European Review for Medical and Pharmacological Sciences 2023; 27: 805-817

Identification and development of a five-gene signature to improve the prediction of mechanical ventilator-free days for patients with COVID-19

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Abstract. – OBJECTIVE: Coronavirus disease 2019 (COVID-19) is a highly contagious infectious disease caused by the newly discovered severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Severe COVID-19 infection causes complications in the respiratory tract, which results in pulmonary failure, thus requiring prolonged mechanical ventilation (MV). An increase in the number of patients with COVID-19 poses numerous challenges to the healthcare system, including the shortage of MV facilities. Despite continued efforts to improve COVID-19 diagnosis and treatment, no study has established a reliable predictive model for the risk assessment of deteriorating COVID-19 cases.

MATERIALS AND METHODS: We extracted the expression profiles and clinical data of the GSE157103, GSE116560 and GSE21802 cohorts from the Gene Expression Omnibus database. Differentially expressed genes (DEGs) were identified as the intersection of the resulting differential genes as analysed *via* limma, edgeR and DESeq2 R packages. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using the R package 'clusterProfiler'. Variables closely related to MV were examined using univariate Cox regression analysis, and significant variables were subjected to least absolute shrinkage and selection operator regression (LASSO) analysis for the construction of a risk model. Kaplan-Meier analysis and receiver operating characteristic (ROC) curves were generated to verify the predictive values of the risk model.

RESULTS: We identified 198 unigenes that were differentially expressed in COVID-19 samples. Moreover, a five-gene signature (BTN3A1, GPR35, HAAO, SLC2A6 and TEX2) was constructed to predict the ventilator-free days of patients with COVID-19. In our study, we used the five-gene signature to calculate the risk score (MV score) for each patient. The results revealed a statistical correlation between the MV score and the scores of the Acute Physiology and Chronic Health Evaluation and Sequential Organ Failure Assessment of patients with COVID-19. Kaplan-Meier analysis revealed that the number of ventilator-free days was significantly reduced in the low-MVscore group compared to the high-MVscore group. The ROC curves revealed that our model had a good performance, and the areas under the ROC curve were 0.93 (3-week ROC) and 0.97 (4-week ROC). The 'Limma' package analysis revealed 71 upregulated genes and 59 downregulated genes in the high-MV score group compared to the low-MV score group. These DEGs were mainly enriched in cytokine signalling in immune system and cellular response to cytokine stimulus.

CONCLUSIONS: This study identified a five-gene signature that can predict the length of ventilator-free days for patients with COVID-19.

Key Words:
COVID-19, Mechanical Ventilation, SARS-CoV-2, Acute respiratory distress syndrome, Predictive model.

Introduction

Coronavirus disease 2019 (COVID-19) has received a great deal of attention because it poses a huge threat to global public health. COVID-19

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infection can range from mild to severe, which may ultimately result in respiratory failure and, in some instances, death[1].

Adult acute respiratory distress syndrome (ARDS) is the main consequence of COVID-19 pneumonia, and mechanical ventilation (MV) remains the cornerstone in the management of patients with ARDS[2-4].

A large clinical study[5] that dealt with COVID-19 characterisation reported a high rate of MV in critically ill patients (88%). Moreover, it was also observed in previous studies[5] that the average mortality rate of mechanically ventilated patients with COVID-19 was approximately 24.5%-28%. Although there are several novel promising treatments for COVID-19, MV is still considered the main control strategy for severe cases[6]. However, as the number of patients with COVID-19 continues to grow, there has been a major shortage of healthcare materials and equipment, including mechanical ventilators[7]. Thus, early evaluation of disease severity and the potential need for a mechanical ventilator is of great significance in clinical settings and will enable better patient care and allocation of medical resources.

In this study, three gene expression data sets and bioinformatics tools were used to establish and verify a five-gene signature risk model, which can accurately estimate the length of ventilator-free days for patients with COVID-19. Therefore, this study provides a new strategy for allocating MV equipment and optimising COVID-19 treatment.

Materials and Methods

Data Acquisition

GSE157103 was prepared from the GPL24676 cohort [Illumina NovaSeq 6000, (San Diego, CA, USA)], which included blood samples from patients with COVID-19 (COVID-19, n=100) and patients in the control group (patients without COVID-19 admitted to the same hospital, n=26)[8]. Normalised gene expression (TPM) data and corresponding clinical information were extracted from the Gene Expression Omnibus database (GSE157103, file: GSE157103_genes.tpm.tsv'). The GSE157103 was used to construct the prediction risk model as the training set. To validate the efficiency of the risk model, we downloaded two additional gene expression profiles with information on MV usage extracted from the GSE116560 and GSE21802 cohorts from the same database[9,10]. Using the GSE116560 cohort, genome-wide transcriptional changes were measured on 68 patients with ARDS. Moreover, the number of ventilator-free days for each patient was also recorded. GSE21802 was prepared from the GPL6102 cohort, which included 40 blood samples from critical patients with positive H1N1 (n=36) and the control group (healthy individuals, n=4). Among the critical patients, 20 were mechanically ventilated.

Functional Enrichment Analysis of COVID-19-Related Genes

Using the data obtained in the GSE157103 cohort, COVID-19-specific differentially expressed genes (DEGs) were identified by comparing blood samples drawn from patients with COVID-19 (n=100) with the remaining samples (n=26) using the R packages 'limma', 'edgeR' and 'DESeq2'[11-13]. Resulting genes with $|\log_2 FC| > 1.0$ and false discovery rate (FDR) < 0.05 were selected as DEGs. Using an online Venn diagram tool (https://bioinfogp.cnb.csic.es/tools/venny/index.html), we identified the intersection of the resulting differential genes from the three analyses and obtained the COVID-19-related genes.

The list of genes was subjected to Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment using the R package 'clusterProfiler' (hypergeometric test, Q<0.1 following Benjamini-Hochberg correction)[14].

Construction of the Five-Gene Signature Model

In the GSE157103 cohort, blood samples obtained from patients with COVID-19 with complete records of MV status were extracted and included in the sampling data.

Univariate Cox regression analysis of COVID-19-related genes was performed to determine which genes were associated with MV probabilities. COVID-19-related genes with p-values < 0.05 were subjected to the Least Absolute Shrinkage and Selection Operator (LASSO) analysis using the 'glmnet' R package[15]. With this analysis, an MV signature (MV score) was calculated to estimate the probability of patients with COVID-19 requiring MV.

The risk score of each patient is calculated using the formula:

$$\mathrm{MVscore} = \sum_{i=1}^{n} Coef_i * x_i$$

where Coefi is the coefficient, and xi is the TPM expression value of each selected gene.

Statistical Analysis

Patients with COVID-19 were classified into high- and low-risk groups depending on their calculated MV scores. Those with MV scores above the overall median (0.429) were considered to be a high-risk group, whereas those with scores below the median comprise the low-risk group. Kaplan-Meier analysis was used to evaluate the MV probabilities of the high- and low-risk groups[16,17]. A log-rank test was used to check for statistical significance between the two groups. The predictive ability of MV score was evaluated by generating time-dependent receiver operating characteristic (ROC) curves[18]. The Kruskal-Wallis test was used to compare the data between the three groups and the Wilcox test was applied to analyse the differences between the two groups[19,20]. Categorical data were tested using the Chi-square test. A nonparametric Spearman test was performed to evaluate the correlation[21]. All statistical analyses were performed using R (version 4.0.4) except for the Chi-square test, which was performed through the GraphPad Prism 8 software (La Jolla, CA, USA). A *p*-value of less than 0.05 was considered significant.

Results

Identification and Evaluation of COVID-19-Specific DEGs

COVID-19-specific DEGs were harvested using the R packages 'DESeq2' [476 upregulated and 263 downregulated genes, (Figure 1A)], 'edgeR' [228 upregulated and 128 downregulated genes, (Figure 1B)] and 'limma' [4774 upregulated and 733 downregulated genes, (Figure 1C)]. The Venn diagram presents the 198 upregulated and 122 downregulated DEGs defined as COVID-19-related genes (Figures 1D, 1E; **Supplementary Table I**).

Figure 1F presents the distribution of clinical features and the differential gene expression between the COVID-19 and non-COVID-19 samples. Numerical clinical features [ferritin, procalcitonin, D-dimer, C-reactive protein (CRP) and fibrinogen] were segregated into high (≥ median value) and low (< median value) level groups. Chi-square test revealed that the COVID-19 group has a higher proportion of patients with high-level ferritin, procalcitonin, D-dimer, CRP, and fibrinogen than the non-COVID-19 group. Meanwhile, the proportion of patients with hospital-free days longer than 30 days was higher in the COVID-19 group than in the non-COVID-19 group. The median hospital-free days of the COVID-19 group (26 days) were lower than that of the non-COVID-19 group (38 days).

Functional Enrichment Analysis of COVID-19-Related Genes

To obtain biological insights from common COVID-19-related genes, we performed GO and KEGG enrichment analyses. The top 20 categories associated with Q values are presented in Figure 2.

Nine enriched KEGG pathways are summarised in Figure 2A. The top three biological processes related to gene counts were nuclear division, organelle fission and mitotic nuclear division (Figure 2B). The top three cellular components in which COVID-19-related genes were mainly involved were the chromosomal region, ribosome, and condensed chromosome (Figure 2C). Two molecular function categories are also enriched, namely the structural constituent of the ribosome and double-stranded RNA binding (Figure 2D).

Construction of a Mechanical Ventilation Signature

Using MV as the endpoint, the number of ventilator-free days of patients in the GSE157103 cohort was recorded during the follow-up (28 days) period. In this cohort, we excluded patients with (n=20) and without (n=26) COVID-19 who have no ventilator-free day in the hospital. We used the expression levels of COVID-19-related genes for the univariate Cox analysis. From this, a total of 102 protective factors and 28 risk factors for ventilator-free days were harvested ($p<0.05$); (**Supplementary Table II**). Through the LASSO Cox regression model, five COVID-19-related genes were obtained to construct the MV signature (MV score). The MV score consisted of four protective factors and one risk factor and was calculated using the formula: MV score = BTN3A1*-0.002256919+GPR35*-0.061471029+HAAO*-0.275842159+SLC2A6*-0.00625967+TEX2*0.097399395; (Figures 3A-C).

Kaplan-Meier analysis revealed that a lower MV score is associated with shorter ventilator-free days ($p<0.0001$). We also observed that none of the patients in the low-MV score group received MV during the first 28 days of follow-up.

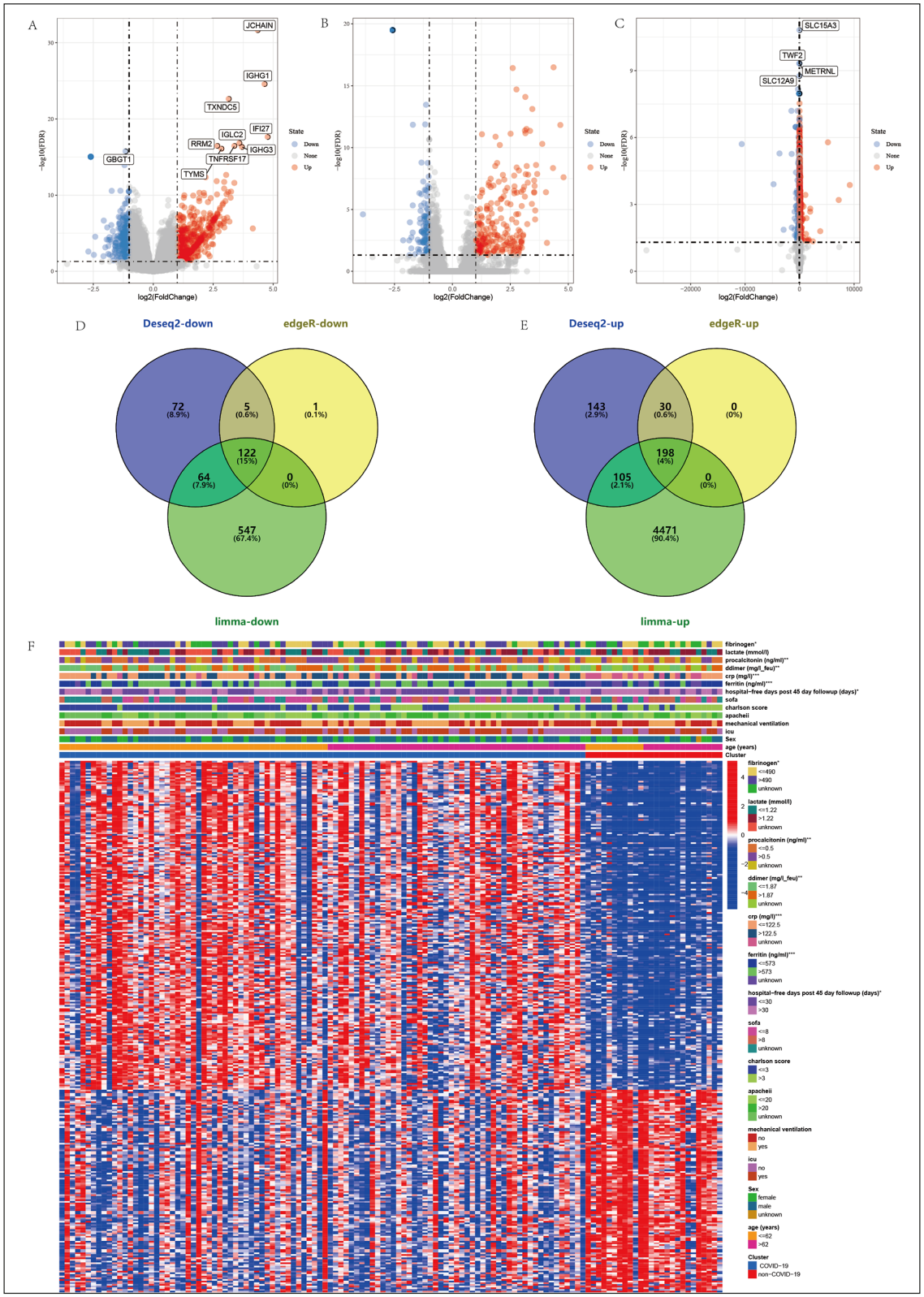

Figure 1. Volcano diagrams depict the differential gene expressions (DEGs) in COVID-19 *vs.* non-COVID-19 samples identified through the (**A**) DESeq2, (**B**) edgR and the (**C**) Limma R Package. Venn diagrams present the number of (**D**) downregulated and (**E**) upregulated COVID-19-specific DEGs. **F,** A Heatmap of the correlation between the DEGs and the clinical phenotypes of COVID-19. *p<0.05 (Chi-square test); **p<0.01 (Chi-square test); ***p<0.001 (Chi-square test).

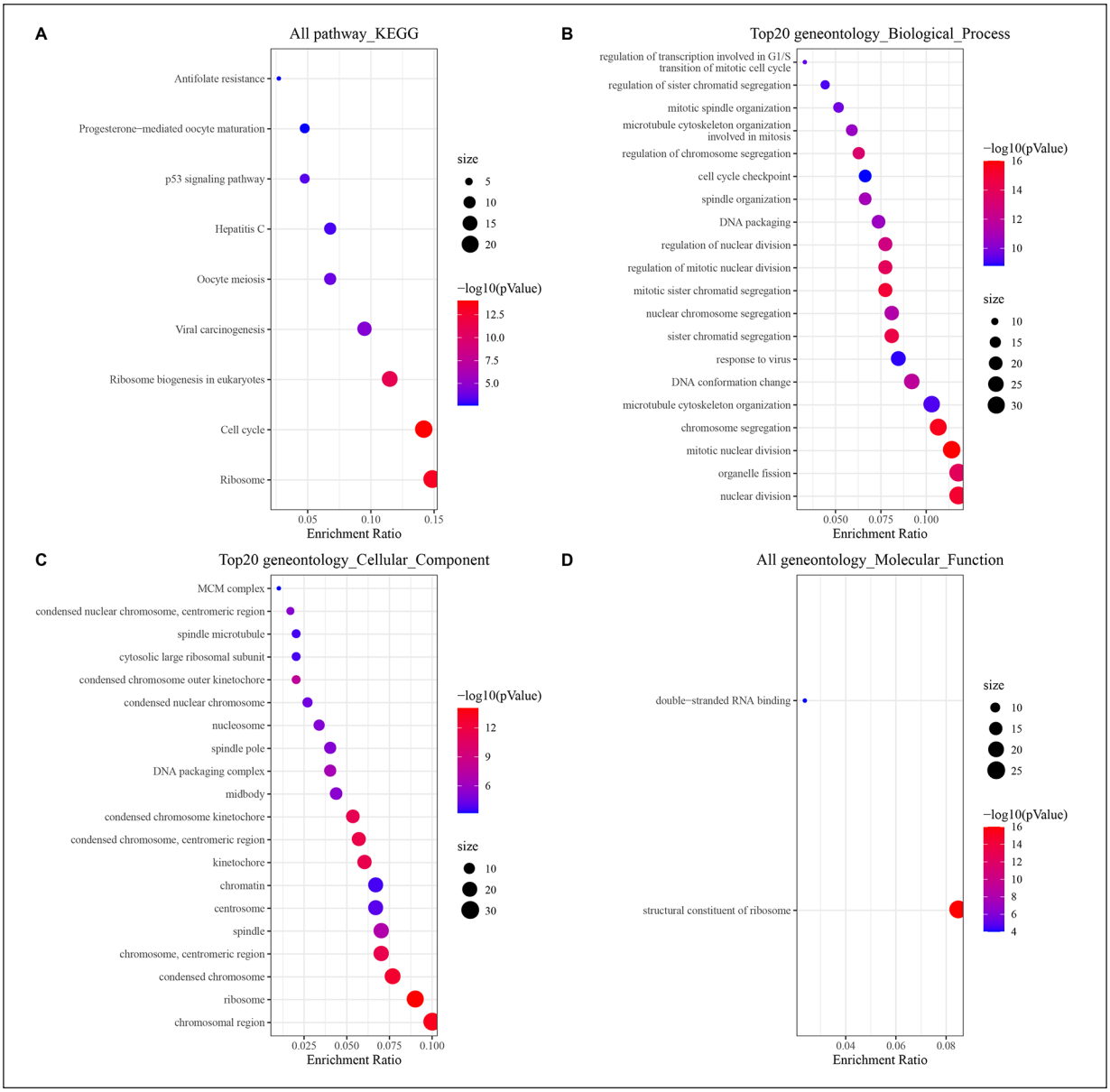

Figure 2. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of DEGs in COVID-19 *vs.* non-COVID-19 samples, including the (**A**) enriched KEGG pathways, (**B**) the GO biological processes, (**C**) cellular components and (**D**) molecular functions.

The median ventilator-free time of patients in the high MV score group was 25.2 days (Figure 3D).

The predictive accuracy of the MV score was evaluated using time-dependent ROC analysis on the 7th, 14th, 21st and 28th days where the corresponding area under the curve (AUC) values were 0.88, 0.91, 0.93 and 0.97, respectively (Figure 3E).

As presented in Figure 4, patients who have been mechanically ventilated are observed mostly in the high-MV score group. Furthermore, TEX2 expression was observed to increase with a higher MV score. MV score was observed to correlate negatively with SLC2A6, HAAO, GPR35 and BTN3A1 expression.

Statistical Analysis of the Correlations Between the MV Score and COVID-19 Phenotypes

Significantly positive associations were observed between the MV score and the APACHE II and SOFA scores (Figures 5A, 5B, 4C, 5D; **Supplementary Table III**). The APACHE II and

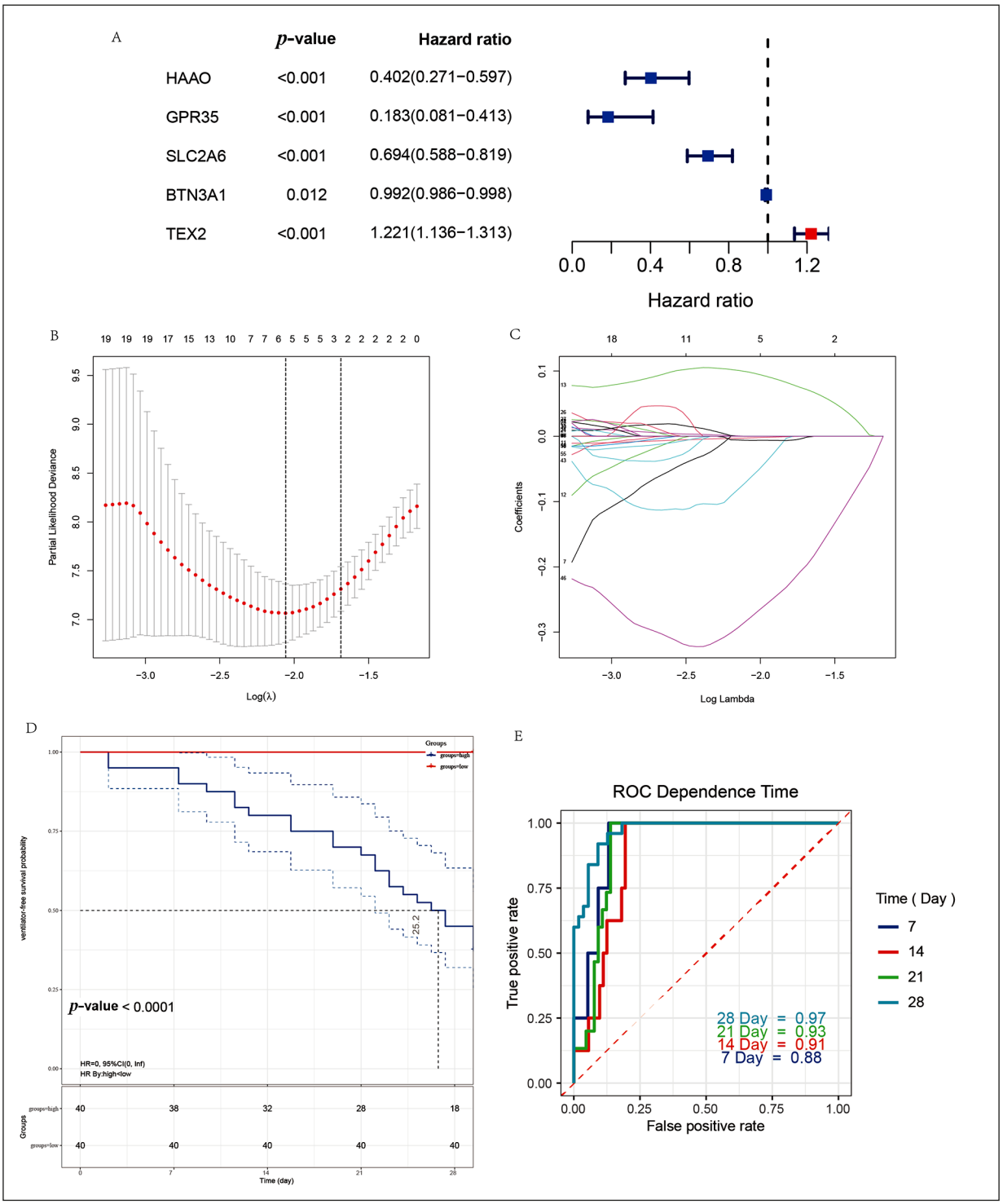

Figure 3. A, Forest plot of the univariate analysis of the five genes selected for the construction of the risk model. **B,** Tuning parameter (and lambda;) selection in the Least Absolute Shrinkage and Selection Operator (LASSO) model using 10-fold cross-validation with minimum criteria. **C,** LASSO coefficient profiles of the 130 texture features. **D,** Kaplan-Meier estimates the ventilator-free time in both the high (*red*) and low (blue) MV score group. **E,** Time-dependent receiver operative characteristics (ROC) curves predict the mechanical ventilation (MV) rate at the 7th, 14th, 21st and 28th days of follow-up.

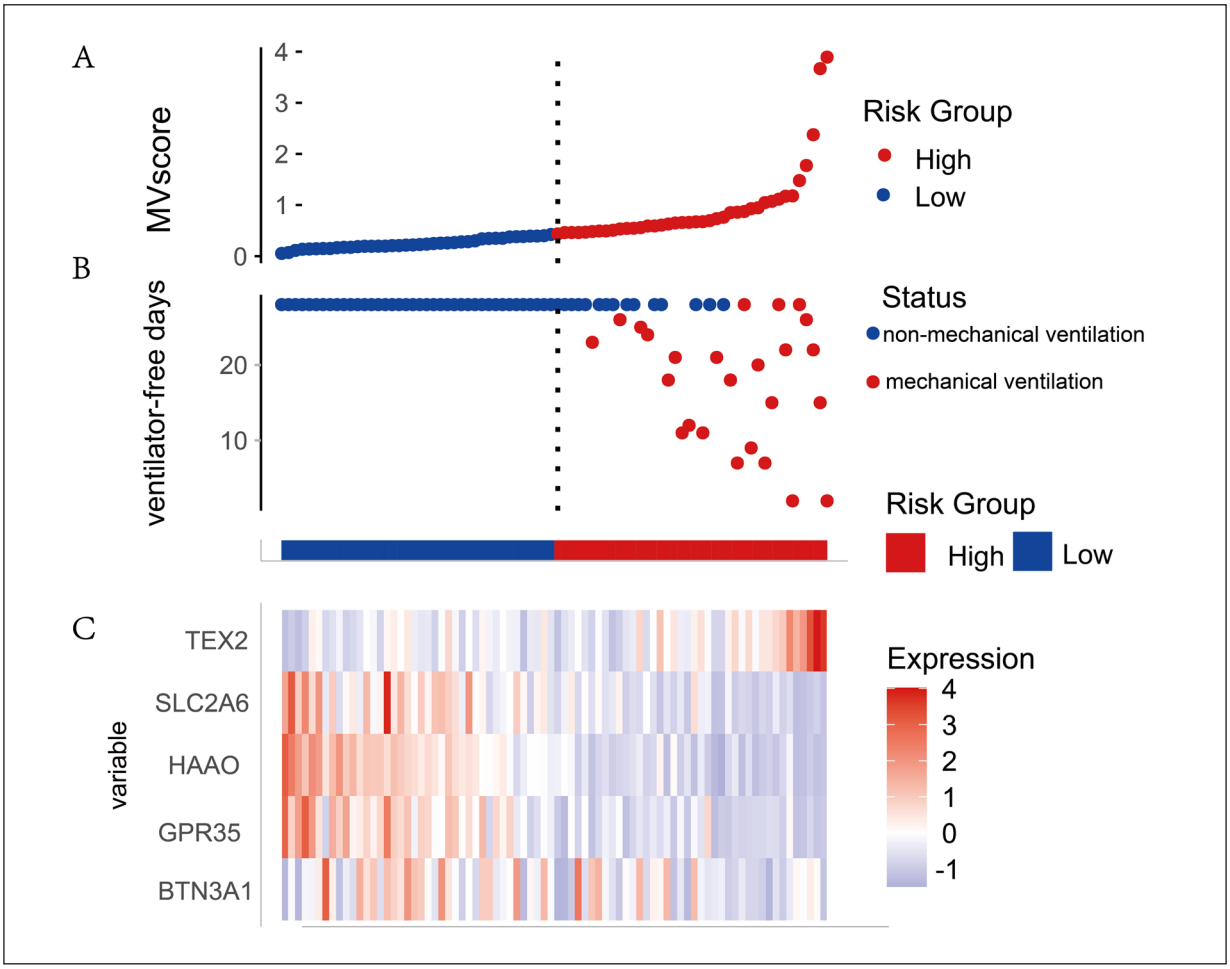

Figure 4. A, Patient risk score distribution according to the five-signature model. **B,** Distribution of mechanical ventilation (MV) status of both the low and the high-risk groups. **C,** Heat map of the five signature genes.

SOFA scores in the high- MV score group were significantly higher (p<0.05) than those in the low-MV score group. Spearman's analysis indicated similar results (Spearman Rho of APACHE II = 0.77; Spearman Rho of SOFA = 0.64). Furthermore, MV score indicated an inverse relationship with hospital-free days 45 days post-follow-up (Spearman Rho = -0.54). The high-MVscore group exhibited fewer hospital-free days than the low-MV score group (Figures 5E, 5F).

In the blood tests, the high-risk group revealed higher levels of procalcitonin and D-dimer (p<0.05). Meanwhile, in the Spearman correlation analysis, procalcitonin and D-dimer levels were positively associated with MV scores (Figures 6A-D). The median CRP level was higher in the high-MV score group. Furthermore, CRP levels were also observed to be positively correlated with MV score. However, the differences between the two were not statistically significant (Figures 6E, 6F).

To determine if the MV score also predict the probabilities of MV in patients with pneumonia in other independent data sets, we validated the model by calculating the MV score of patients with records of MV in the GSE116560 (n=68) and GSE21802 (n=36) cohorts. The MV score of each patient in the validation set is summarised in **Supplementary Table IV**.

Both cohorts were subdivided into high- and low-MV score groups using the same median value as the cut-off.

Figure 7A indicates that the MV scores of patients with no ventilator-free days (n=37) were significantly higher than that of patients with ventilator-free days ≥7 (n=31) [GSE116560 cohort, p=0.036]. Patients with ventilator-free days ≥7 accounted for a higher proportion in the low-

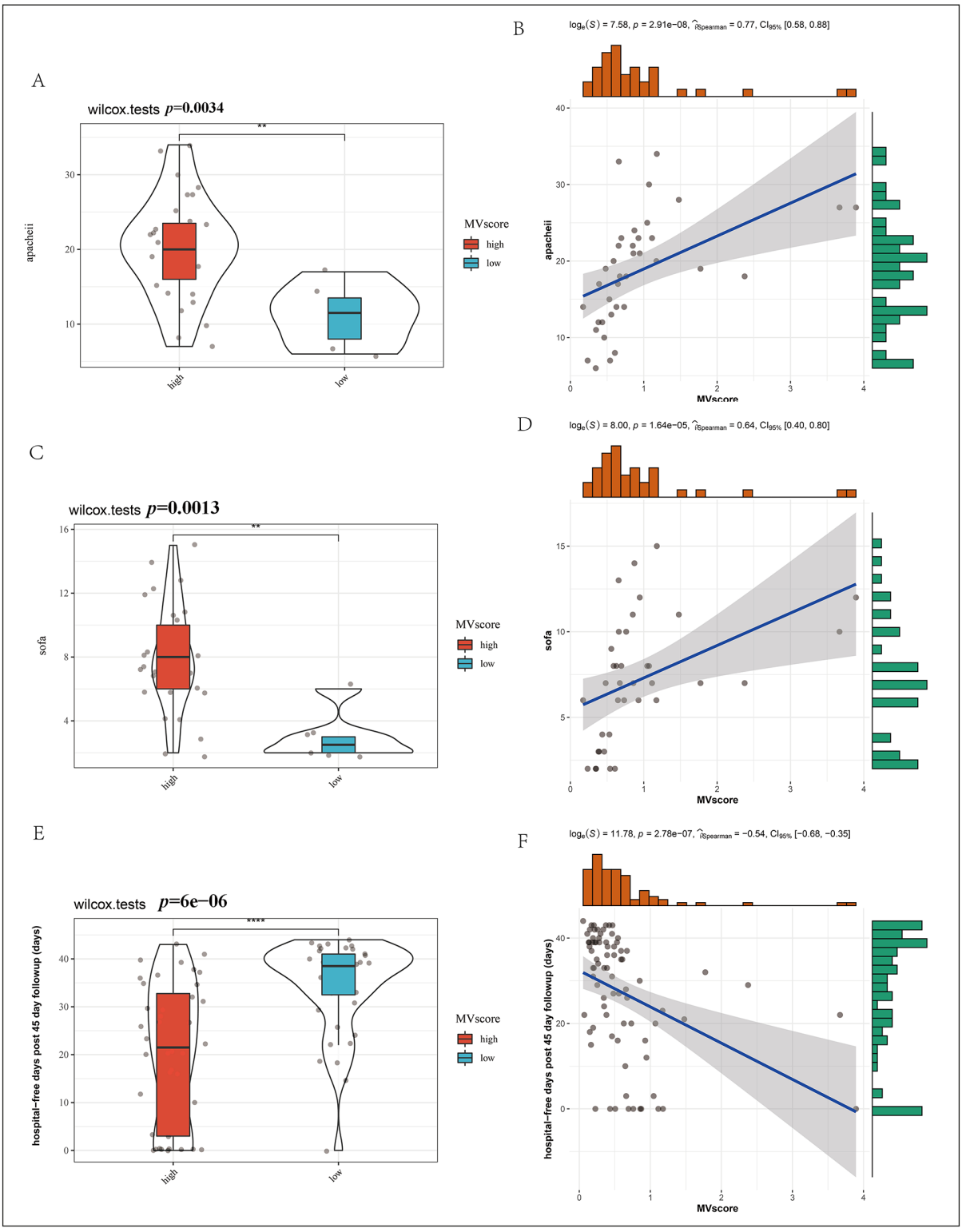

Figure 5. **A**, Violin plots of the Acute Physiology and Chronic Health Evaluation (APACHE) II scores of the low-risk group (blue) and the high-risk group (red) (p=0.0034). **B**, Spearman correlations between the MV scores and the APACHE II scores. C, Violin plots of Sequential Organ Failure Assessment (SOFA) scores (p=0.0013) of the low-risk group (blue) and the high-risk group (red). **D,** Spearman correlations between the MV scores and the SOFA scores. **E,** Violin plots of hospital-free days (p=0.0013) of the low-risk group (blue) and the high-risk group (red). **F,** Spearman correlations between the MV scores and the hospital-free days. The top panels exhibit the Spearman correlation coefficients and corresponding p-values.

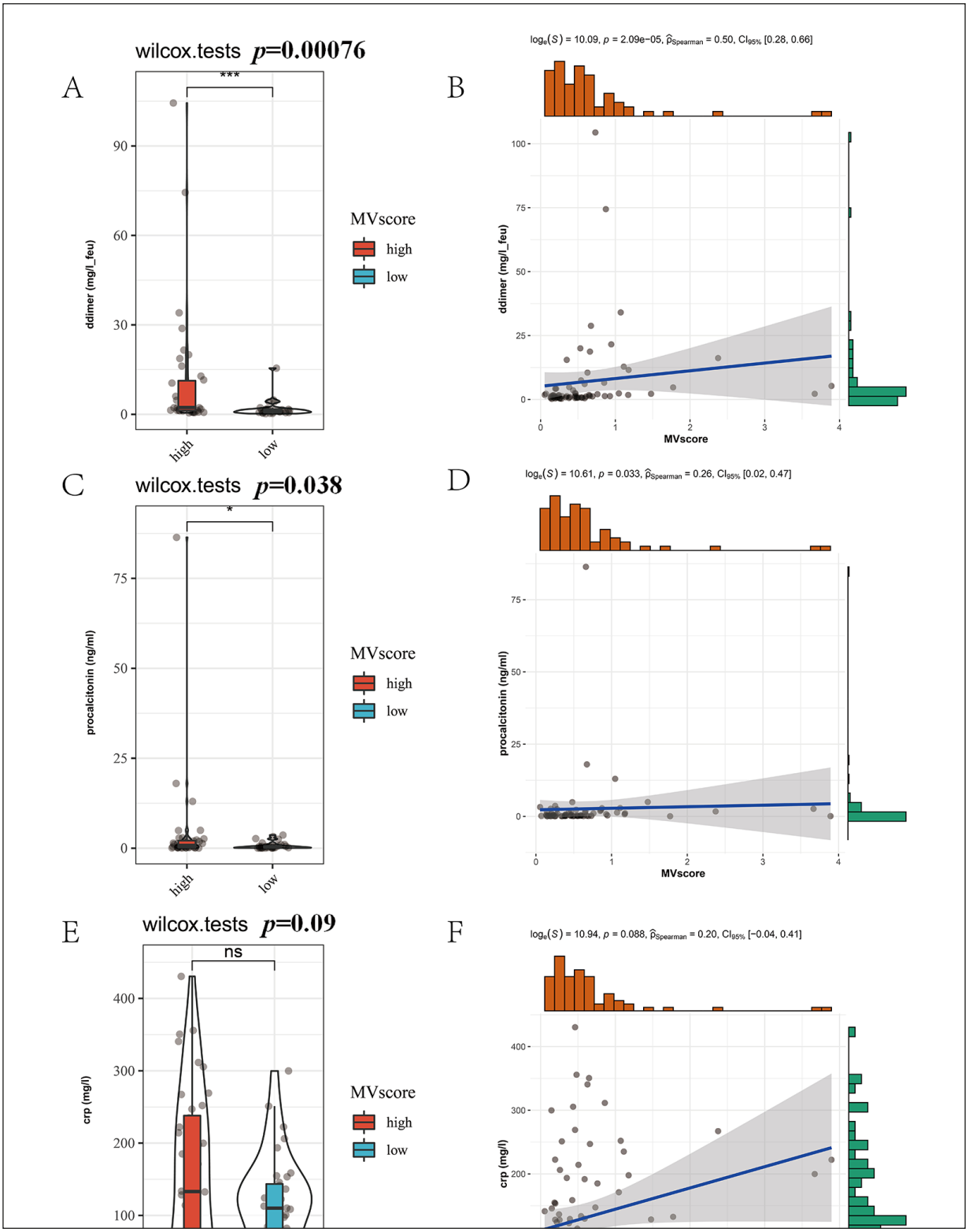

Figure 6. **A**, Violin plots of the D-dimer (p=0.00076) of the low-risk group (blue) and the high-risk group (red) (p=0.0034). **B**, Spearman correlations between the MV scores and the D-dimer. **C,** Violin plots of procalcitonin (p=0.038) of the low-risk group (blue) and the high-risk group (red). **D,** Spearman correlations between the MV scores and procalcitonin. **E,** Violin plots of CRP (p=0.09) of the low-risk group (blue) and the high-risk group (red). **F,** Spearman correlations between the MV scores and the CRP. The top panels exhibit the Spearman correlation coefficients and corresponding p-values.

MV score group, whereas patients with no ventilator-free days were mostly observed in the high-MVscore group (Chi-square test p=0.0074); (Figure 7B).

Similar trends are observed in Figures 7C and 7D. Most patients in the high-MV score group received MV, unlike the low-MV score group (72.2% *vs.* 38.9%). Further, the MV scores of the patients who received MV were significantly higher than those of the patients who did not receive MV (p=0.0083).

One hundred and thirty DEGs (71 up-regulated and 59 down-regulated) were obtained between the high- and low-MV score group (**Supplementary Table V**). The results of the gene enrichment analysis for these 130 DEGs are presented in **Supplementary Table VI**. These DEGs were mainly enriched in cytokine signalling in the immune system and cellular response to cytokine stimulus.

Discussion

The drastic growth of COVID-19 cases is accompanied by many other challenges, including the exhaustion of supplies and services in healthcare systems[22]. Particularly, many hospitals worldwide do not have sufficient MV equipment

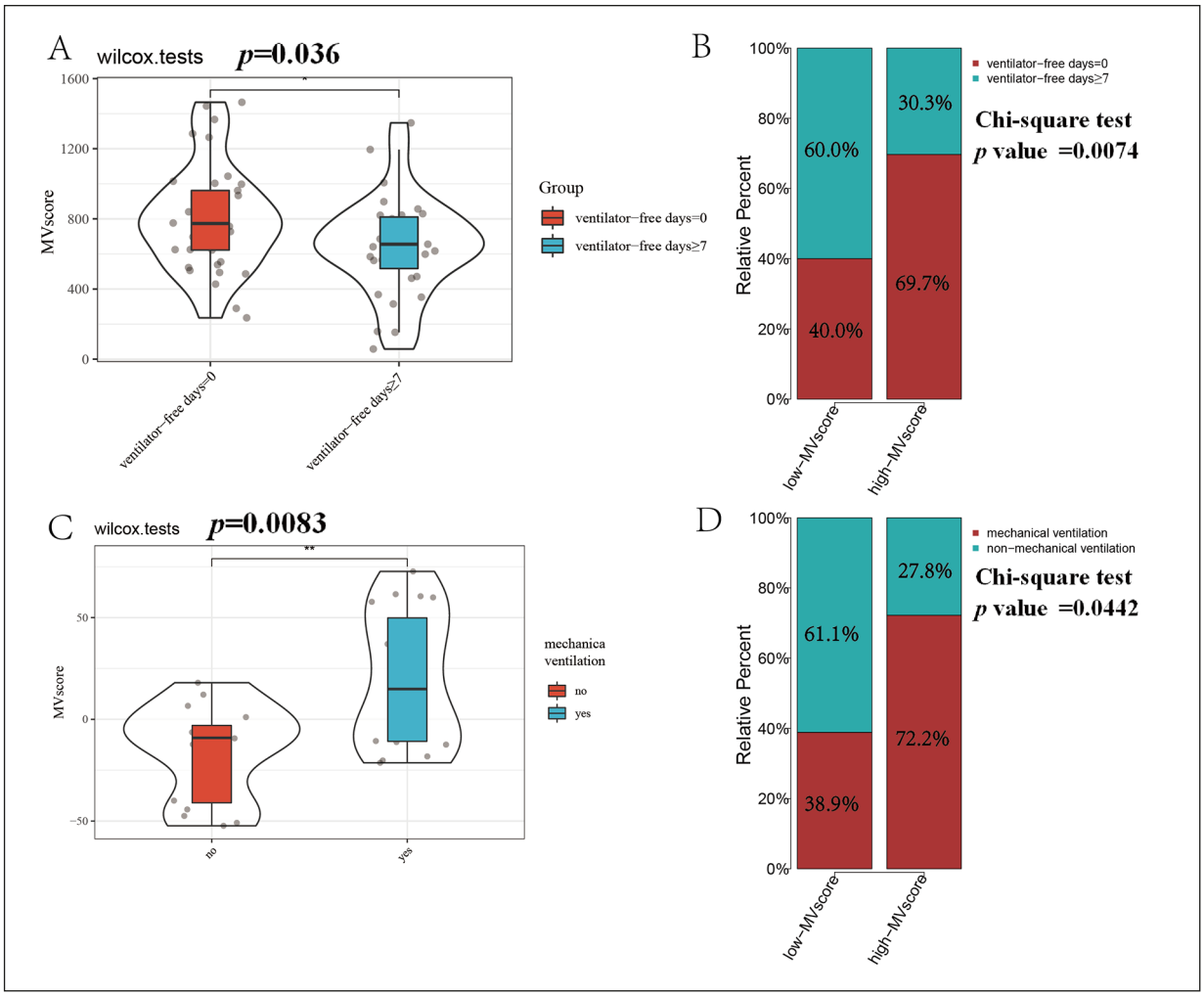

Figure 7. A, Violin plots demonstrate the distributions of MV scores (p=0.036) between patients with ventilator-free days ≥7 (blue) and patients with no ventilator-free days (red), with data extracted from the GSE116560 cohort. **B,** The low MV score group comprises patients with ventilator-free days ≥7 whereas the high MV score group mostly consists of patients with zero ventilator-free days (p=0.0074). **C,** Violin plots demonstrate the distributions of MV scores between patients who required mechanical ventilation (blue) and patients who did not (red) (p=0.0083), with data extracted from the GSE21802 cohort. **D**, Similarly, patients who did not require MV mostly had lower MV scores than those who required mechanical ventilation and mostly had relatively higher MV scores (p=0.0442).

to satisfy the demands of patients with severe COVID-19. This explains the need to better estimate the urgency of patients needing MV and prepare the required equipment in advance. However, a suitable classifier has not been established previously. Our study proposed a signature risk model that can accurately assess the number of ventilator-free days in patients with COVID-19. Patients with a high-risk score are predicted to demand MV in the future as their disease progresses. The constructed model is therefore a promising tool that allows for the ample preparation of MV equipment.

Using LASSO analysis, five genes (BTN3A1, GPR35, HAAO, SLC2A6 and TEX2) were included in the model construction. It has been previously reported that BTN3A1 can promote the activation and proliferation of Vγ9Vδ 2T cells in the peripheral blood, induced by the *Mycobacterium tuberculosis* heat resistant antigen[23]. In contrast, GPR35 is a class A, rhodopsin-like G protein-coupled receptor[24]. Previous studies[25] have demonstrated that the agonism of GPR35/CXCR8 causes a significant decrease in interleukin (IL)-4 as released by α-galactosylceramide-activated human invariant natural killer T cells. SLC2A6, a lysosomal transporter regulated by inflammatory stimuli, affects the metabolic shift in macrophages to modulate the inflammatory response[26]. Macrophages are known to contribute to excessive inflammation and disease progression in some COVID-19 cases[27]. Increased levels of IL-2, IL-7, interferon-inducible protein (IP)-10, granulocyte-colony stimulating factor (G-CSF), macrophage inflammatory protein (MIP)-1, monocyte chemoattractant protein (MCP)-1 and tumour necrosis factor (TNF) are also observed to be positively correlated with COVID-19 severity[28]. We, therefore, speculate that the signature is closely related to the immune response, and it may be the physiological basis of the constructed model. These results were supported by the enrichment analysis of the differential gene expression between high and low MV score groups (**Supplementary Table VI**).

Patients with COVID-19 with short ventilator-free days had higher MV scores, with the AUC of the time-dependent ROC curves >0.8. This indicated that our signature was effective in estimating the need for MV. To further verify the robustness of the signature, we calculated the MV score for each patient in two separate validation sets. Since publicly available COVID-19 cohorts are limited, we selected cohorts of patients with ARDS and critical patients with positive H1N1 as the validation sets. In severe cases, respiratory failure caused by COVID-19 fulfils the criteria for ARDS[29]. In addition, H1N1 infections may also cause ARDS and be included in the validation set. Furthermore, respiratory support in both COVID-19 and H1N1 cases should be according to the therapeutic strategies for ARDS[30]. Therefore, this indicates that the choice of validation sets is reasonable enough to validate the risk model. Similarly, high MV scores revealed a strong correlation with patients who used mechanical ventilators. Aside from validating the robustness of the five-signature model, the test results also suggested that the model has the potential to be extrapolated to all patient populations with ARDS.

MV scores revealed a strong positive correlation with the APACHE II and SOFA scores. APACHE II and SOFA scores have been widely employed to estimate the outcome of critically ill patients[31]. Therefore, APACHE II and SOFA scores usually indicate a more severe disease outcome and a worse prognosis[32]. In this study, it was observed that patients with higher MV scores had a higher elevation of D-dimer, procalcitonin and CRP in their blood. Previous studies[33,34] have demonstrated that high levels of D-dimer, CRP and procalcitonin may be used as independent factors to predict the severity of COVID-19. These findings, thus, suggest that the MV score has the potential to be an indicator of COVID-19 severity.

Conclusions

Through bioinformatics analysis, we established a five-gene signature that successfully evaluates the risk of severe COVID-19 pneumonia. In addition, this signature demonstrates efficient predictive capability in two independent verification sets. Hence, our study provides a reliable method for the efficient allocation of MV equipment for patients with COVID-19 and has a substantial potential to optimise the clinical treatment for COVID-19.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

We thank all those participants in this study. We thank Dr. Sonia Zebaze Dongmo for carefully reading the manuscript and editing the language.

Authors' Contribution
All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Informed Consent
Not required.

Ethics Approval
Not required.

Funding
This study did not receive any funding.

Availability of Data and Materials
The raw data of this study are derived from the GEO data portal (https://www.ncbi.nlm.nih.gov/geo/), which are publicly available databases.

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