Comprehensive analyses of immunoglobulin proteome and clinical variables identify biomarkers to predict mortality in patients with influenza-associated lower respiratory tract infection

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Abstract

Background: Influenza-associated lower respiratory tract infection (I-LRTI) brings a heavy clinical burden, and clinicians lack an effective prognostic evaluation system to control disease progression.

Methods: This was a prospective, observational study, and the endpoint was 28-day mortality. Plasma microarrays were used for quantitative analysis of immunoglobulin (Ig) and its subclasses. Prognostic factors from Ig and clinical variables in the patients with I-LRTI were identified to create a prediction model.

Results: To address this issue, we prospectively and observationally studied the difference of immunoglobulin proteome and clinical variables between survivors and non-survivals in 107 patients with influenza-associated lower respiratory tract infection (I-LRTI) selected from four hospitals affiliated to Capital Medical University. The results identified 17 variables with significant or marginally statistical differences by univariate analysis, including lymphocyte count (LY), monocytes count (MO), CD3 + CD4 + T-cell count, CD3 + CD8 + T-cell count, IgA, IgA1, IgG2, IgG4, CRP, PCT, D-dimer, oxygenation index, glycosylated hemoglobin, lactic acid (LAC), base excess of blood, lactic dehydrogenase, and α-hydroxybutyrate dehydrogenase. Furthermore, we analyzed the correlations of all the variables by hierarchical clustering analysis in which different functional modules were formed between survival and non-survival groups that are associated with the immunity and severe infection. At last, we built a prediction model with nine variables (D-dimer, days from onset to ED, IgA, IgG2, LAC, LY, MO, Staphylococcus aureus co-infection and age), with which the AUC value of 0.810 (95% CI 0.755-0.839) was achieved with the evaluation of LOO cross validation. The predictive model was further validated by disease severity evaluation.

Conclusion: Lethal bacterial (especially S. aureus) co-infection was associated with cellular immunity, oxygenation index, HbA1C and age. The combined prediction model with D-dimer, Days from onset to ED, IgA, IgG2, LAC, LY, MO, S.aureus co-infection and age demonstrate the predictive mortality powerfully in patients with I-LRTI.

Background

Seasonal influenza epidemic accounted for an estimated 145 000 (95% uncertainty interval [UI] 99 000–200 000) deaths among all ages worldwide annually.[1] Although influenza vaccine is an available countermeasure to mitigate the considerable influenza-related mortality burden,[2] vaccine effectiveness is low compared with other viral vaccines, and the induced immune response is narrow and short-lived.[3] Current influenza vaccines are imperfect and the expected benefits of vaccination programs might be overstated, especially in elderly people.[4, 5] For people who are not effectively prevented and are infected with influenza, neuraminidase inhibitors (NAIs) are recommended as the most effective antiviral agents.[6] However, in clinical practice, anti-influenza treatment for hospitalized patients with suspected influenza is not always initiated at admission and may be delayed while patients await results of diagnostic tests.[7] Even more, not all observed studies have confirmed the benefits of NAIs treatment in hospitalized influenza patients.[8] Therefore, vaccines or antiviral agents alone tend not to reduce the burden of deaths caused by

influenza, comprehensive evaluation based on clinical indicators and individual immune status remains critical.

Severe influenza originated from a respiratory infection but involved multiple organ systems, so it should also be caused by highly interacting networks of parameters in body instead of the isolated ones.[9–12] Particularly notable is the importance of omics analysis and the interrelationship between these multiple indicators, which can reflect both physical and non-physical associations between them.[13] There is no widely accepted clinical system for evaluating the prognosis of influenza patients. Although commonly used clinical evaluation systems, such as pneumonia severity index (PSI)[14] and Sepsis-related Organ Failure Assessment (SOFA) score[15] were multi-index evaluation systems, they were based on clinical data of patients with community-acquired pneumonia or sepsis, and lacked an assessment of the immune system and co-regulation network analysis. Therefore, the efficacy of these systems are limited in evaluating the prognosis of influenza patients. There have been no new reports of physicians exploring evaluation systems in recent years to assess the prognosis of patients with severe influenza.

Protein microarray has the advantages of high sensitivity and accuracy, which makes it possible to detect low abundant proteins in clinic.[16] In this work, we developed and applied a high-throughput and ultra-micro plasma microarray platform to measure the expression of nine immunoglobulin isotypes (IgG, IgG1-4, IgA, IgA1-2, IgM) in hundreds of clinical plasma samples, which proved to be of high reproducibility and stability. The purpose of this study was to perform comprehensive analysis of the changes of immunological proteome and clinical variables, to explore novel pathological mechanisms through the immunoglobulin proteome-clinical variable co-regulation networks and to establish a prediction model for evaluating the prognosis of influenza patients.

Methods

Study Design and Participants

We conducted a prospective observational study, and collected patients with suspected influenza-associated lower respiratory tract infection (I-LRTI) in emergency departments (EDs) of four hospitals (Beijing Chao-Yang Hospital, Capital Medical University; Beijing Di-Tan Hospital, Capital Medical University; Mi-Yun Teaching Hospital, Capital Medical University and Beijing Huai-Rou Hospital, Capital Medical University) during the winter of 2018-2019 in Beijing, China. All consecutive patients admitted to the EDs (including 24-hour fever clinics) for observation or hospitalization with suspected I-LRTIs were experienced rapid antigen detection and/or reverse transcription polymerase chain reaction (RT-PCR) test for influenza A and pulmonary imaging examination for LRTI. Those patients with insufficient evidence of influenza or LRTI and those who received hormone therapy within the last three months were excluded. All selected patients followed clinical guidelines for standardized management.[17, 18] Medical records were independently reviewed by two physicians. The endpoint was 28-day mortality.

Definitions

LRTI was defined as a group of acute clinical diseases, including community-acquired pneumonia (CAP), acute exacerbation of chronic obstructive pulmonary disease (COPD), or acute bronchitis.[19] I-LRTI was defined in LRTI patients with confirmed influenza infection. The definition of bacterial co-infection refers to our previous research.[20] Virus co-infection was defined in patients with one or more isolates by RT-PCR test obtained from a sputum, tracheal or bronchoscopic sample.

Clinical Data Collection

At admission, data regarding medical identification, demographic characteristics, medical history, co-morbidities, clinical symptoms, physical examinations, laboratory results and radiological findings were collected. During hospitalization stay, microbiologic investigations, therapeutic management, invasive mechanical ventilation and vasopressor requirements were also recorded. The bloodwork results and microbial findings were provided by the Clinical Laboratory Department and Microbiology Laboratory Department of each hospital, respectively.

Plasma Sample Preparation

At the same time, 2 mL of peripheral blood was collected in an EDTA anticoagulant tube and thoroughly mixed. At 4 ℃, blood samples were centrifuged at 1300×g for 10 minutes. After centrifugation, supernatant (plasma) was taken and loaded into a 1.5 mL centrifuge tube. Mark the sample number, date on the centrifugal tube and record the sample information. After processing the sample to save to -80 ℃ refrigerator, avoid freeze and thaw.

Plasma Microarrays

Properly diluted plasma samples (from 10 to 500-fold) and serially diluted standard immunoglobulins and then printed onto a 3D modified slide surface (Capital Biochip Corp, Beijing, China) in two replicates using an Arrayjet microarrayer (Roslin, UK). Phosphate-buffered saline (PBS) and bovine serum albumin (BSA, 1 mg/mL) (Sigma-Aldrich, MO, USA) were used as negative controls. After printing, the plasma microarrays were stored at - 20 ℃ until use.

After equilibration to room temperature, the plasma microarrays were assembled into a microarray incubation tray and blocked with 500 μL 1% BSA in each well for 1 hour at room temperature. After removing the BSA, the arrays were incubated with the corresponding fluorescently-labeled antibody combinations for 1 hour at room temperature. The resulting slides were washed three times with PBS containing 0.05% (w/v) Tween 20 (PBST), 5 min/each, and H_2O twice, 5 min/each. The slide was scanned using a Genepix 4000A microarray scanner (Molecular Devices, CA, USA). The fluorescent images were analyzed and the signal intensity was extracted using a GenePix Pro image analysis software (Molecular Devices, CA, USA). For each Ig subclass, the 4-paramter logistic standard curve between the absolute concentration and the signal intensity was fitted with properly diluted plasma samples by least-squares method and thus the absolute concentration of Ig subclass in plasma samples was imputed by Newton-GC method according to the fitted standard curve.

Statistical Analysis

Mann Whiney *U* test was used to compare the difference of the means of two groups as the univariate values do not conform to the normal distribution. The exact Fisher's test was used to perform the independence statistical test. If *p*-value is under 0.05, then the result is considered statistically significant. The Spearman correlation coefficient was calculated and the hierarchical correlation clustering heatmap was plotted with the R package corrplot. Logistic regression-based model was adopted for the discrimination of the survival/non-survival groups of the influenza patients with the recursive feature selection in the LOO cross-validation procedure. ROC curve was plotted and AUC was used to assess the discriminative efficacy of the trained classifier model. The statistical analyses were performed with R. The logistic regression-based prediction and evaluation was performed with the Python packages SciKit-Learn and SciPy.

Results

Patient Characteristics

In the winter of 2018–2019, 280 patients with suspected influenza and accompanied LRTI from the four hospitals were screened. Of these patients, 173 were excluded: 166 had no laboratory positive test evidence for influenza, and 7 had recently been treated with hormones or immunosuppressants for autoimmune disease or solid organ transplantation. The final enrolled cohort consisted of 107 patients with I-LRTI, 85 from Beijing Chao-Yang Hospital, 9 from Beijing Di-Tan Hospital, 11 from Mi-Yun Teaching Hospital, and 2 from Beijing Huai-Rou Hospital. All the 107 patients were diagnosed with influenza A, 71 (66.4%) of them with the H1N1 subtype. None of them were documented as having received an influenza vaccine. According to the 28-day mortality, the study cohort was divided into survival (80/107) and non-survival (27/107) groups. Flow chart was showed in Fig. 1.

Differential Regulation of Immunoglobulin Proteome and Clinical Indicators between Survival/Non-survival Groups

Each patient in the study cohort was investigated for demographic characteristics (age, sex, and body mass index (BMI)), days from onset to ED, co-morbidities (active cancer, chronic respiratory disease, coronary artery disease, chronic heart failure, chronic hepatopathy, chronic kidney disease, diabetes mellitus), vital signs (heart rate (HR), respiratory rate (RR), mean arterial pressure (MAP), and Glasgow Coma Scale (GCS)), clinical laboratory tests (blood routine examination, blood biochemistry, D-dimer, arterial blood gas analysis, Glycosylated hemoglobin (HbA1C), C-Reactive Protein (CRP), procalcitonin (PCT), and T-cell subset counts), microbiological detections, antiviral administrations, organ supports, and immunoglobulin quantification (IgG, IgG1-4, IgA, IgA1-2, IgM). All of the above indicators were compared between the two groups (Table 1), and the indicators with significant or marginal differences (lymphocyte count (LY), monocytes count (MO), $CD3^+CD4^+$T-cell count, $CD3^+CD8^+$T-cell count, IgA, IgA1, IgG2, IgG4, CRP, PCT, D-dimer, oxygenation index (OI), HbA1C, lactic acid (LAC), base excess of blood (BEB), lactic dehydrogenase (LDH), and α-hydroxybutyrate dehydrogenase (HBDH)) were also described in Fig. 2. The

KS test indicated that LY, BEB, D-dimer, HbA1C, LDH, HBDH, CRP, PCT and IgG2 between survival and non-survival groups are statistically differentially distributed, and meanwhile MO, LAC and IgA are of marginally statistically differentially distributed. To measure effects of age, sex and days from onset on the concentration of Ig subclasses, generalized multivariate linear analysis was performed, in which the concentration of Ig subclass is taken as the dependent variable and age, sex, days from onset and the survival/non-survival group together as the independent variables. The results suggested that IgG2 concentration is statistically impacted with age, and IgA concentration with both age and days from onset.

Table 1

Comparison of clinical features and immunoglobulin proteome quantification between the survivors and non-survivors in patients with l-LRTI

Variables	Total n = 107	Survivors n = 80	Non-survivors n = 27	p value
Age, years, median(IQR[a])	64(50,79)	63(49,78)	67(56,81)	0.254
Age group, n(%)				
age < 45	15(14.0)	13(16.3)	2(7.4)	0.410
45 ≤ age < 65	42(39.3)	32(40.0)	10(37.0)	0.785
age ≥ 65	50(46.7)	35(43.8)	15(55.6)	0.288
Sex (male), n(%)	67(62.6)	51(63.8)	16(59.3)	0.677
BMI, (kg/m^2), median(IQR)	23.7(21.6,26.6)	23.7(21.5,26.6)	24.2(22.0,26.6)	0.769
Co-morbidities, n(%)				
Active cancer	3(2.8)	3(3.8)	0(0)	0.570
Chronic respiratory disease [b]	13(12.1)	11(13.8)	2(7.4)	0.595
Coronary artery disease	25(23.4)	19(23.8)	6(22.2)	0.871
Chronic heart failure	5(4.7)	4(5.0)	1(3.7)	0.999
Chronic hepatopathy	3(2.8)	2(2.5)	1(3.7)	0.999
Chronic kidney disease	10(9.3)	7(8.8)	3(11.1)	0.999
Diabetes mellitus	27(25.2)	20(25.0)	7(25.9)	0.924

[a] IQR: inter-quartile range;

[b] Chronic respiratory disease: asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, bronchiectasis;

[c] NAIs: Neuraminidase inhibitors.

Variables	Total n = 107	Survivors n = 80	Non-survivors n = 27	p value
Days from onset to ED, median(IQR)	4 (2,7)	3.5(2,7)	5(3,7)	0.124
NAIs [c] administration, n(%)	90(84.1)	70(87.5)	20(74.1)	0.178
NAIs administration within 48 hours, n(%)	10(9.3)	9(11.3)	1(3.7)	0.434
Bacterial co-infection, n(%)	17(15.9)	11(13.8)	6(22.2)	0.461
S. aureus co-infection, n(%)	7(6.5)	3(3.8)	4(14.8)	0.066
Virus co-infection, n(%)	23(21.5)	16(20.0)	7(25.9)	0.517
Mechanical ventilation, n(%)	27(25.2)	9(11.3)	18(66.7)	< 0.001
Vasoactive agents, n(%)	18(16.8)	8(10.0)	10(37.0)	0.003
Heart rate, beats/min, median(IQR)	97(81,112)	97(81,112)	104(81,118)	0.403
Respiratory rate, breaths/min, median(IQR)	22(20,27)	21(20,26)	22(20,29)	0.690
Mean arterial pressure, mmHg, median(IQR)	95(85,109)	95(85,107)	96(82,110)	0.971
Glasgow Coma Scale, median(IQR)	15(15,15)	15(15,15)	15(15,15)	0.574

[a] IQR: inter-quartile range;

[b] Chronic respiratory disease: asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, bronchiectasis;

[c] NAIs: Neuraminidase inhibitors.

Variables	Total n = 107	Survivors n = 80	Non-survivors n = 27	p value
Clinical parameters, median(IQR)				
White blood cell, WBC, (× 10^9/L)	7.72(4.79,10.79)	7.94(5.01,10.55)	6.86(4.43,11.33)	0.846
Neutrophil, NE, (× 10^9/L)	6.19(3.77,8.81)	6.20(3.75,8.75)	6.07(3.85,10.30)	0.578
Lymphocyte, LY, (× 10^9/L)	0.71(0.44,1.24)	0.86(0.50,1.37)	0.50(0.38,0.79)	0.012
Monocyte, MO, (× 10^9/L)	0.36(0.16,0.62)	0.40(0.19,0.62)	0.26(0.09,0.61)	0.050
NK cell, (/μL)	9.60(5.73,13.15)	9.55(7.20,16.43)	9.75(5.43,13.15)	0.652
$CD3^+CD4^+$T cell, (/μL)	199.50(114.25,355.00)	201.50(130.75,386.50)	169.00(87.50,238.00)	0.077
$CD3^+CD8^+$T cell, (/μL)	117.50(70.25,208.00)	123.50(78.50,278.75)	107.00(45.75,174.50)	0.080
D-dimer, (mg/L FEU)	1.48(0.89,3.54)	1.21(0.72,2.18)	2.43(1.85,13.34)	< 0.001
Lactate dehydrogenase, LDH, (U/L)	355.0(223.0,576.7)	302.0(216.5,511.1)	482.0(302.0,642.0)	0.019
α-hydroxybutyrate dehydrogenase, HBDH, (U/L)	275.5(182.0,423.3)	236.0(181.0,367.3)	375.5(259.5,473.0)	0.008
Glycosylated hemoglobin, HbA1C, (%)	6.1(5.8,6.9)	6.1(5.7,6.7)	6.6(6.1,7.7)	0.015
Lactic acid, LAC, (mmol/L)	1.2(1.0,1.7)	1.1(0.9,1.7)	1.5(1.2,1.9)	0.023
Oxygenation index, OI	263(151,381)	268(162,393)	194(116,294)	0.059

[a] IQR: inter-quartile range;

[b] Chronic respiratory disease: asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, bronchiectasis;

[c] NAIs: Neuraminidase inhibitors.

Variables	Total n = 107	Survivors n = 80	Non-survivors n = 27	p value
HCO_3^-, (mmol/L)	21.1(18.6,23.9)	22.5(20.0,24.9)	20.0(17.1,22.5)	0.023
Base Excess of Blood, BEB, (mmol/L)	-1.0(-3.8,0.9)	-0.6(-3.3,1.2)	-3.7(-5.0,-0.4)	0.010
C-Reactive Protein, CRP, (mg/dL)	12.1(4.2,22.1)	9.6(3.0,22.8)	19.6(14.2,25.8)	0.018
Procalcitonin, PCT, (ng/mL)	0.22(0.08,1.01)	0.16(0.06,0.60)	0.91(0.23,6.43)	0.001
Plasma immunoglobulin quantification, (μg/mL), mean ± SD				
IgA	4277.955517 ± 1751.127383	4495.494011 ± 1799.218731	3633.397017 ± 1445.182929	0.053
IgA1	3251.871483 ± 1575.089408	3411.345318 ± 1646.654368	2779.356417 ± 1251.261989	0.087
IgA2	326.738305 ± 148.035244	330.15051070 ± 139.051213	316.628064 ± 174.505309	0.347
IgG	22497.252537 ± 10921.188428	22082.874174 ± 10369.593861	23725.040279 ± 12547.880435	0.596
IgG1	6682.518079 ± 3470.015276	6569.600710 ± 2892.282678	7017.088061 ± 4849.447558	0.628
IgG2	167.928721 ± 121.118981	189.688541 ± 129.383245	103.455179 ± 56.304086	< 0.001
IgG3	24.179282 ± 33.797908	25.921704 ± 36.743783	19.016550 ± 22.755197	0.168
IgG4	21.765171 ± 25.443257	21.888248 ± 20.932293	21.400501 ± 36.162335	0.056
IgM	3515.750491 ± 2696.014576	3578.764165 ± 3087.582291	3329.043310 ± 786.461713	0.683

[a] IQR: inter-quartile range;

[b] Chronic respiratory disease: asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, bronchiectasis;

[c] NAIs: Neuraminidase inhibitors.

Co-regulatory Network Analysis of Clinical Indicators and Immunoglobulin Proteome Quantification in the Survival/Non-survival Groups

Co-regulatory network was used to analyze and compare the correlation characteristics across clinical indicators and immunoglobulin quantification for the survivors and the non-survivors respectively, and was showed by hierarchical correlation clustering heatmap (Fig. 3). Red color represents a positive correlation, blue color represents a negative correlation, and the darker the color, the stronger the correlation. In the heatmap of the survival group, different variables were respectively separated into two red-colored positively correlated clusters (Fig. 3A). Cluster 1 included IgA, IgA1-2, IgG, IgG1-4, WBC, NE, MO, NK cell, and age, and these variables were related to immunity. Cluster 2 included CRP, PCT, D-dimer, LDH, LBDH, HR and RR, and these variables revealed the severity of infection. In the heatmap of the non-survival group, the indicator clusters were re-clustered (Fig. 3B). Cluster 1 included days from onset to ED, IgM, sex, and GCS. Cluster 2 contained indicators of the severity of the infection (LAC, LDH, HBDH, MAP, HR, RR, D-dimer, CRP and PCT). Cluster 3 included partial immunoglobulin family molecules (IgA, IgA1, IgG, IgG1, IgG2, IgG4) and BMI. Cluster 4 included both cell-immune-related indicators (age, WBC, NE, MO, LY, $CD3^+CD4^+$T-cell, and $CD3^+CD8^+$T-cell), OI, HbA1C and bacterial co-infection. Cluster 5 included NK cell, IgG3, IgA2, BEB and virus co-infection.

Predictive Model of Clinical Outcomes

We investigated the potential of these variables in the prediction of survivors/non-survivors, and calculated the AUCs of these differentially regulated variables. Based on the statistical difference, we performed iterative recursive feature selection and found the optimal combination of features. Logistic regression-based classifier was trained to discriminate survivors and non-survivors. To evaluate the predictive efficacy of the trained model objectively, LOO cross validation was employed to ensure the mutual independence between the training dataset and the testing dataset. In LOO cross validation, the training and validation steps were repeated N times, and for each step one sample was selected for validation and the rest were used for model training iteratively (N denoted with the sample number). Then random sampling (sampling ratio 95%, iteration times 1000) was performed to calculate the 95% confidence intervals (CIs) of the AUC of the prediction model. The AUCs of IgG2, IgA and the combination the two were 0.725 (95% CI 0.702–0.748), 0.575 (95% CI 0.404–0.694) and 0.737 (95% CI 0.714–0.760), respectively (Fig. 4A). The combined prediction model with nine variables (D-dimer, days from onset to ED, IgA, IgG2, LAC, LY, MO, Staphylococcus aureus (S.aureus) co-infection and age) reached the maximum AUC value of 0.810 (95% CI 0.755–0.839) (Fig. 4B). The AUCs of D-dimer, LAC, LY, and MO were 0.722 (95% CI 0.690–0.751), 0.662 (95% CI 0.634–0.684), 0.644 (95% CI 0.607–0.673), and 0.572 (95% CI 0.536–0.609), respectively. All the pairwise comparisons across each of the single-factor models with the combination prediction model were of statistical significance.

To further evaluate the selected optimal indicators for the prediction model, the unsupervised participant-feature hierarchical correlation clustering was also performed and the participants were separated into two clusters. The majority of the actual non-survivors were discriminated into the same cluster, which indicated

the consistency between the clustered results and the actual grouping (Fig. 4C). This proved the feature combination's discriminative efficacy of the survivors/non-survivors.

Validation of the Model by Disease Severity Evaluation

The risk of progression to critical conditions, including mechanical ventilation requirements or vasoactive agent requirements, can also be evaluated using the predictive model and for mechanical ventilation or vasoactive agent apparently there exists statistical difference between survivors and non-survivors in practice. For the requirement of mechanical ventilation, 15 (15/37) predicted non-survivors and 12 (12/70) predicted survivors should receive mechanical ventilation respectively, exhibiting the statistical difference between two predicted groups (Fig. 5A), which is in consistency with the actual facts (In the study cohort, 18 (18/27) non-survivors and 9 (9/80) survivors actually received mechanical ventilation (Fig. 5B)).For the requirement of vasoactive agent, 10 (10/37) predictive non-survivors and 8 (8/70) predicted survivors should receive vasoactive agents respectively, exhibiting the marginally statistical difference between two predicted groups (Fig. 5C), which is in accordance with the actual facts (In the study cohort, 10 (10/27) non-survivors and 8 (8/80) survivors received vasoactive agents, respectively (Fig. 5D)). The results of both mechanical ventilation and vasoactive agents demands proved the potential of the predictive model.

Discussion

This study evaluated the prognosis of patients with I-LRTI by investigating clinical indicators and immunoglobulin quantification, all of which were taken from the initial assessment of the patient's ED visit. For the reason that corticosteroids and immunosuppressants have multiple anti-inflammatory and immunosuppressive effects and influence virtually all immune cells,[21] patients who had recently received immunosuppressive or hormone therapy were excluded, but patients with other underlying diseases were also included. Chronic pulmonary, cardiovascular, renal, hepatic, hematologic, metabolic, or neurologic disorders put influenza patients at high risk of complications, but our study did not identify these underlying conditions as risk factors for mortality. Current findings were consistent with the results of a multicenter study in Spain involving 2,901 patients, showing that underlying diseases and physiological status were not independent risk factors for mortality in severe influenza patients.[22]

Although there was a high proportion of NAIs administration at the time of admission, only a few patients were administrated within 48 hours from the onset of influenza-like symptoms. Both the proportion of patients who had started NAIs at the time of admission and the proportion of patients who had started NAIs within 48 hours from onset were lower in the non-survivors than that in the survivors, in spite of no statistical differences. Our results still supported previous observational studies that antiviral treatment of critically ill adult influenza patient with a NAI was associated with survival benefit.[23, 24] However, problems such as delayed administration of NAIs (> 48 hours), drug resistance and induced cytokine storms still put severe cases at risk of early mortality[25, 26]. Another study argued that outpatient data on patients with relatively mild illnesses should not form the basis for policies on the management of more severe disease.[27] Therefore, comprehensive clinical evaluation and multi-system support are still crucial and indispensable.

Clinicians tend to evaluate the prognosis of I-LRTI patients with multiple factors rather than single factor, but there is currently no efficacious evaluation system, which could reflect disturbances in the physiological system. Host immunity, especially humoral immunity, plays an important role in the defense and elimination of influenza viruses. Influenza virus infection, as an initiating factor, can lead to multiple organ dysfunction and usually co-infect with pathogenic bacteria. This process is associated with higher mortality rate than individual viral infection.[28] The complex interaction of initial influenza virus, co-pathogen invasion and host immune response complicates the pathophysiological process, and its molecular biological mechanism needs to be explored urgently.[29] In order to facilitate clinical application, we explored the changing pattern of simple and easily available biomarkers to reveal the underlying pathophysiological signals in I-LRTI status and their changes prior to death. In this study, co-regulation networks and a multi-factor model were established by combining immune cell count, immunoglobulin quantification and clinical indicators routinely measured in clinic.

We developed a large-scale, ultra-micro plasma immunoglobulin quantification chip, which has high reproducibility (Pearson's $r > 0.9$) and high sensitivity (~ attomolar). Through the platforms, we screened the immunoglobulin concentration of hundreds of plasma samples within 20 minutes with low cost. Our results showed that decreased IgA, IgA1, and IgG2 were associated with mortality. Two studies demonstrated respectively that impairment of the antibody response to specific microbial antigens predisposed patients with IgG2, IgG3 and IgA deficiencies to recurrent infections.[30, 31] Shield JP et al. investigated the immunology of eight patients with recurrent bacterial infections in infancy or early childhood and attributed this phenomenon to their lower IgG2 levels and poor production of specific antibodies against common pathogens.[32] In these studies, all enrolled patients had normal serum total IgG range, but had selective IgG-subclass deficiency. IgG2 is a weak mediator of FcγR and complement mediated effector functions, and is involved in the response to polysaccharide antigens.[33] Influenza virus elicits a vigorous non-virus-specific IgG2a response, which is dependent on cognate T-B interactions. A robust virus-specific IgA response with virus-neutralizing activity is crucial for eliminating influenza virus.[34] Low concentrations of IgG2 often occur in association with a deficiency in IgG4 and/or IgA1 and IgA2.[35] Thus, inadequate IgG2 and IgA response may be the underlying cause of infection progression and poor prognosis.

The detection of immunoglobulin subclass has not been routinely carried out in clinical practice, but our results showed that many laboratory indicators provided strong evidence for prognosis in patients with I-LRTI. First, elderly patients still have the highest mortality rate.[36] In the age stratification of our cohort, mortality rate increased with age, which may be related to body's organ systems aging, especially immune system aging. Hernandez-vargas EA et al. confirmed in mouse experiments that immune activation capacity was weakened in elderly mice after influenza virus infection, and thus the efficiency of clearing influenza virus was decreased than it was in younger mice.[37] Second, a decrease in immune cell count predicts a poor prognosis. Lymphocytopenia was considered as a risk factor for mortality in patients with pneumonia caused by influenza virus.[38] Decreased monocytes was found to have a marginal difference between the survivors and the non-survivors, which may also be a prognostic factor. So far, few studies have been reported, and further large sample validation is required. The decrease of $CD3^+CD4^+$T-cell and

$CD3^+CD8^+$T-cell had been proved to be closely associated with increased mortality and deteriorated primary and subsequent infections in influenza A virus infected animals.[39, 40] Similar results were found in our cohort study, where decreased $CD3^+CD4^+$T-cell and $CD3^+CD8^+$T-cell counts were associated with mortality and bacterial co-infection (especially S. aureus co-infection). Third, some biomarkers such as D-dimer, LDH, HBDH, HbA1C, LAC, HCO_3^-, BEB, CRP and PCT were also strong prognostic factors. A surge of LDH suggested metabolic reprogramming, and was associated with increased mortality in patients with sepsis.[41] Currently, we found a similar phenomenon in the influenza cohort. Our previous study confirmed that elevated PCT levels in influenza patients reflected bacterial co-infection, which was an independent risk factor for mortality.[42]

We further comprehensively analyzed the immunoglobulin profile and laboratory parameters in patients with I-LRTI between survivors and non-survivors and established corresponding co-regulatory network models for different groups. In the cluster heatmap, we found that in the survival group, indicators reflecting the body's immune status, including IgA, IgA1-2, IgG, IgG1-4, WBC, NE, MO, NK cell, and age, were nearly clustered together. In the same way, indicators reflecting the severity of infection, including CRP, PCT, D-dimer, LDH, and LBDH, were clustered together. There always be positive correlations between IgG1 and IgG, since IgG1 constitutes the main component (60%-75%) of IgG.[43] However, we found that correlation patterns between indicators were apparently modified in the non-survival group compared with the survival group. The association between bacterial co-infection and T cell counts was present in the non-survival group, suggesting a potential role of their interaction between impaired cellular immune and bacterial co-infection. A recent study similarly showed that the nosocomial co-infection of A. baumannii was associated with the dysregulation of immune responses including deranged T-cell counts.[44] In our previous studies, patients with influenza was found to be susceptible to community-acquired bacterial co-infections, and was characterized by a synergistic lethal effect of influenza and S. aureus.[20] In view of the low sensitivity and lagging results of bacteriological investigation, our another study have quantified bacterial co-infection in influenza patients using PCT and identified an independent association between PCT and 28-day mortality.[42] However, the correlation between bacterial co-infection in hospitalized patients with influenza and their immune indicators has not been revealed. In the current study, we further elucidated the correlation between this phenomenon and T cell mediated immunity. This finding is of great clinical significance. One is that T cell counts should be instantly detected to assess the risk stratification when a severe influenza patient visits ED. The other is to predict the risk of bacterial co-infection in patients with I-LRTI based on the T cell counts, and then to manage antibiotics and regulate immune function as early as possible.

In this study, we have created an assessment model to predict mortality due to I-LRTI by combining nine variables, including IgG2, D-dimer, LAC, LY, MO, IgA, days from onset, age, and S. aureus co-infection. The first to the eighth variables in the model were continuous, and they were ordered according to the contribution of each single variable to the model, which was quantified as area under ROC curve. To make the evaluation of the prediction model closer to the real world, LOO cross validation was adopted to implement the mutual independence between model training and validation. The prediction model was more powerful than the isolated use of each of the original variables based on an AUC comparison.

The current study has several limitations that prevent broad generalization of the prediction model. First, the relatively small number of patients may limit external validity, and large sample validation is required. Second, IgG2 quantification cannot be implemented in clinical practice, and the predictive power of the model without IgG2 may be weakened in clinical application. It is suggested that the testing of Ig subclass should be carried out in hospitals in the future. Third, influenza vaccination rates in our country were low, and the information was usually poorly documented in medical history, preventing any conclusion on this point. Fourth, all the variables mentioned in this study were dynamic changes after the body was infected with influenza virus. This study only captured a single time point at the time of admission, which failed to fully reflect the progress of patients' illness.

Conclusions

The main conclusions and innovations of this study were as follows: I) Quantitative analysis of Ig and Ig-subclass by proteomics constitutes part of immune evaluation, which is especially important for the prognosis assessment in patients with I-LRTI. Proteomics, with its superiorities of high throughput, high sensitivity, high accuracy and low cost, may become an important technique in medicine in the future. II) Cluster analysis revealed that the body's homeostasis in the non-survivors had been disrupted by the time the patients were admitted to the hospital. Moreover, an important finding was that lethal bacterial (especially Staphylococcus aureus) co-infection was closely associated with cellular immunity (WBC, NE, MO, LY, $CD3^+CD4^+$T-cell, and $CD3^+CD8^+$T-cell), OI, HbA1C and age. III). So far, no accurate and effective prediction model for prognosis in patients with I-LRTI has been reported. This study created a multi-dimensional and cross-verified prediction model for clinical application.

Abbreviations List

NAIs, neuraminidase inhibitors; Ig, immunoglobulin; I-LRTI, influenza-associated lower respiratory tract infection; ED, emergency department; RT-PCR, reverse transcription polymerase chain reaction; ROC, receiver operating characteristics; AUC, area under receiver operating characteristics curve; A(H1N1), pandemic 2009 influenza A(H1N1); BMI, body mass index; HR, heart rate; RR, respiratory rate; MAP, mean arterial pressure; GCS, Glasgow Coma Scale; HbA1C, Glycosylated hemoglobin; CRP, C-Reactive Protein; PCT, procalcitonin; WBC, white blood cell; NE, neutrophil; LY, lymphocyte; MO, monocytes; OI, oxygenation index; LAC, lactic acid; BEB, base excess of blood; LDH, lactic dehydrogenase; HBDH, α-hydroxybutyrate dehydrogenase; *S.aureus*, *Staphylococcus aureus*; CI, confidence interval; LOO, leave-one-out; KS test, Kolmogorov-Smirnov test

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Review Board and Medical Ethics Committee of Beijing Chao-Yang Hospital (Ethical approval number: 2019-ke-301), which was also recognized by ethics committees at the three other hospitals. Verbal consent had been obtained for all forms of personally identifiable data

including biomedical, clinical, and biometric data from study participants or their legal guardian. As this study is an observational study, no written consent was obtained. Verbal informed consent had been approved by the Ethics Committee in this study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FT and DW had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis, including and especially any adverse effects. X-MZ, J-HL and J-YD contributed to immunoglobulin quantification, data collection and analysis. FT, DW, Y-DY, LG, Y-FC, S-FM, X-TL, W-PY, X-JY and XM contributed to data collection and analysis and manuscript preparation and review. S-BG and X-BY conceived and designed the research, guided the experiment, analyzed and interpreted the data, helped to draft the manuscript and revised it critically for important intellectual content and was responsible for the overall content as guarantor. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figures

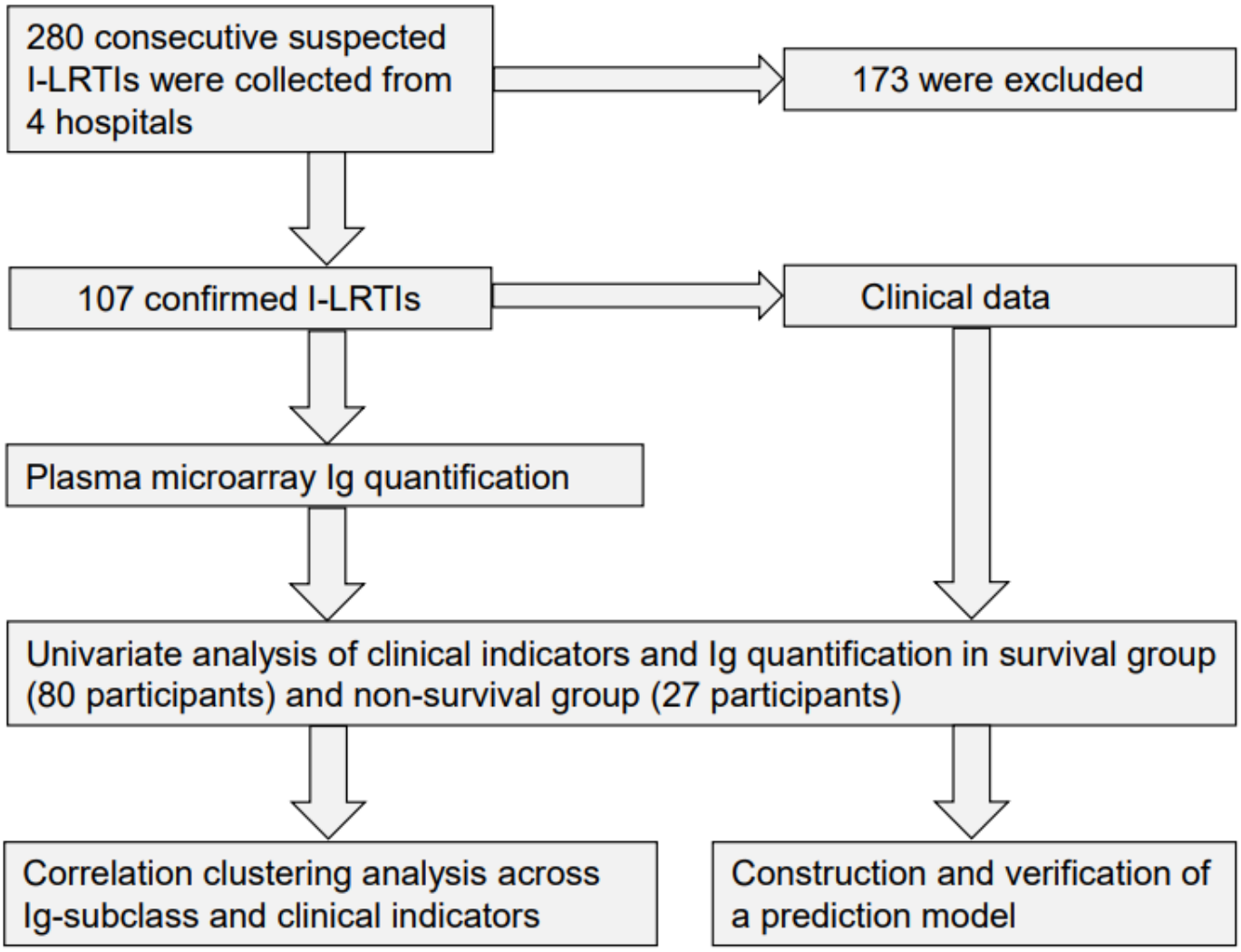

Figure 1

Flow chart. In the winter of 2018-2019, participants were selected from consecutive influenza patients who visited EDs in four teaching hospitals by pre-established inclusion criteria and exclusion criteria. When patients arrived at the EDs, the first clinical evaluation and plasma samples collection were achieved. The amount of immunoglobulin in the plasma was quantified using the standard curve generated by the purified immunoglobulin standards. According to the final prognosis, all participants were divided into a survival group and a non-survival group, and the differences of variables between the two groups were compared by univariate analysis. Variables with significant differences and marginal differences were used in correlation network analysis to observe the characteristics of the coordinated changes of indicators of the homeostasis imbalance in the non-survivors. A comprehensive correlation analysis between the immunoglobulin proteome expression and clinical data helped construct a prediction model, to distinguish patients at high risk of mortality at the ED arrival.

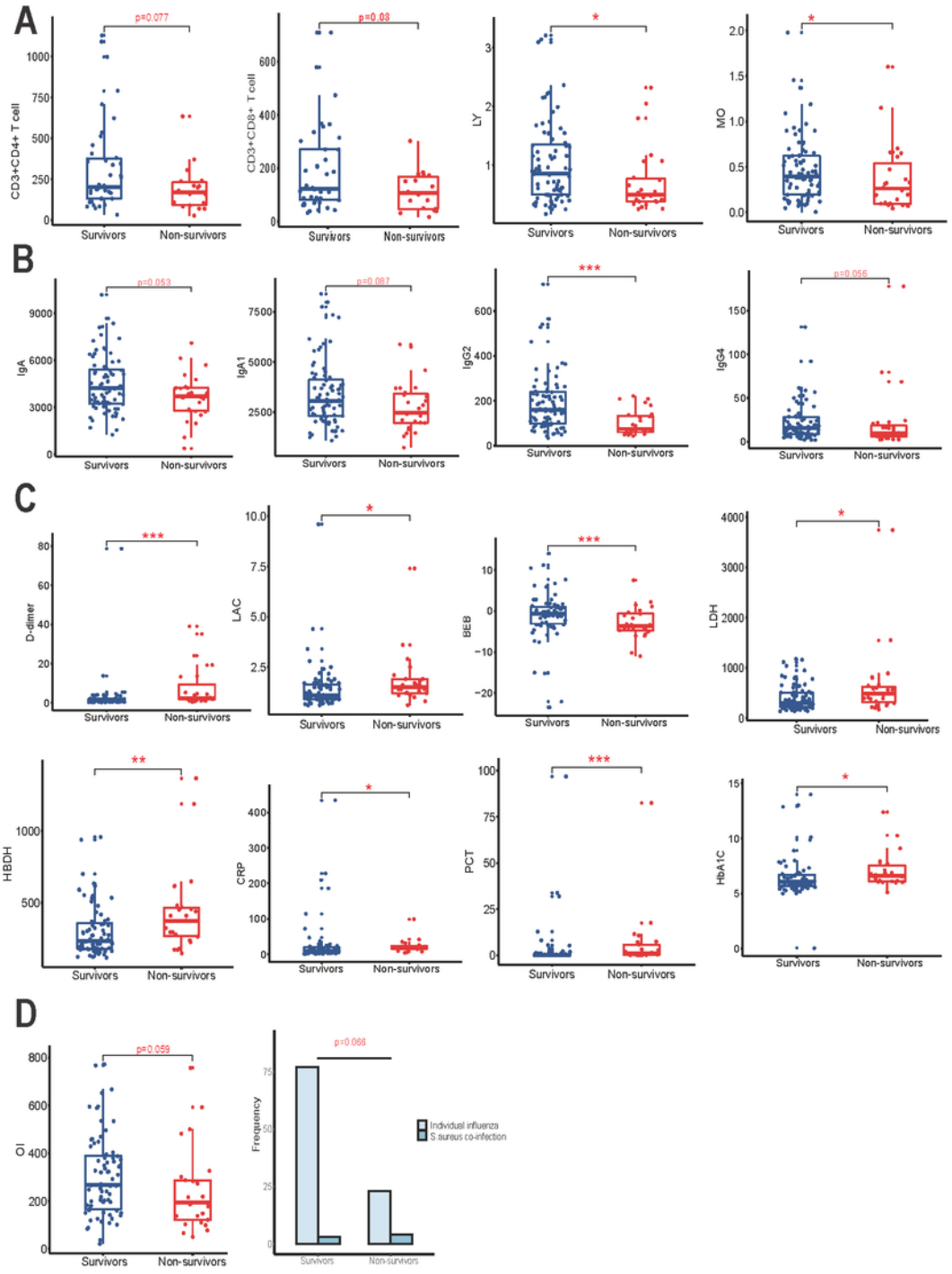

Figure 2

Univariate analysis of variables with significant statistical differences and marginal statistical differences between the survivors and non-survivors. A. Immune cells. B. Immunoglobulin and subclass. C. Biomarkers related to severe infection. D. Other clinical factors associated with prognosis. Mann Whitney U test was used for quantitative variables, and Fisher's accurate test was used for qualitative variables. Different statistical significance levels were marked (p <0.001 ***, p <0.01 **, p <0.05 *, p < 0.1).

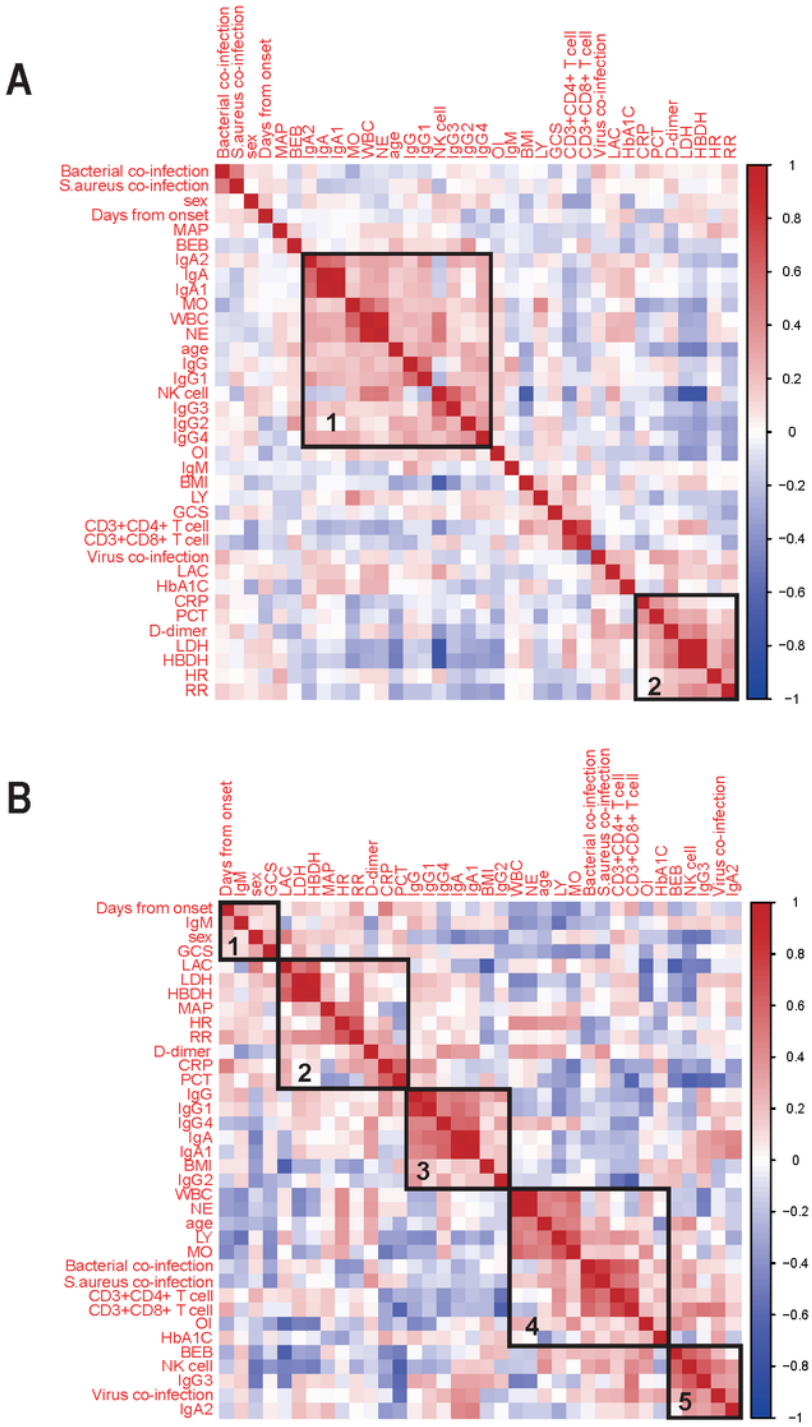

Figure 3

Construction of co-regulation networks described by hierarchical correlation clustering heatmap in the survival and non-survival groups. Spearman correlation coefficient was used to measure the correlation between the indicator pairs, and hierarchical clustering was used to cluster the highly correlated clinical indicators into modules. Red color represents a positive correlation, blue color represents a negative correlation, and the brighter the color, the stronger the correlation. A. Different variables were respectively

clustered into two red modules with boundary. Cluster 1 contained IgA, IgA1-2, IgG, IgG1-4, WBC, NE, MO, NK cell, and age. Cluster 2 contained CRP, PCT, D-dimer, LDH, LBDH, HR and RR. B. Different variables were respectively clustered into five red modules with boundary. Cluster 1 contained days from onset to ED, IgM, sex, and GCS. Cluster 2 contained LAC, LDH, HBDH, MAP, HR, RR, D-dimer, CRP and PCT. Cluster 3 contained IgA, IgA1, IgG, IgG1, IgG2, IgG4 and BMI. Cluster 4 contained age, WBC, NE, MO, LY, CD3+CD4+T-cell, and CD3+CD8+T-cell, OI, HbA1C and bacterial co-infection. Cluster 5 contained NK cell, IgG3, IgA2, BEB and virus co-infection.

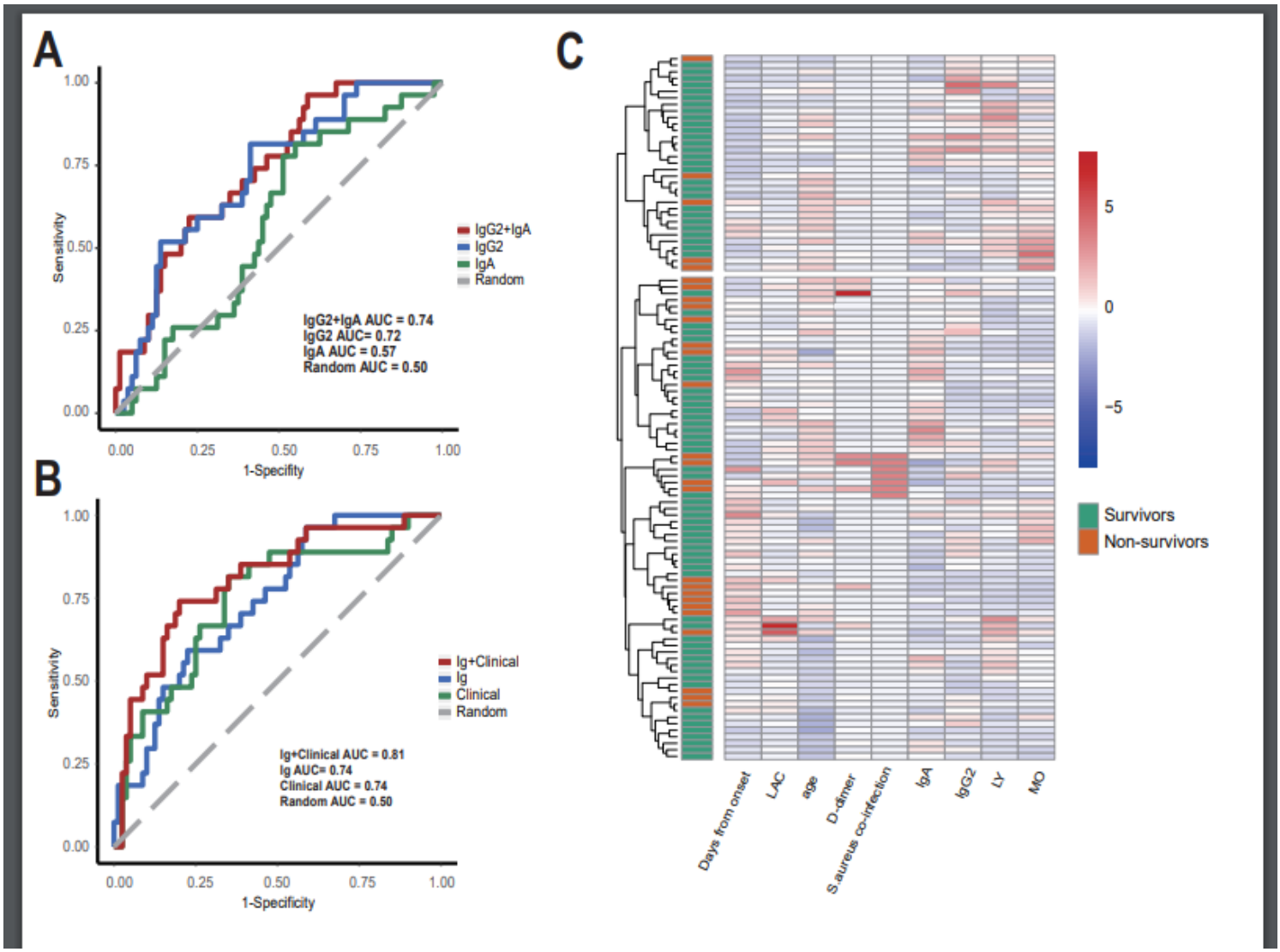

Figure 4

A. Receiver operating characteristic curves of prediction for prognosis of immunoglobulin based on multivariate Logistic regression. The blue line represented IgG2. The green line represented represented IgA. The red line represented the combination of immunoglobulin quantification (including IgA and IgG2). B. Receiver operating characteristic curves of prediction model based on multivariate Logistic regression. The blue line represented the combination of immunoglobulin quantification (including IgA and IgG2). The green line represented the combination of clinical indicators (including D-dimer, Days from onset to ED, LAC, LY, MO, S.aureus co-infection and age). The red line represented the combination of immunoglobulin

quantification and clinical indicators (including D-dimer, Days from onset to ED, IgA, IgG2, LAC, LY, MO, S.aureus co-infection and age). C. Participants - indexes hierarchical correlation clustering heatmap of composite features based on immunoglobulin quantification and clinical indicators (including D-dimer, Days from onset to ED, IgA, IgG2, LAC, LY, MO, S.aureus co-infection and age). Columns were indexes characteristics and rows were participants.

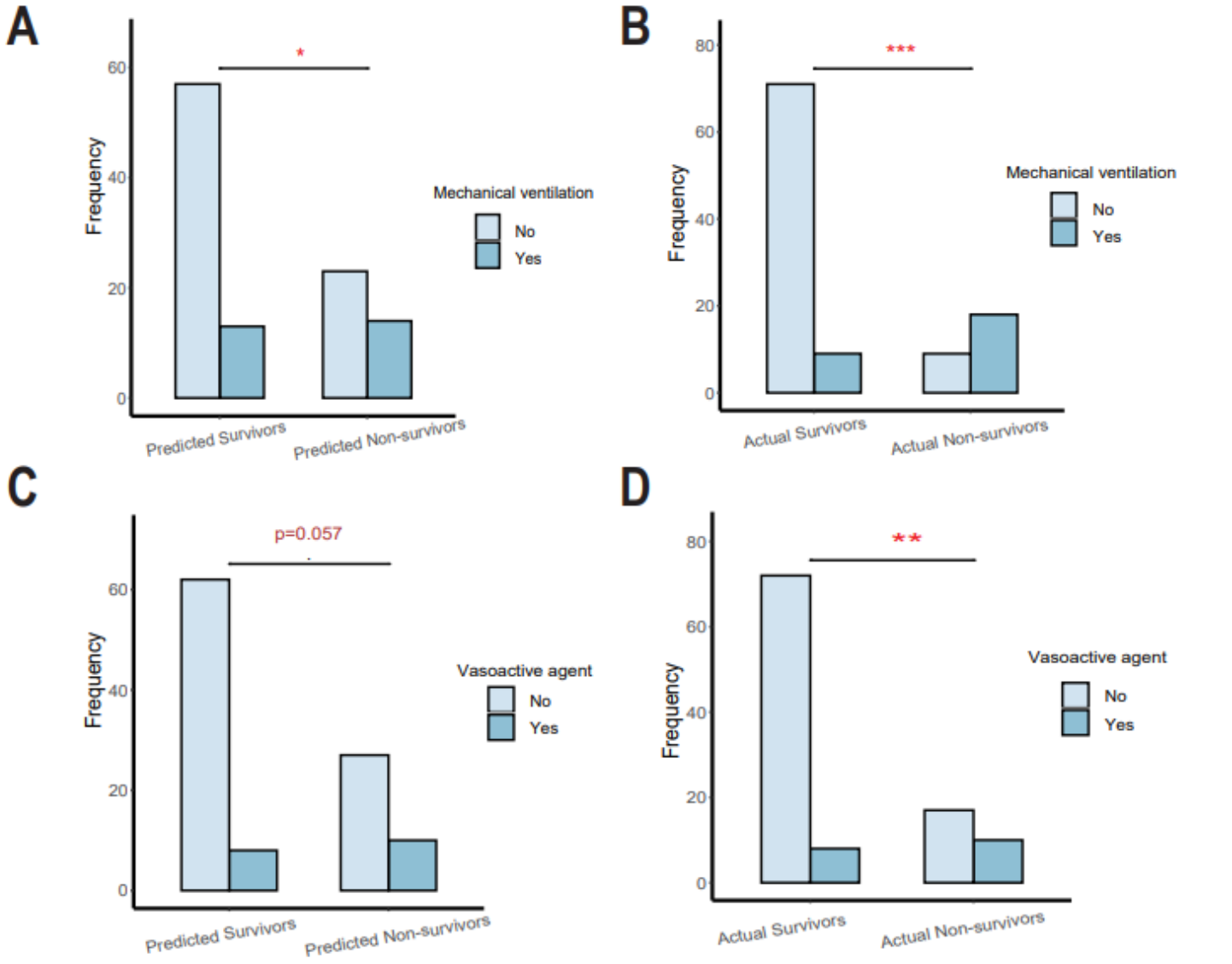

Figure 5

The efficacy of the model in predicting critical condition (mechanical ventilation and vasoactive agents). Fisher's accurate test was used to calculate the p value, and different statistical significance levels were noted (p <0.001 ***, p <0.01 **, p <0.05 *, p < 0.1).