Enhancing-ARDS diagnostics for ICU patients: a retrospective, nested case-control study to develop a biomarker-based model

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Research Article

Keywords: Acute respiratory distress syndrome, Biomarkers, Club cell protein 16, Angiopoietin 2, Soluble receptor for advanced glycation end-products, High-mobility group box 1 protein, Surfactant protein D, Intensive care unit

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Enhancing-ARDS diagnostics for ICU patients: a retrospective, nested case-control study to develop a biomarker-based model

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Background: To investigate whether a series of biomarkers including club cell protein 16 (CC16), angiopoietin 2(Ang-2), soluble receptor for advanced glycation end-products (sRAGE), high-mobility group box 1 protein (HMGB1), and surfactant protein D (SPD) could be utilized for identifying patients, thereby increasing the diagnostic value of acute respiratory distress syndrome(ARDS) in intensive care unit (ICU).

Methods: 211 ICU admissions were enrolled in this retrospective, nested case-control study. These patients were then divided into ARDS (n=79) and non-ARDS (n=132) groups according to the Berlin criteria on ICU day 1. Patient characteristics, vital signs, and laboratory examinations were collected within three hours of admission. Five inflammatory associated plasma biomarkers, as well as lung epithelial and endothelial injury which included CC16, Ang-2, sRAGE, HMGB1 and SPD were measured in the morning of day two in the ICU. Diagnostic values were analyzed with receiver operating characteristic (ROC) curves. Pearson's product-moment correlation coefficient and multivariate logistic regression analysis were applied for predictive purposes.

Results: C-reactive protein (CRP), NT-proBNP, and PH values for traditional

indicators and five biomarkers were analyzed with an objective ARDS indicator, the PaO_2/FiO_2 ratio. Evidence suggests that only four of potential indicators analyzed here, and CRP hold high diagnostic value. The area under curve (AUC) for each were as follows: CC16 (AUC: 0.752; 95%CI0.680-0.824), Ang-2 (AUC: 0.695; 95%CI 0.620 -0.770), HMGB1 (AUC: 0.668; 95%CI 0.592-0.744), sRAGE (AUC: 0.665; 95%CI 0.588-0.743), CRP (AUC: 0.701; 95%CI 0.627-0.776). No single indicator surpassed the diagnostic capability of the PaO_2/FiO_2 ratio which had an AUC: 0.844(95%CI 0.789-0.898), especially in terms of sensitivity. However, when the binary logistic model was transformed and the model was built, the AUC increased from 0.647(95%CI 0.568-0.726) to 0.911(95%CI 0.864-0.946). Among the combinations tested, PaO_2/FiO_2+CRP+Ang-2+CC16+HMGB1 resulted in an AUC of 0.910 (95%CI 0.863-0.945), while PaO_2/FiO_2+CRP+Ang-2+CC16+HMGB1+sRAGE+SPD have an AUC of 0.911(95%CI 0.864-0.946).

Conclusions: A combination of the assessed biomarkers could enhance ARDS diagnostics, which has obvious ramifications for patient care and prognosis. It may be possible to develop a predictive ARDS nomogram; however, of the combinations tested here, we would recommend PaO_2/FiO_2+CRP+Ang-2+CC16+HMGB1 for clinical practice. This is because of the cost implications in contrast with the benefit involved in utilizing the more elaborate model. Although, further health economics research is required to consider this opportunity cost for emergency care policy.

Keywords： Acute respiratory distress syndrome; Biomarkers; Club cell protein 16; Angiopoietin 2; Soluble receptor for advanced glycation end-products; High-mobility

group box 1 protein; Surfactant protein D; Intensive care unit

Introduction

Acute respiratory distress syndrome (ARDS) is a common disease characterized by permeability pulmonary edema and refractory hypoxemia in intensive care unit (ICU)[1]. ARDS is associated with high morbidity and mortality rates although, it remains underdiagnosed and therefore all too often untreated[2]. The traditional diagnostic methods mentioned in the Berlin definition[3], such as the ratio of partial pressure in arterial oxygen over the fraction of inspired oxygen (PaO_2/FiO_2) and standard X-rays could perhaps be updated. The prevalence of ARDS and underdiagnosis with delays in identification of cases limits the effect of any intervention/s once administered. Physicians must have the most advanced diagnostics in order to improve clinical decision-making and at present this may not be the case for ARDS.

Biomarkers are indicators of pathophysiological processes, and prove insight into the biological responses to therapeutics. Biomarkers are increasingly becoming common place in both clinical research for participant selection but also for clinical practice such as triage. For example, biomarkers such as procalcitonin (PCT), C-reactive protein (CRP), and interleukin-6 (IL-6) are frequently measured for sepsis, and Cardiac Troponin-I (cTnI) is now commonly used for acute myocardial infarction. These biomarkers provide timely information which can be effectively

used to tailor therapeutic strategies around individual characteristics[4,5]. Therefore, having an appropriate biomarker (or combination of biomarkers) can help to determine disease characteristics as well as the sensitivity and specificity of an intervention/s.

To date, more than 20 biomarkers have proven useful for diagnosing or predicting ARDS[1]. Fremont[6] and Ware[7]have also proposed that combining biomarkers will enhance ARDS diagnostics compared to a single biomarker. This is, because a specific combination is likely to improve both the accuracy and reliability of diagnosis and therefore prognostics. However, when developing biomarker-based models, it is necessary to avoid repeating measures for those with dissimilar pathophysiologies. Therefore, biomarkers derived through basic research into different pathophysiological pathways for inflammation, lung epithelial and endothelial injury were chosen for this study. These included club cell protein 16 (CC16), angiopoietin 2(Ang-2), soluble receptor for advanced glycation end-products (sRAGE), high-mobility group box 1 protein (HMGB1) and surfactant protein D (SPD)[2].

Methods

Study population

ICU patients were enrolled between March 2013 and March 2017. The following eligibility criteria were necessary for inclusion: 1) patient age > 18 and < 75; 2)

expected ICU stay > 24 h; 3) blood samples were collected < 6 h after admission; 4) diagnosis had been conformed prior to discharge. Those who did not meet all of the criteria were excluded. Written informed consent was then requested from potential participants or their legal representatives. The institutional human ethics committee of the affiliated Baoan Hospital of Shenzhen, Southern Medical University approved our study protocols (BYL 20141007). Research involving human participants, human material, or human data have been performed in accordance with the Declaration of Helsinki. All methods were carried out in accordance with relevant guidelines and regulations.

Data collection and outcome of patients

Values at baseline were recorded within 3 h of admission to the ICU, including individual characteristics (i.e., age, gender, comorbidities, and risk factors of ARDS), Acute Physiology and Chronic Health Evaluation II score (APACHE II), vital signs (i.e., blood pressure, body temperature, respiratory rate, and heart rate). Physiological variables for the PaO_2/FiO_2 ratio, as well as C-reactive protein (CRP), white blood cell count (WBC), the N-terminal of the prohormone brain natriuretic peptide (NT-proBNP), PH value, serum total protein (TP), D-Dimer, serum creatinine concentration (Scr), and lactic acid (Lac) were determined synchronously within 3 h of admission. Duration of mechanical ventilation (MV), mortality at Day-7, and Day-28 were recorded for all participants.

Diagnosis criteria and subgroups

ARDS is diagnosed according to the Berlin definition[3]which stipulates: 1)ARDS has an acute onset, of less than 7 days; with 2) bilateral opacity (consistent with pulmonary edema), as detected by CT or X-ray; and 3) PaO_2/FiO_2 ratio of less than 300 mmHg, with ventilation support (Positive End Expiratory Pressure or Continuous Positive Airway Pressure$\geqslant$5 mmH_2O). Two senior physicians make a diagnosis based on patients' conditions within the first 48 hours of admission. All participants were divided into an ARDS or non-ARDS groups for further retrospective analysis.

Measurement of serum CC16, Ang-2, sRAGE, HMGB1 and SPD

Blood samples at Day-1 and Day-2 were separately collected from the radial artery within 3 hours and 24 hours of admission to ICU. Blood samples were then centrifuged at 3000 rpm for 10 min and upper serum stored in EP tubes at an ultra-low temperature refrigerator (-80℃) until required for analysis. Serum CC16, Ang-2, sRAGE, and SPD concentrations were determined using ELISA kits (R&D Systems, Minneapolis, USA) and HMGB1 concentration were determined using ELISA kits (Elabscience Biotechnology Co., Ltd, Wuhan, Hubei, China), following the manufacturer's instructions. Each sample was measured in duplicate and assaying was conducted using the ELISA kits. The researchers who performed these analyses were

blinded to group assignment.

Statistical analysis

Data are presented as the means with standard deviations or numbers (proportion) as indicated. Student's t-test was then performed to compare serum concentrations for Ang-2, CC16, sRAGE, SPD, and HMGB1 between the two groups, when distribution was considered to be normal. Conversely, when Guassian distribution was not evident, we adopted Mann-Whitney's U-test. Categorical data were compared using standard Chi-square tests. Correlations between Ang-2, CC16, sRAGE, SPD, HMGB1, PaO_2/FiO_2, PH, WBC, CRP, NT-proBNP, TP, D-Dimer, albumin, and Scr were estimated using Pearson's linear regression coefficients.

Receiver operating characteristic (ROC) curves were utilized to assess the optimal area under the curves (AUC) with corresponding 95% confidence intervals (CI). The optimal cut-off value, sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) were calculated after taking Youden's maximum (i.e., sensitivity+specificity-1). Statistical analysis was performed using R (v3.5.1, R Foundation for Statistical Computing, Vienna, Austria); A p-value <0.05 was considered the threshold for statistical significance. Graphs were created using GraphPad Prism software (v 3.0, GraphPad Software Inc., La Jolla, CA, USA) and MedCalc statistical software (v19.0.7, MedCalc Software Ltd, Ostend,

Belgium). *Corrplot* was used to calculate correlation coefficient and to measure the significance of each correlation.

Results

Participants and demographics

Between 1st March, 2013, and 1st March, 2017, 1,121 ICU patients were initially considered eligible. Please see Fig. 1 for a complete flowchart of the process from screening to analysis. Those discharged within 24 hours (n =253), those beyond the age range (n = 198) and those who did not provide informed consent within the pre-specified 12-hour window (n =78), were excluded. A further 381 potential candidates were excluded due to incomplete blood samples (n =198), incomplete clinical data (n =152) and discharge with unclear diagnosis (n =31). A final cohort of 211 patients was recruited, of whom 37.4% (n=79) had been diagnosed with ARDS within the first day, and 62.6% (n=132) who did not meet the Berlin criteria (Fig.1).

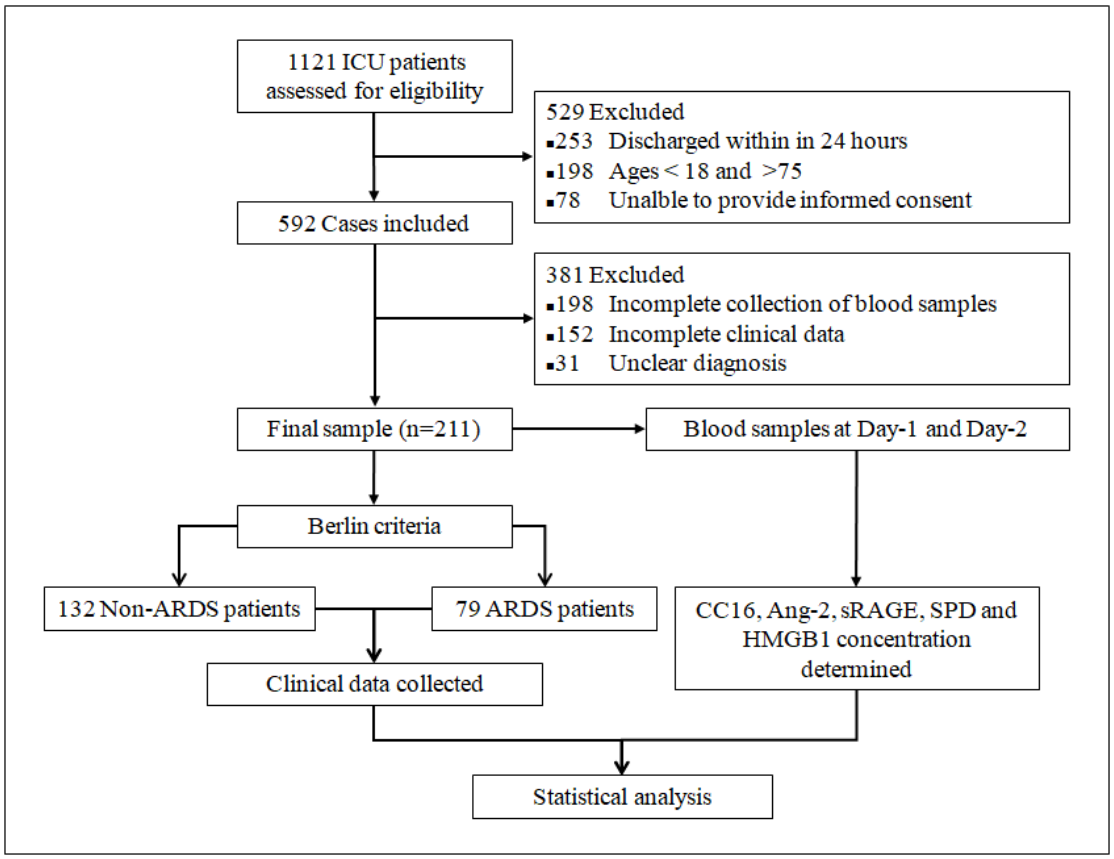

Fig.1 Flowchart of enrolment and study processes

Reasons for exclusion were not mutually exclusive or exhaustive because there may have been a number of reasons for exclusion.

Abbreviations: ICU, intensive care unit; ARDS, acute respiratory distress syndrome; CC16, club cell protein 16; Ang2, angiopoietin 2; sRAGE, soluble receptor for advanced glycation end-products; SPD, surfactant protein D; HMGB1, high mobility group box 1

Demographics and clinical characteristics of two groups are presented in Table 1. The *p* values provided are the result of comparisons between the ARDS and Non-ARDS groups using Student's t test, or Chi-square test. The median age was 54.68 (±18.94) years and 63.3% (50/79) were male in ARDS group. At baseline, patients in the ARDS group had higher CRP levels and lower PaO_2/FiO_2 ratios than patients

in non-ARDS group. Comorbidities were similarly distributed in both groups. Pneumonia had a higher rate in ARDS group although; no other major risk factors were significantly different between the groups. APACHE II scores for the severity of illness, ventilation time, length of ICU stay and overall hospitalization were not significantly different between the two groups. However, 7-day mortality and 28-day mortality were higher in ARDS group.

Table 1. Demographics and clinical characteristics

Variables	ARDS(n=79)	Non-ARDS(n=132)	*P*value
Age, years	54.68±18.94	49.79±17.05	0.058
Male, n (%)	50(63.3%)	81(61.4%)	0.780
Comorbidities			
Hypertension, n (%)	24(30.4%)	41(31.1%)	0.917
Diabetes, n (%)	17(21.6%)	27(20.5%)	0.854
Coronary heart disease, n (%)	11(13.9%)	8(6.1%)	0.053
Chronic lung disease, n (%)	1(1.8%)	4(5.2%)	0.446
Hepatitis B, n (%)	3(3.8%)	3(2.3%)	0.413
Chronic renal disease, n (%)	7(8.9%)	13(9.8%)	0.813
Risk Factors of ARDS			
Pneumonia, n (%)	36(72.0%)	25(34.7%)	0.001*
Inhalation injury, n (%)	3(3.8%)	0(0.0%)	0.024
Pulmonary contusion, n (%)	1(1.3%)	11(8.3%)	0.032
Sepsis, n (%)	19(24.1%)	19(14.4%)	0.077
Cardiogenic shock, n (%)	1(1.3%)	1(0.8%)	0.712
Post-resuscitation, n (%)	8(10.1%)	5(3.8%)	0.064
Post-surgery, n (%)	24(30.4%)	57(43.2%)	0.064
Operative complication, n (%)	20(25.3%)	22(16.7%)	0.128
DIC, n (%)	5(6.3%)	8(6.1%)	0.937
Severe acute pancreatitis, n (%)	5(6.3%)	7(5.3%)	0.755
Poisoning, n (%)	2(2.5%)	3(2.3%)	0.905
Tuberculosis, n (%)	3(3.8%)	3(2.3%)	0.519
Pulmonary embolism, n (%)	1(1.3%)	3(2.3%)	0.604
Vital Signs			
T(°C)	36.99±1.03	36.88±0.98	0.704
HR, per min	110.39±27.88	101.64±27.21	0.026
RR, per min	24.31±8.21	23.18±7.17	0.310

SBP (mmHg)	125.37±27.98	127.25±24.97	0.614
DBP (mmHg)	76.73±17.69	77.57±17.64	0.741
MAP (mmHg)	92.83±19.87	94.07±18.48	0.650
Physiological Variables			
PaO_2/FiO_2 ratio	180.64±99.12	359.73±143.63	0.001*
CRP (mg/L)	121.01±73.99	67.76±63.79	0.001*
PH value	7.34±0.14	7.38±0.12	0.032
Lac (mmol/L)	4.87±8.24	3.02±3.47	0.061
WBC ($\times 10^9$/L)	14.37±8.57	13.94±8.16	0.717
TP(g/L)	57.93±14.20	59.24±12.87	0.493
Alb (g/L)	31.67±24.58	31.95±7.53	0.903
D-Dimer (mg/L)	12.95±4.31	5.53±9.94	0.067
Cr (μmol/L)	164.40±289.79	142.66±197.22	0.520
NT-proBNP (pg/mL)	712.17±924.59	634.86±1055.74	0.595
Evaluation index			
APACHE II score	19.40±6.88	18.51±6.03	0.573
Time of ventilation, days	7.81±7.53	6.56±9.67	0.327
Length of ICU stay, days	10.24±8.32	10.84±11.26	0.377
Length of hospitalization, days	22.50±22.47	29.08±63.68	0.681
7-day mortality, n (%)	18(22.8)	10(7.6)	0.002*
28-day mortality, n (%)	30(38.0)	23(17.4)	0.001*

Data shown as mean with corresponding standard deviations; n (%);

* Statistically significant *p* value.

Abbreviations: DIC, disseminated intravascular coagulation; APACHE II, Acute Physiology and Chronic Health Evaluation II; PaO_2 /FiO_2, partial pressure of arterial oxygen to fraction of inspired oxygen

Validation of a biomarker model for diagnosis of ARDS

Levels of the five plasma biomarkers recorded from the entire cohort are shown in Table 2. Bar graphs were used to compare CC16, Ang-2, sRAGE, HMGB1 and SPD levels between the ARDS and non-ARDS groups. Of the five biomarkers, four significantly differed between groups under univariate analysis at ICU Day 1 and

Day 2, i.e., CC16, Ang-2, sRAGE and HMGB1. See Figure 2 for confirmation.

Table 2. Serum levels of CC16, Ang-2, sRAGE, SPD and HMGB1 in ARDS groups and non-ARDS groups at ICU Day 1 and Day 2

Biomarker	Day 1		Day 2	
	ARDS(n=79)	non-ARDS (n=132)	ARDS(n=79)	non-ARDS(n=132)
CC16 (ng/mL)	50.43±24.05*	29.30±20.06	43.40±27.33*	28.98±21.38
Ang-2(pg/mL)	5597.20±3902.87*	3307.24±2477.35	5405.13±3709.28*	3438.32±2650.91
sRAGE(pg/mL)	2280.20±1946.72*	1371.95±1690.66	2088.90±2364.73*	1400.18±2254.02
HMGB1(pg/mL)	2749.73±2128.15*	1606.92±1525.98	2861.68±2148.54*	1595.75±1498.42
SPD (ng/mL)	25.13±38.39*	15.51±19.35	29.09±41.49	19.92±26.31

* the difference between two groups was statistically significant

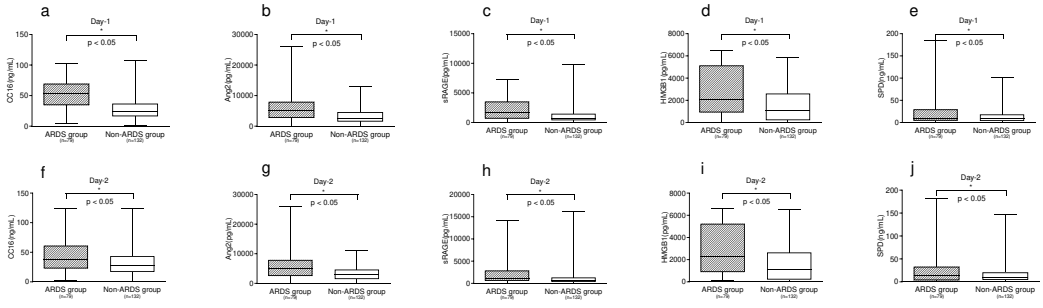

Fig. 2 Bar graph for comparison of CC16, Ang-2, sRAGE, HMGB1 and SPD levels between groups.

The *p* values were derived from using student's t-test or Mann Whitney U test.

A logistic regression model was developed for ARDS diagnosis using all 5 biomarkers, and model performance was assessed using AUCs. As can be seen in Table 3, the AUCs for each the biomarkers were as follows: CC16 0.752 (95% CI 0.680-0.824), Ang-2 0.695 (95% CI 0.620 - 0.770), HMGB1 0.668 (95% CI 0.592-0.744), sRAGE 0.665 (95% CI 0.588- 0.743).

Table 3. ROC analysis of Serum CC16, Ang-2, sRAGE, HMGB1, SPD, PaO_2/FiO_2, CRP at diagnosing ARDS among critical care patients

Values	AUC	S. E	95%CI	Cut-off	Sensitivity	Specificity	NPV	PPV
CC16 (ng/ml)	0.752[†]	0.037	0.680, 0.824	>33.67	77.2	69.7	83.62	60.39
Ang2 (pg/ml)	0.695	0.038	0.620, 0.770	>3943.13	62.0	69.7	75.39	55.04
sRAGE (pg/ml)	0.665	0.040	0.588, 0.743	>1283.83	62.0	71 .2	75.79	65.30
HMGB1 (pg/ml)	0.668	0.039	0.592, 0.744	>823.9	87.3	42.4	84.79	47.56
SPD (ng/ml)	0.537	0.042	0.454, 0.620	>24.48	31.6	83.3	67.05	53.10
PaO_2/FiO_2	0.844[#]	0.028	0.789, 0.898	<296.5	93.7	69.7	94.86	64.92
CRP (mg/ml)	0.701	0.038	0.627,0.776	>88.0	63.5	73.5	77.08	58.91

Diagnostic values were assessed by the receiver operating characteristic curve (ROC). In the univariate logistic regression analyses, the diagnostic value of each factor was studied individually, introducing only one factor at a time into the model.

[†] Among 5 biomarkers, CC16 provided the highest AUC of 0.752 with a sensitivity of 77.2% and a specificity of 69.7% at the optimal cut-off point of 33.67ng/mL in patients with ARDS.

[#] Among PaO_2/FiO_2, CRP and 5 biomarkers, PaO_2/FiO_2 provided the highest AUC of 0.844 with a sensitivity of 93.7% and a specificity of 69.7% at the optimal cut-off point of 296.5 in patients with ARDS.

Abbreviations: AUC, area under the curve; S.E, standard error; CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value

Correlations of biomarkers with traditional parameters and their diagnostic value

As an objective indicator of ARDS, PaO_2/FiO_2 resulted in the largest AUC of

0.844 (95% CI 0.789-0.898), as can be seen in Table 3. In Figure 3a, *corrplot* was used to plot the data series of PaO_2/FiO_2, five biomarkers, laboratory parameters and vital signs. CRP, NT-proBNP, PH value of traditional indicators and five biomarkers all correlated with PaO_2/FiO_2 although, only four (i.e., CC16, Ang-2, HMGB1, sRAGE) and CRP (AUC0.701, 95% CI 0.627-0.776) had high diagnostic values, but no single marker was high as the PaO_2/FiO_2 ratio (please see table 3 for further analysis).

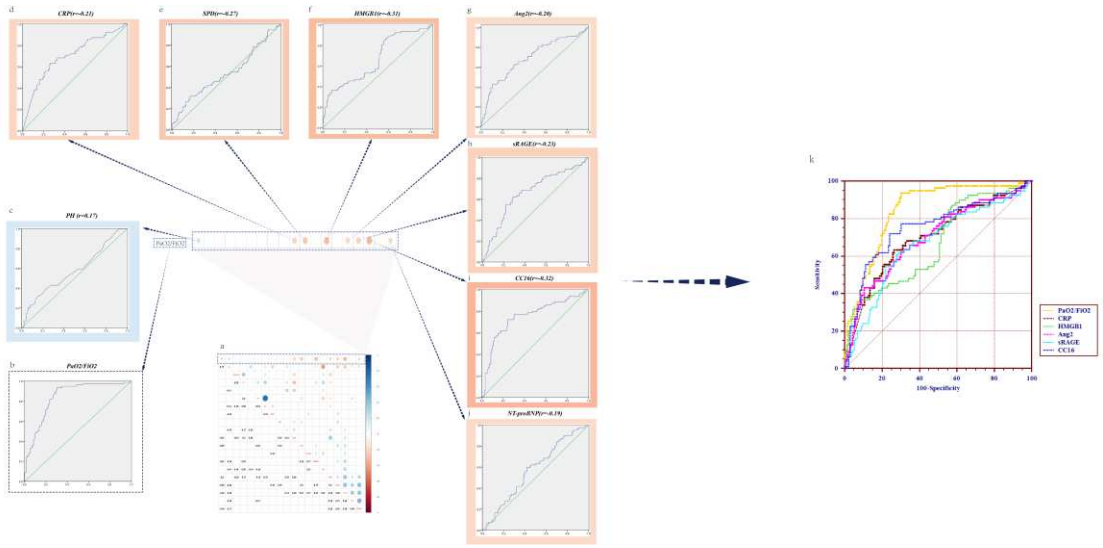

Fig. 3 (a) Correlations between P/F, PH, Albumin, TP, T, SP, DP, WBC, RR, H, CRP, SPD, D-dimer, HMGB1, Lac, Ang-2, sRAGE, CC16, Scr and NT-proBNP were investigated using Pearson's correlation coefficient. The ratio R was calculated by using Pearson's correlation coefficients ranging from -1 to 1. Negative correlations have been highlighted in blue and positive correlations in red. Highlighted correlation coefficients indicate that correlations significantly differ from zero, and an emptyspace indicates the r ratio had no significant difference. (b-j) ROC curves of P/F, PH, CRP, SPD, HMGB1, Ang-2, sRAGE, CC16 and NT-proBNP in diagnosing ICU patients with ARDS. The Pearson's correlation between P/F and other indicators were referred to the r value above. A negative value of r indicated a negative correlation, or vice versa. (k) ROC

curves analysis showed the AUC of P/F, CRP, HMGB1, Ang-2, sRAGE and CC16, respectively. A logistic regression model was fit for diagnosis performance of ARDS using P/F, CRP and four biomarkers; the model performance was assessed by the AUC of the ROC

Abbreviations: P/F, PaO_2/FiO_2; TP, serum total protein; T, temperature; SP, systolic blood pressure; DP, diastolic blood pressure; WBC, white blood cell count; RR, respiratory rate; H, heart rate; CRP, c-reactive protein; Lac, lactic acid; Scr, serum creatinine; CC16, club cell protein 16; Ang-2, angiopoietin 2; sRAGE, soluble receptor for advanced glycation end-products; HMGB1, high-mobility group box 1 protein; SPD, surfactant Protein D; NT-proBNP, the N-terminal of the prohormone brain natriuretic peptide; ICU, intensive care unit; ARDS, acute respiratory distress syndrome; AUC, area under the curve; ROC, receiver operator characteristic.

Improvement of ROC value for the diagnosis of ARDS

By translating from a binary logistic model, it was possible to build a model using various biomarker combinations (Table 4). As a result, the AUC increased from 0.647 (95% CI 0.568-0.726) to 0.800 (95% CI 0.737-0.863). With two biomarkers i.e., CC16+Ang-2 providing the highest AUC of 0.787 with a sensitivity of 73.4% and a specificity of 77.3% in patients with ARDS. However, higher AUCs of 0.796 were provided in both CC16+Ang-2+HMGB1 and with the CC16+Ang-2+HMGB1+sRAGE combination. When combining five biomarkers, we observed the highest AUC value of 0.800 with a slightly lower sensitivity of 70.9% but a specificity of 79.5%.

Table 4. ROC analysis of combining CC16, Ang-2, sRAGE, HMGB1 and SPD at diagnosing ARDS with different combination methods among critical care patients

Value	AUC	SE	95%CI	Sensitivity	Specificity	NPV	PPV
CC16+Ang-2	0.787*	0.034	0.721,0.854	73.4	77.3	82.92	65.92
CC16+sRAGE	0.754	0.036	0.682,0.825	73.4	74.2	82.33	62.99
CC16+HMGB1	0.765	0.035	0.696,0.833	67.1	77.3	79.69	63.88
CC16+SPD	0.761	0.035	0.692,0.830	74.7	72.0	82.62	61.48
Ang-2+sRAGE	0.723	0.036	0.653,0.794	81.0	50.8	81.71	49.62
Ang-2+HMGB1	0.742	0.036	0.670,0.813	67.1	72.0	78.52	58.91
Ang-2+SPD	0.724	0.037	0.650,0.797	63.3	75.8	77.53	61.01
sRAGE+HMGB1	0.696	0.037	0.623,0.769	57.0	73.5	74.06	56.27
sRAGE+SPD	0.647	0.04	0.568,0.726	63.3	65.9	75.00	52.62
HMGB1+SPD	0.670	0.039	0.593,0.747	43.3	87.1	71.96	66.76
CC16+Ang-2+sRAGE	0.787	0.034	0.721,0.854	74.7	75.8	83.35	64.87
CC16+Ang-2+HMGB1	0.796†	0.033	0.732,0.860	74.7	72.0	82.62	61.48
CC16+Ang-2+SPD	0.794	0.033	0.730,0.858	78.5	68.9	84.26	60.16
Ang-2+sRAGE+HMGB1	0.754	0.035	0.685,0.823	74.7	66.7	81.49	57.31
Ang-2+sRAGE+SPD	0.740	0.036	0.670,0.809	73.4	65.9	80.54	56.29
sRAGE+HMGB1+SPD	0.695	0.038	0.620,0.769	58.2	77.3	75.55	60.54
CC16+Ang-2+ HMGB1+sRAGE	0.796&	0.033	0.732,0.860	62.0	85.6	79.00	72.04
CC16+Ang-2+ sRAGE+SPD	0.794	0.033	0.730, 0.858	75.9	72.5	83.40	62.28
Ang-2+sRAGE+ HMGB1+SPD	0.764	0.034	0.696, 0.831	79.7	60.6	83.30	54.76
CC16+Ang-2+sRAGE+ HMGB1+SPD	0.800#	0.032	0.737, 0.863	70.9	79.5	82.03	67.42

* CC16+Ang2 showed the highest AUC of 0.787 with a sensitivity of 73.4% and a specificity of 77.3% using two joint biomarkers.

† CC16+Ang2+HMGB1 showed the highest AUC of 0.796 with a sensitivity of 74.7% and a specificity of 72% using three biomarkers combination.

& CC16+Ang2+sRAGE+HMGB1+SPD showed the highest AUC of 0.796 with a sensitivity of 62% and a specificity of 85.6% using four biomarkers combination.

[#] Combination of all 5 biomarkers showed the AUC of 0.800 with a sensitivity of 70.9% and a specificity of 79.5%.

The PaO_2/FiO_2 ratio is the traditional indicator for the diagnosis of ARDS, whereas CRP is an index that is relatively easy to obtain. This evidence also confirms that CRP has a significant influence in the diagnosis of ARDS (Table 3), despite negatively correlating with PaO_2/FiO_2 (Figure 3). We tried to combine PaO_2/FiO_2, CRP, and panels of biomarkers (Table 5) which garnered higher diagnostic significance. The PaO_2/FiO_2+CRP+Ang-2+CC16+HMGB1 combinations yielded an AUC of 0.910 (95% CI 0.863-0.945), whereas the PaO_2/FiO_2+CRP+Ang-2+CC16+HMGB1+sRAGE+SPD had the highest an AUC of 0.911(95% CI 0.864-0.946).

Table 5. ROC analysis of combining CC16, Ang-2, sRAGE, HMGB1 and SPD at diagnosing ARDS with different combination methods among critical care patients

Value	AUC	SE	95%CI	Sensitivity	Specificity	NPV	PPV
PaO_2/FiO_2	0.844	0.028	0.789, 0.898	93.7	69.7	69.7	94.8
CRP (mg/ml)	0.701	0.038	0.627,0.776	63.5	73.5	77.08	58.91
CC16+Ang-2+sRAGE +HMGB1+SPD	0.800	0.032	0.737, 0.863	70.9	79.5	67.4	82.03
PaO_2/FiO_2+CRP+Ang-2 +CC16+HMGB1	0.910[*]	0.0207	0.863, 0.945	87.3	84.1	76.66	91.71
PaO_2/FiO_2+CRP+Ang-2 +CC16+HMGB1 +sRAGE+SPD	0.911[#]	0.0206	0.864, 0.946	86.1	83.3	75.5	90.9

[#] The highest AUC was 0.911 with a sensitivity of 86.1% and a specificity of 83.3% when combined all five biomarkers with PaO_2/FiO_2 and CRP.

* The combination of PaO_2/FiO_2, CRP, Ang-2, CC16 and HMGB1 showed an AUC of 0.910 with a sensitivity of 87.3% and a specificity of 84.1%.

Relationship of biomarkers with outcomes

Across the entire ICU sample, higher serum levels of CC16 (Fig.4a to Fig.4d) and Ang-2 (Fig.4e to Fig.4h) were found in non-survivors. Consistently, CC16 at Day-1(r=0.189, Fig.5a), Ang-2 at Day-1 (r=0.158, Fig. 5b) and Day-2 (r=0.143, Fig. 5c) showed positive correlation with MV duration in the entire ICU patients. However, only CC16 on day 1 and day 2 showed higher mortality and statistical significance in 7-day and 28-day mortality in ARDS group. See Figure 4i to 4l for further details.

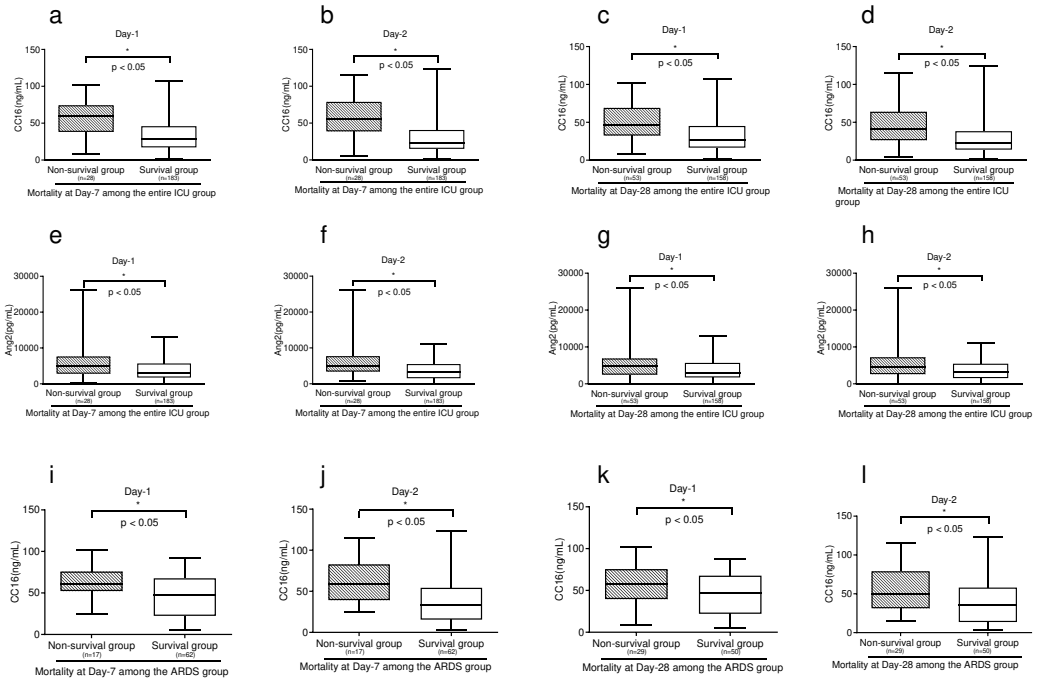

Fig. 4(**a-h**) Comparative bar charts of CC16 and Ang-2 between non-survival groups and survival groups across entire cohort. (**i-j**) Comparative bar charts of CC16 between non-survival groups

and survival groups in ARDS only patients.

p values were generated using Student's T-test.

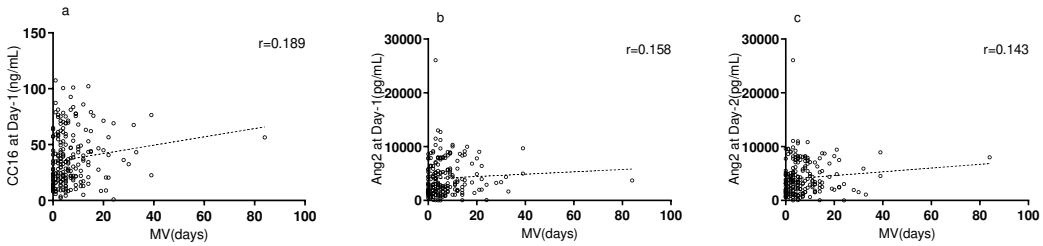

Fig.5 Scatter plots of a) the relationship between CC16 levels at Day-1 and duration of mechanical ventilation; b) the relationship between Ang-2 levels at Day-1 and duration of mechanical ventilation; c) the relationship between Ang-2 levels at Day-2 and duration of mechanical ventilation.

r values were accounted from using Pearson correlation coefficient.

Abbreviations: MV, mechanical ventilation duration; r, correlation coefficient

Discussion

The primary objective was to consider whether intercalating series of biomarkers into traditional ARDS diagnostics would increase diagnostic accuracy for critically ill patients. We initially sought to confirm whether biomarkers such as CC16, Ang-2, HMGB1 and sRAGE would prove useful for patients with ARDS. We then began to analyze potential correlations between theafore mentioned biomarkers and traditional indicators. We found that these biomarkers can supplement routine

indicator such as the PaO_2/FiO_2 ratio and could prove useful in clinical practice. We also found that CC16, Ang-2 and HMGB1 detection when combined with PaO_2/FiO_2 and CRP can improve the diagnostic accuracy of traditional methods. Our evidence also suggests that CC16 and Ang-2 may prove useful for assessing lung function recovery, which positively correlates with mechanical ventilation time. Further, we speculate that CC16 might be useful as a prognostic indicator for ARDS patients who have higher comparative 7-day and 28-day mortality.

The relatively new Berlin definition made a number of improvements over previous definitions of acute lung injury as opposed to acute respiratory distress syndrome. As such, the newer Berlin definition removing acute lung injury and other ancillary indicators, categorizing ARDS into three levels of severity, which actually lowers the diagnostic criteria for ARDS. People have reason to doubt whether a large number of patients without diffuse alveolar damage pathological changes will be diagnosed as ARDS according to the Berlin definition, thereby increasing the incidence of ARDS and the cost of treatment. While according to the LUNG SAFE study, this increase ARDS prevalence which actually appears necessary because only 51.3–78.5% of ARDS cases are identified in clinical practice[8]. As mentioned within the introduction, one of the greatest problems is underdiagnose which could mean there is a lack of clinical awareness. Although, the main reason appears to be the lack of simple, accurate diagnostic tests, which leads to an over-reliance on consensus definitions. This necessitates the development of more objective measures for ARDS to ensure patients are treated more appropriately and in a timely manner.

Researchers have suggested[9] it necessary to establish a set of biomarkers in order to further develop specific medicaments and tailor interventions to individual needs. Evidence also demonstrates that clinically established biomarkers, such as white blood cell count, blood creatinine, inflammation indicators, etc., have less diagnostic value than plasma protein-based biomarkers. Therefore, plasma protein biomarkers are more likely to provide insight into the pathophysiology of ARDS and indeed into the distinct levels of severity as described in the newest Berlin definition. Fortunately, number of biomarkers identified through different pathophysiological pathways of inflammation, lung epithelial and endothelial injury, have been studied and have been found to be relevant in the diagnosis of ARDS[1]. However, ARDS is not a single disease, but rather a syndrome with complex etiology, pathogenesis, pathophysiology and therefore testing may lack specificity.

There are however a wide array of biomarkers which show promise and some studies have investigated the potential of combining several biomarkers into ARDS diagnostics. For example, Fremont et al.[6] found seven biomarkers including RAGE, PCPIII, BNP, Ang-2, IL-10, TNF-α and IL-8 had high diagnostic accuracy for ARDS diagnosis. Likewise, Ware et al.[7] also tested a panel of biomarkers including vWF, SPD, TNFR, IL-6, IL-8, ICAM-1, PROTC and PAI-1, and found that combinations enhanced prognostic values compared to any single biomarker. More recently, Ware et al.[10] studied a different set of biomarkers which included SPD, RAGE, IL-6, IL-8 and CC16, and found that this had a higher AUC for ARDS

diagnosis. Although, one could argue due to sheer number of plasma protein-based biomarkers, we need a more systematic process for inclusion and exclusion based upon previous evidence. Additionally, there is evidence which suggests there are genetics in terms of thrombomodulin and endothelial protein C receptors which are associated with ARDS mortality[11]. This makes it necessary to investigate biomarkers across different ehicities because they are associated with dyregulated coagulation and therefore ARDS outcomes.

Through previous investigation[12], we found that CC16 could be applied as an efficacious diagnostic biomarker for ARDS. In the present study, we attempted to add to this knowledge base by adding other biomarkers which have proven promising elsewhere. The hope was to extend the detection range and avoid repeat determinations for those with dissimilar pathophysiologies. As such, CC-16, sRAGE and SPD, which are associated with epithelial lung damage, were derived on an individual basis from club cells, type I and II alveolar epithelial cells. Likewise, Ang-2 wasderived from individual endothelial cells and represent endothelial lung damage. HMGB1 were recorded from macrophages which relate to inflammatory changes. When the immune system is infected, inflammatory biomarkers change, including HMGB1[13], CRP and PCT change. Similarly, lung biomarkers including CC16[14], Ang-2[15], sRAGE[16] and SPD[17] which are generally low in healthy individuals, are found to elevate in patients with a severely compromised blood gas barrier. There is evidence throughmouse modelling studies, that this complement of biomarkers synchronously increase[18]. However, this is the first study to observe this in the development of

ARDS. We also found that the AUC values increased with varying degrees after adding various biomarkers. This is consistent with the results of the study by Ware et al[6,10], although the complement of biomarkers involved in this study is not the same.

At present, the PaO_2/FiO_2 ratio is the gold standard in ARDS diagnostics; however, the diagnostic performance is not ideal andit has limitation when considering other cardiac factors, as shown by the negative correlation with NT-proBNP. Consequently, we theorized that the observed improvement was related to a reduction in the influence of cardiogenic factors. In the past, biomarker research in this field has predominantly focused on how to distinguish severe ARDS patients from cardiogenic pulmonary edema, with no biomarker being identified as capable of replacing the PaO_2/FiO_2 ratio in terms of sensitivity. We found that CRP not only has diagnostic value, but also negative correlates with PaO_2/FiO_2. Therefore, we iteratively tested various combinations of biomarkers with CRP and PaO_2/FiO_2, and found little difference between three and five in diagnostic sensitivity and specificity. Although of course, this means we are able to recommend the Ang-2, CC16, HMGB1, CRP and PaO_2/FiO_2 for clinical practice in order to save medical resources.

We also found that there are physiological interrelations between some of the investigated biomarkers and traditional indicators. Evidence from our previous research[19] and other[20,21] suggests that CC16 positively correlates with Scr, Lac and NT-proBNP. This might related to the anti-inflammatory function, immune system

activation, and lactate metabolism pathway related to prephosphorylation. Likewise, the relationship between Ang-2 and albumin, and CRP, is partly consistent with previous results[22], most of which were obtained in clinical samples. It was found that the pro-inflammatory properties may affect the leukocyte adhesion mechanism in inflammation. The association between sRAGE and NT-proBNP we observed might be also affected by cardiac remodeling and anti-inflammatory effects. Both BNP and NT-pro-BNP levels elevate in patients with chronic renal insufficiency, which are closely related to left ventricular hypertrophy and abnormal systolic function. Therefore, both have the potential to predict heart failure and mortality. This hypothesis seems to be supported by the results obtained in other types of patients[23-26], where elevated levels of sRAGE were considered a sign of worsening cardiac function and mortality.

From a prognostic perspective, the association between serum Ang-2 and mortality is consistent with previous findings in patients with sepsis[27,28]. Consistently, multivariate regression analysis has shown that non-survivors are more likely to have biological dysfunction when admitted, which may help integrate biomarker-based predictive models to support clinicians assessing critically ill patients before confirming diagnosis. SPD appears to have diagnostic value for ARDS and shows superiority in prognostic value of interstitial lung disease[29]. However, previous research has found that the level of SPD is not related to lung contusion associated with ARDS[30]. In that study, the researchers also found that serum CC16 level is related to the volume of lung contusion and may not be affected by the overall

severity of injury, age, gender or ventilation. This supports our findings that SPD should not be used alone to diagnose and evaluate the prognosis of ARDS. However, we found that the CC16 levels on Day-1 and Day-2 correlated with 7-day and 28-day mortality. Based on the pathophysiology of ARDS, prognosis appears to be reflected in CC16 levels which we suggest related to the blood gas barrier repair process. In our previous study[31], we discovered the prognostic value of CC16 for non-invasive ventilation in critically ill patients. The findings of this study elaborate on this; suggesting that serum CC16 and Ang-2 in critically ill patients are closely related to the duration of mechanical ventilation. However, this is a tentative notion because subsequent lung infections such as hospital-acquired pneumonia, or ventilator-associated pneumonia, may have influenced our findings.

Strength and limitations

To the best of our knowledge, this is one of the few studies which combine plasma-based biomarkers and traditional indicators to evaluate ARDS in more than 200 patients[32,33]. We also think by recruiting various critically ill patients, our research can be repeated at the local emergency and critical care center. The recommended combination could help clinicians identify ARDS in critically ill people although the findings will need to be externally validated across a larger Chinese population.

The patients enrolled in this study were also pretreated, prior to receiving ICU

treatment. This of course creates, inconsistencies across an otherwise genetically population. Future research might try to develop a nomogram for this population although effort should be taken to reduce selection bias, where possible. There are a number of prospective advantages in intercalating plasma-based biomarkers, yet remain difficult to adopt these in clinical practice without commercially produced instanteous testings. Given this, we were only able to perform testing at the early stage of patient admission, not throughout the entire period of hospitalization. However, we hope this evidence adds to a necessary, growing body of evidence and will help those considering the design of large-scale, prospective clinical studies in this field.

Conclusion

We investigated the detection of serum CC16, Ang-2, sRAGE, SPD and HMGB1 to identify patients with ARDS in those considered to be critically ill. Using an iterative approach to developing a combined detection method, we found that Ang-2+CC16+HMGB1+CRP +PaO_2/FiO_2 enhances ARDS diagnostics substantially. However, Ang-2+CC16+HMGB1+CRP +PaO_2/FiO_2 *plus* sRAGE+SPD was superior. We also found that CC16 and Ang-2 might effect mortality and mechanical ventilation time. Further research into the underlying mechanisms is currently underway, which we hope will improve our understanding of ARDS physiology. We would also suggest there may be a need to develop a predictive nomogram as well as for health

economics research to consider the trade-offs between the more basic model recommended here and the more sophisticated model, which is marginally more accurate.

Declarations

Ethics approval and consent to participate

Research involving human participants, human material, or human data have been performed in accordance with the Declaration of Helsinki. All methods were carried out in accordance with relevant guidelines and regulations. The Institutional Human Ethics Committee of affiliated Baoan Hospital of Shenzhen, Southern Medical University approved the study protocols employed in this observational study. Written informed consent was obtained from each subject or their legal guardians.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author in response to reasonable requests.

Competing interests

The authors declare that they have no competing interests.

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All of funders equally contribute to this investigation.

Authors' contributions

Conceived and designed the study: XF, JL. Study design Assessment: WZ, YX and WJ. Data acquisition: WT, JW, SZ, YL and YS. Blood collection and handling: XF, XZ and JY. Analyzed the data: JL, XF and SS. Wrote the paper: XF and JL. Provided critical appraisals of the study: XJ, QD, ZH and LW. Revised the manuscript: SS, WZ and YX. All authors read and approved the final manuscript.

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Figures

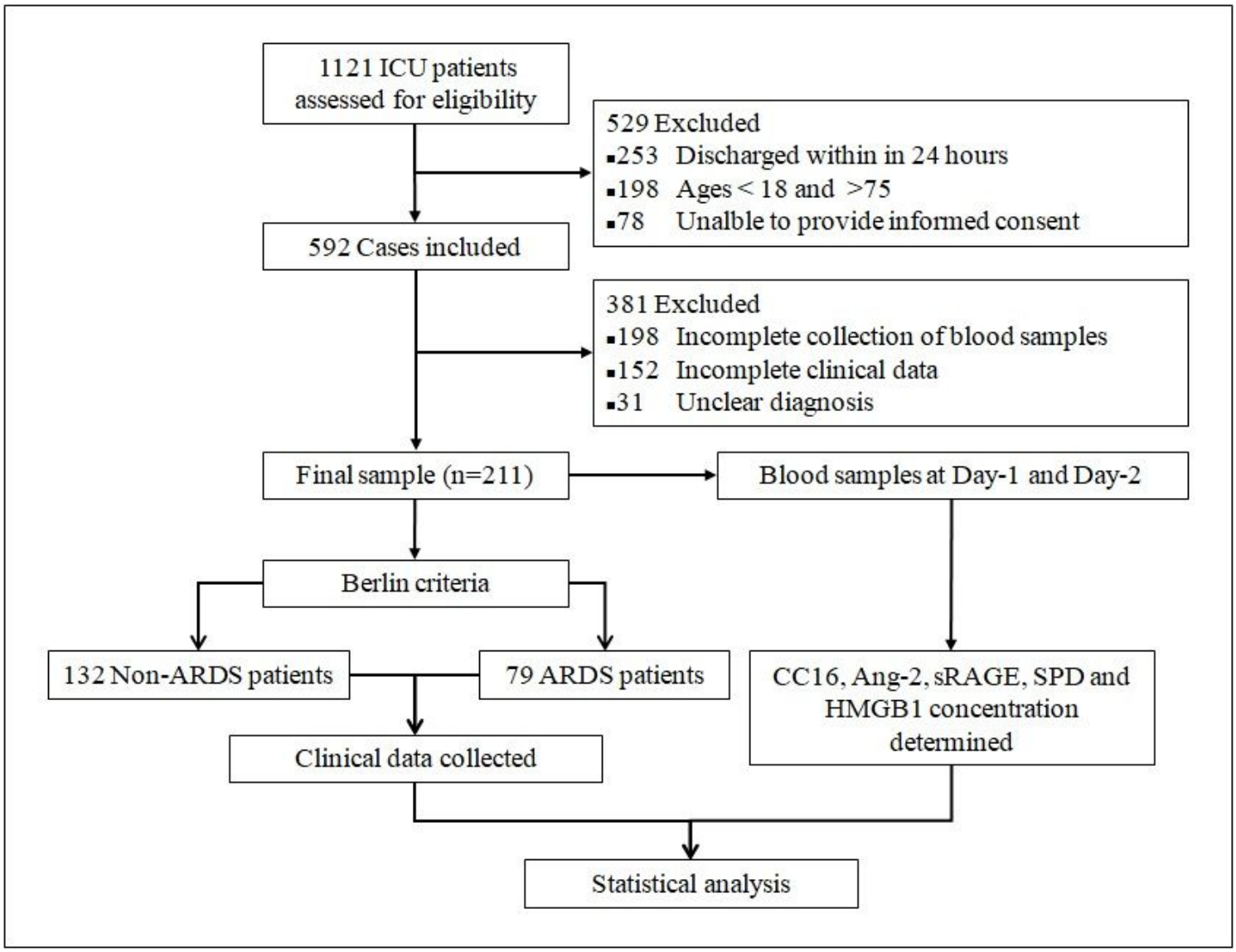

Figure 1

Flowchart of enrolment and study processes Reasons for exclusion were not mutually exclusive or exhaustive because there may have been a number of reasons for exclusion. Abbreviations: ICU, intensive care unit; ARDS, acute respiratory distress syndrome; CC16, club cell protein 16; Ang2, angiopoietin 2; sRAGE, soluble receptor for advanced glycation end-products; SPD, surfactant protein D; HMGB1, high mobility group box 1

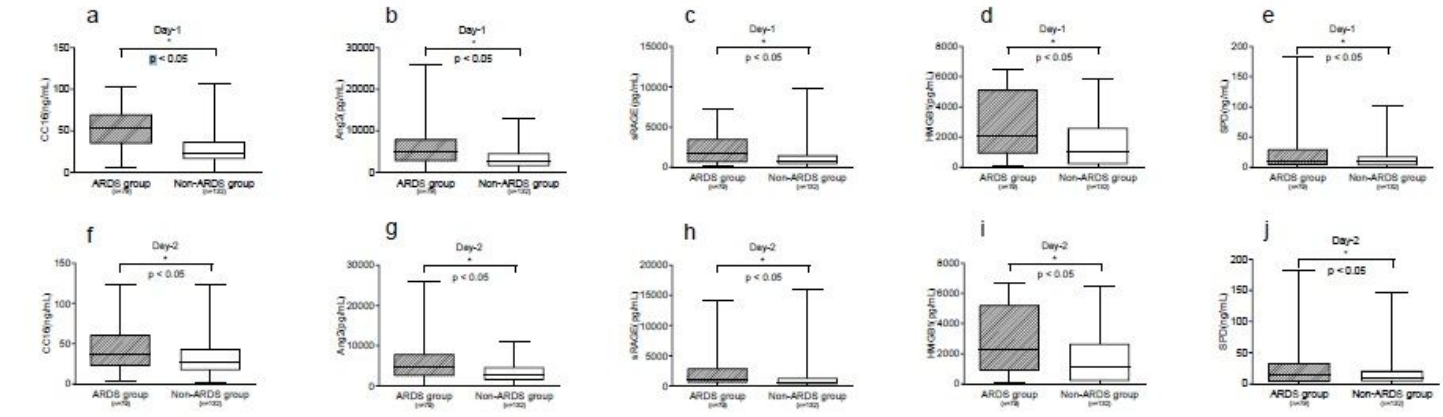

Figure 2

Bar graph for comparison of CC16, Ang-2, sRAGE, HMGB1 and SPD levels between groups. The p values were derived from using student's t-test or Mann Whitney U test.

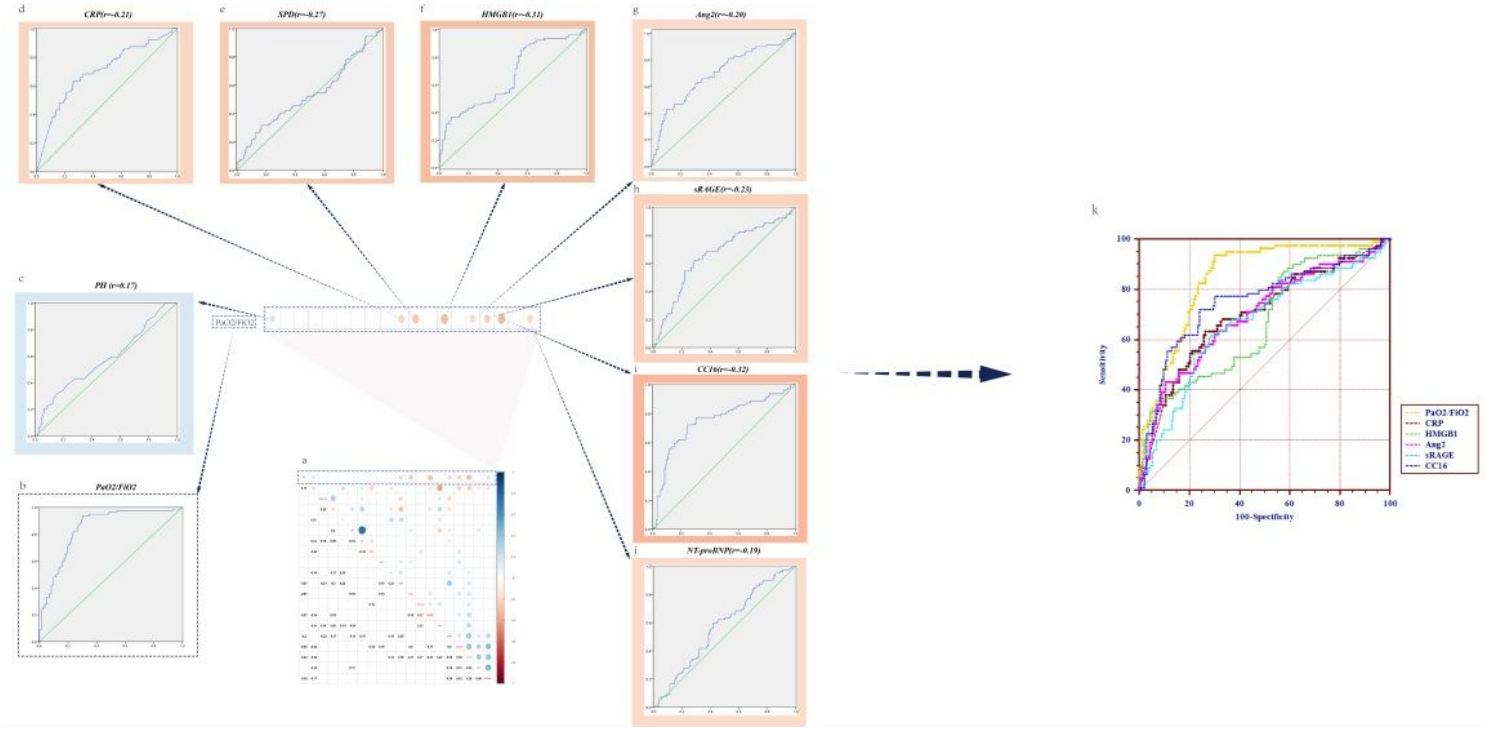

Figure 3

(a) Correlations between P/F, PH, Albumin, TP, T, SP, DP, WBC, RR, H, CRP, SPD, D-dimer, HMGB1, Lac, Ang-2, sRAGE, CC16, Scr and NT-proBNP were investigated using Pearson's correlation coefficient. The ratio R was calculated by using Pearson's correlation coefficients ranging from -1 to 1. Negative correlations have been highlighted in blue and positive correlations in red. Highlighted correlation coefficients indicate that correlations significantly differ from zero, and an emptyspace indicates the r ratio had no significant difference. (b-j) ROC curves of P/F, PH, CRP, SPD, HMGB1, Ang-2, sRAGE, CC16 and NT-proBNP in diagnosing ICU patients with ARDS. The Pearson's correlation between P/F and other indicators were

referred to the r value above. A negative value of r indicated a negative correlation, or vice versa. (k) ROC curves analysis showed the AUC of P/F, CRP, HMGB1, Ang-2, sRAGE and CC16, respectively. A logistic regression model was fit for diagnosis performance of ARDS using P/F, CRP and four biomarkers; the model performance was assessed by the AUC of the ROC. Abbreviations: P/F, PaO2/FiO2; TP, serum total protein; T, temperature; SP, systolic blood pressure; DP, diastolic blood pressure; WBC, white blood cell count; RR, respiratory rate; H, heart rate; CRP, c-reactive protein; Lac, lactic acid; Scr, serum creatinine; CC16, club cell protein 16; Ang-2, angiopoietin 2; sRAGE, soluble receptor for advanced glycation end-products; HMGB1, high-mobility group box 1 protein; SPD, surfactant Protein D; NT-proBNP, the N-terminal of the prohormone brain natriuretic peptide; ICU, intensive care unit; ARDS, acute respiratory distress syndrome; AUC, area under the curve; ROC, receiver operator characteristic.

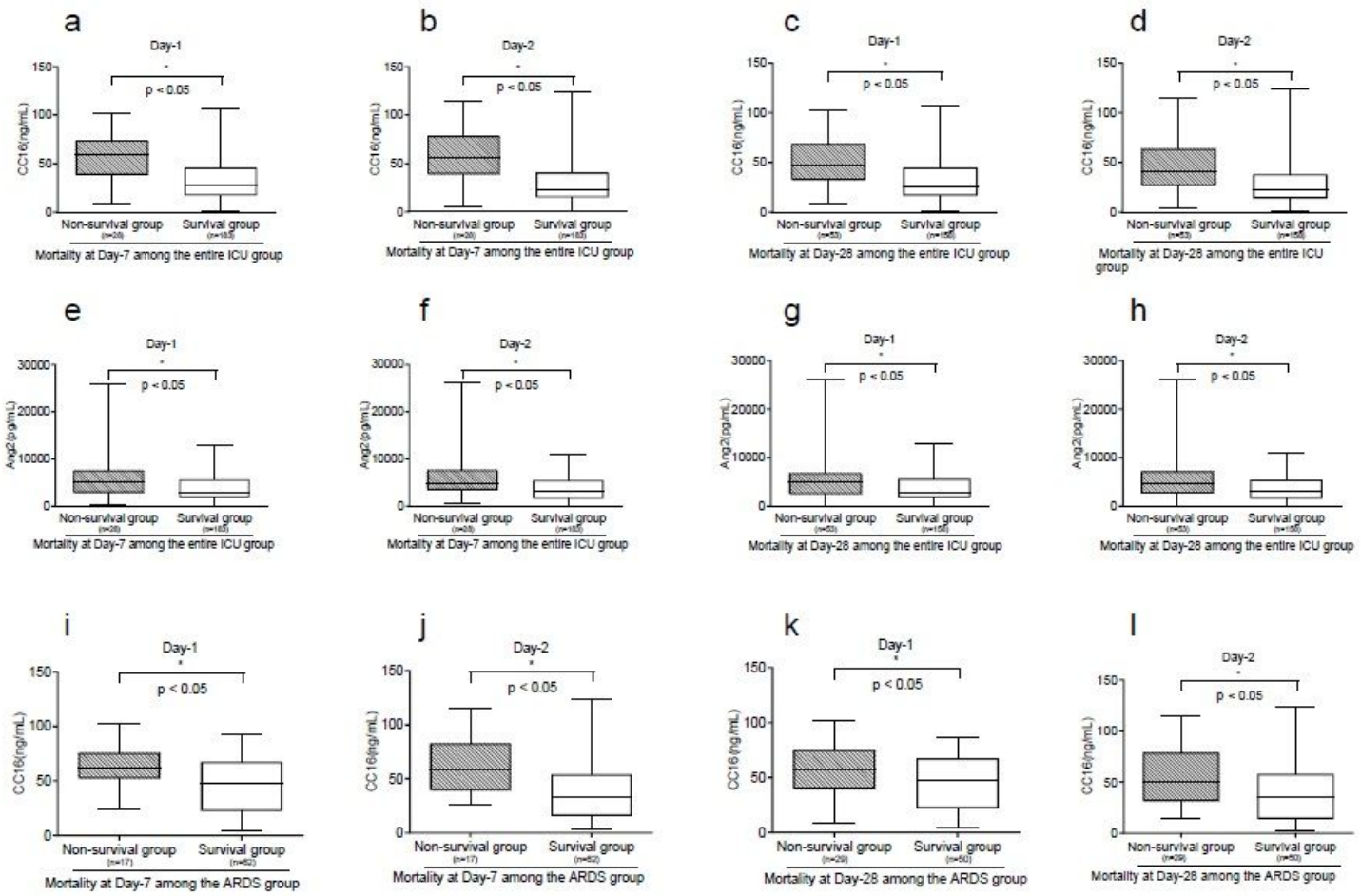

Figure 4

(a-h) Comparative bar charts of CC16 and Ang-2 between non-survival groups and survival groups across entire cohort. (i-j) Comparative bar charts of CC16 between non-survival groups and survival groups in ARDS only patients. p values were generated using Student's T-test.

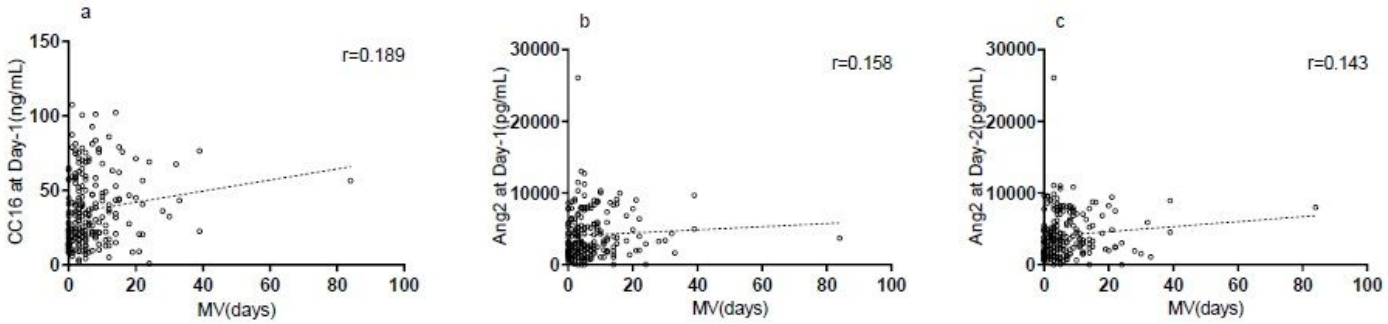

Figure 5

Scatter plots of a) the relationship between CC16 levels at Day-1 and duration of mechanical ventilation; b) the relationship between Ang-2 levels at Day-1 and duration of mechanical ventilation; c) the relationship between Ang-2 levels at Day-2 and duration of mechanical ventilation. r values were accounted from using Pearson correlation coefficient. Abbreviations: MV, mechanical ventilation duration; r, correlation coefficient

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AbbreviationList.docx