A12 are key mediators linking neutrophil-dominant airway inflammation to mucin overproduction.⁴⁶ From

a clinical perspective, we believe the screened markers have a specific clinical potential and should be investigated further.

The current study has some limitations due to the limited data and available techniques. The existing public database lacks sufficient VAP-related samples, resulting in statistical bias. Further validation by large-scale clinical studies is required. Moreover, the pathogenic microorganisms reported for the patients in the database were not mentioned. Therefore, we can only confirm that an infection occurred but cannot provide etiological information to guide antibiotic use. If S100A8 can provide prognostic information, it has the potential to serve not only as a diagnostic marker but also as a prognostic marker, and further exploration of this aspect would enhance its clinical applicability and significance as a VAP biomarker.

Conclusions

This study utilized integrated bulk RNA-seq and scRNA-seq analyses to explore potential biomarkers for Ventilator-Associated Pneumonia (VAP). Our comprehensive analysis identified S100A8 as a promising biomarker exhibiting significant differential expression in VAP patients relative to controls. The strong correlation of S100A8 with inflammatory responses and its heightened expression levels in VAP patients indicate its potential involvement in the disease's pathogenesis. These results emphasize the significance of S100A8 as both a diagnostic biomarker for VAP. Future studies should focus on validating these findings in larger cohorts and exploring the underlying mechanisms of S100A8 in VAP.

Data Sharing Statement

The datasets presented in this paper can be found in online repositories. Data are available upon reasonable request made to the corresponding author.

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Disclosure

The authors declare that they have no conflict of interests.

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