Research Square

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Circulating rather than alveolar extracellular DNA levels predict outcomes in influenza: a cohort study

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Abstract

Background: We have reported that high levels of circulating neutrophil extracellular traps (NETs) are associated with a poor prognosis in patients with severe influenza A infection. It remains unclear whether NETs in the bronchoalveolar lavage fluid (BALF) are a superior biomarker for predicting clinical outcomes in influenza.

Methods: One hundred eighteen patients who were diagnosed with H1N1 influenza in 2017-2018 were recruited. NETs were assessed in plasma and BALF samples by quantifying cell-free DNA (cfDNA) and protein-DNA complexes. Predictions of severe illness and 60-day mortality were analyzed with receiver operating characteristic curves.

Results: The NET levels were significantly elevated in the BALF and contributed to the pathological injury of lungs, yet it was not associated with disease severity or mortality in patients severely infected with H1N1. Plasma NET levels were significantly increased in the patients with severe influenza and positively correlated with the oxygen index and sequential organ failure assessment scores. High levels of plasma cfDNA (> 286.6 ng/ml) or histone-bound DNA (> 9.4 ng/ml) on the day of admission predicted severe influenza, and even higher levels of cfDNA (> 306.3 ng/ml) or histone-bound DNA (> 23.1 ng/ml) predicted fatal outcomes in severely ill patients.

Conclusions: Alveolar NETs contribute to lung damage but do not correlate with outcomes in patients with severe H1N1 infection. Plasma cfDNA and histone-bound DNA represent early predictive biomarkers for the prognosis of influenza.

Introduction

Seasonal influenza is an acute respiratory infection caused by influenza viruses that spread worldwide. Although most infected patients recover from the high fever and other symptoms of influenza infection without medical intervention, a proportion of individuals develop acute respiratory distress syndrome and eventually progress to multiple organ dysfunction syndrome (MODS) and even death[1–3]. Early antiviral treatment may improve patient outcomes; however, mortality remains high in patients with severe disease[4–7]. According to the World Health Organization, annual epidemics of influenza are estimated to cause 3 to 5 million cases of severe illness worldwide and 290,000 to 650,000 deaths[8].

Neutrophils, the most abundant circulating immune cell type in humans, have been recognized as a double-edge sword in the immune response against influenza virus infection[9–11]. In response to influenza virus infection, large amounts of neutrophils are released from the bone marrow into the peripheral blood and then migrate to the lungs. Neutrophils are capable of reducing the viral load and guiding antiviral adaptive immunity in the early phase (within days) of infection[12, 13]. However, these cells may also play detrimental roles in tissues and organs by amplifying immune reactions. Published studies in human and animal models have revealed that atypical or sustained activation of neutrophils contributes significantly to influenza-related mortality[14–16].

Neutrophils cause inflammatory damage by generating reactive oxygen species and proteolytic enzymes or releasing neutrophil extracellular traps (NETs)[17–19]. Recently, NETs have drawn considerable attention in the field of neutrophil-mediated tissue damage. A NET is a meshwork-like DNA structure released by activated neutrophils that contains chromatin complexes of cell-free DNA (cfDNA) and histones decorated with granule proteins such as myeloperoxidase (MPO), neutrophil elastase (NE) and lactotransferrin (LTF)[20–22]. In a number of lung diseases, excessive formation and poor degradation of NETs have been demonstrated to be associated with exacerbated tissue damage[19, 23, 24]. In 2018, we reported a correlation between high levels of NETs and a poor prognosis for severe influenza A infection in two independent cohorts of patients with H7N9 or H1N1 influenza by measuring two surrogate markers, cfDNA and myeloperoxidase-DNA complexes[25]. Our study is supported by a recent large-scale high-throughput analysis, which reported that many NET-related genes were strongly associated with the severity of influenza infection[26]. In addition, a randomized, controlled trial also identified NETs as a marker for clinical instability, prolonged length of hospital stay and mortality from community-acquired pneumonia, suggesting that NETs are a possible target for adjunct treatments for pneumonia[27].

Since the lungs are the primary target organ of influenza viruses, NETs in the bronchoalveolar lavage fluid (BALF) have been speculated to be a better marker than circulating NETs for evaluating disease severity and predicting outcomes in patients with influenza. Although large amounts of NETs in the BALF and lung tissue have been detected in H1N1-infected mice[25, 28], direct evidence for the formation of NETs in the lungs or lower airways of patients with influenza is still lacking. Here, we evaluated clinical samples from H1N1-infected patients (n = 118) collected during the 2017–2018 season to address this question.

Materials And Methods

Study population

From December 2017 to March 2018, 118 patients with H1N1 virus infection and 40 healthy donors (HCs), were recruited at Beijing Ditan Hospital, Capital Medical University and China-Japan Friendship Hospital, China. For validating the predictive analysis, we adopted a cohort of 66 H1N1 influenza patients which was collected from November 2009 to January 2010 from Beijing Ditan Hospital and had been described in the previous study[25]. This study was approved by the local ethics committee. Written informed consent was obtained from all the individuals involved in the study.

All patients were confirmed to be positive for H1N1 infection by real-time PCR analysis of nasopharyngeal swabs. According to the diagnostic criteria for severe influenza described as previous reports[25, 29], 30 patients were diagnosed with severe illness, and 27 of them underwent bronchoalveolar lavage within 7 days post admission to ICU. The healthy control BALF were collected the unilateral healthy lung from 12 patients with benign lung nodules or unilateral pulmonary hemorrhage. A fatal outcome among these patients was defined as death from any cause within 60 days of hospitalization for influenza.

Sample collection, Cytokine Detection and the Permeability Assay

Bronchoalveolar lavage was performed during bronchoscopy. The detailed procedure of sample collection, cytokine detection and permeability assay were described in Additional file 1.

Evaluation of NET formation by measuring cfDNA and histone-DNA, MPO-DNA and LTF-DNA complexes

CfDNA in plasma and BALF samples was quantified using a Quant-iT PicoGreen dsDNA assay (Invitrogen, Darmstadt, Germany) as previously[30]. For quantification of protein-DNA complexes, a capture antibody against histone (Cell Death ELISA^{PLUS} kit, Roche Diagnostics, Mannheim, Germany), MPO (MilliporeSigma, Burlington, MA, USA) or LTF (Abcam, Cambridge, MA, USA) was used to coat a 96-well flat-bottom plate at a 1:200 dilution, and the amount of protein-bound DNA was quantified using the Quant-iT^{TM} PicoGreen® dsDNA assay as above.

Statistical analysis

Data were expressed as the mean ± standard deviation for normally distributed data, or the median and interquartile range for nonnormally distributed data. All statistical analyses were calculated using GraphPad Prism 6.01 software (San Diego, CA, USA). Comparisons among three groups were performed by a one-way Kruskal-Wallis test or one-way ANOVA followed by Bonferroni's test. Correlation analysis was performed by calculating Spearman correlation coefficient. Counting data comparisons were analyzed by the Chi-square (χ^2) test. Optimum cut-off values of receiver operating characteristic (ROC) curves were defined based on their maximum Youden index (sensitivity+specificity−1). The predictive accuracy was shown along with the associated 95% confidence intervals (CIs).

Results

Plasma NETs are associated with disease severity and outcomes in H1N1 infection

The characteristics of all subjects in the present study including 30 individuals with severe H1N1 infection and 88 individuals with mild H1N1 infection are presented in Table 1 and Table 2. Consistent with our previous findings[25], the severely infected patients had higher levels of NETs (cfDNA, histone-DNA, MPO-DNA and LTF-DNA) in the plasma than the patients with mild infection and healthy controls (all $p < 0.001$; Figure 1a).

Among the 30 severely infected patients, the levels of both plasma cfDNA and histone-bound DNA were negatively correlated with the oxygen index (Figure 1b). The patients who received extracorporeal membrane oxygenation (ECMO) support (n=14) displayed significantly higher levels of cfDNA and histone-bound DNA than those without undergoing ECMO support (cfDNA: median: 501 ng/ml [IQR: 348-711 ng/ml] *vs* 310 ng/ml [240-403 ng/ml], $p = 0.005$; histone-bound DNA: 25.9 ng/ml [21.1-39.3 ng/ml] *vs* 17.2 ng/ml [12.0-23.5 ng/ml], $p = 0.012$; Figure 1c).

In addition, the levels of both cfDNA and histone-bound DNA were positively correlated with sequential organ failure assessment (SOFA) scores (Figure 1d). Accordingly, the patients with MODS had greater

levels of NETs than those without MODS (cfDNA: 572 ng/ml [405- 676 ng/ml] *vs* 306 ng/ml [248-354 ng/ml], $p < 0.001$; histone-bound DNA: 31.5 ng/ml [23.4-35.9 ng/ml] *vs* 17.1 ng/ml [11.6-23.0 ng/ml], $p < 0.001$; Figure 1e). Furthermore, higher levels of NETs were also observed in the plasma of the 17 patients who died within 60 days than in that of the 13 patients who survived (cfDNA: 400 ng/ml [333-622 ng/ml] *vs* 298 ng/ml [244-461 ng/ml], $p = 0.020$; histone-bound DNA: 24.1 ng/ml [19.1-34.9 ng/ml] *vs* 16.6 ng/ml [11.2-23.0 ng/ml], $p = 0.012$; Figure 1f). Thus, the data from the present cohort confirmed our previous finding that plasma NET levels correlated with disease severity and outcomes in H1N1 infection.

Enhanced levels of BALF NETs are not associated with disease severity or outcomes in patients with severe H1N1 infection

Next, we evaluated NET levels in BALF samples from the patients with severe H1N1 infection. Significant increases in the levels of cfDNA, histone-DNA, MPO-DNA and LTF-DNA were observed in the BALF from the severe H1N1 patients compared with those from the healthy controls (all $p < 0.001$; Figure 2a). Although the NET content in alveoli was diluted during the lavage (120 ml saline per person), the concentrations of cfDNA, histone-DNA, MPO-DNA and LTF-DNA in BALF samples were still 13.3-, 9.5-, 2- and 9.7-fold higher, respectively, than those in plasma samples from the same patients (all $p < 0.001$; Figure 2b). Further analysis showed that the cfDNA concentrations in the BALF were not correlated with those in the plasma (Figure 2c). Additionally, the cfDNA levels in the BALF were not correlated with the proportions of neutrophils in the BALF or the levels of the cytokines IL-6, IL-8, MIP-1α and MIP-1β in the BALF (Figure 2c, Table 3) and additional tables show these in more detail (see Additional files 2 and 3). Notably, we found distinct increases in permeability of alveolar epithelial cells when co-cultured with the supernatant of BALF from severe patients, compared with those from HCs. (Figure 2d).

Next, we investigated whether the cfDNA levels in the BALF correlate with disease severity. Surprisingly, we observed no correlation between the BALF cfDNA levels and the oxygen index (Figure 3a). Among the severely infected patients, those who received ECMO support had levels of cfDNA and histone-bound DNA in the BALF comparable to the levels in the patients who did not receive ECMO support (Figure 3b). Furthermore, the BALF cfDNA levels were not correlated with SOFA scores (Figure 3c).

The patients with MODS and those who did not progress to MODS had similar levels of BALF NETs (Figure 3d). Accordingly, the NET levels in the BALF were comparable between the 17 patients who died within 60 days and the 13 survived patients (Figure 3e).

The levels of cfDNA and histone-bound DNA in the plasma predict disease severity and clinical outcomes in H1N1 infection

Next, we plotted a ROC curve for plasma levels of cfDNA to predict severe illness of H1N1 influenza. With an optimal cut-off value of 286.6 ng/ml, the AUC of cfDNA was 0.922 (95% CI, 0.872-0.972; $p < 0.001$), and the sensitivity and specificity of cfDNA were 80.0% (95% CI, 61.4%-92.3%) and 89.8% (95% CI, 81.5%-95.2%), respectively (Figure 4a). The cfDNA levels in the plasma were measured in the H1N1 cohort 2009 in our previous study[25]. Using the cut-off value of cfDNA obtained from cohort 2017 (286.6

ng/ml), we performed a separate validation study with cohort 2009. The sensitivity and specificity were 87.5% (95% CI, 71.0%-96.5%) and 76.5% (95% CI, 58.8%-89.3%), respectively (Figure 4a).

In addition to cfDNA, another marker of NETs, the histone-bound DNA levels in the plasma, also accurately predicted severe illness with an AUC of 0.996 (95% CI, 0.987-1.005; $p < 0.0001$) in cohort 2017. With a cut-off value of 9.4 ng/ml, the sensitivity and specificity were 100.0% (95% CI, 88.4%-100.0%) and 98.0% (95% CI, 89.2%-100.0%), respectively (Figure 4b).

We further performed ROC curve analysis to predict influenza mortality in cohort 2017. As shown in Table 4 and an additional figure (see Additional file 4), the AUC of plasma cfDNA was 0.751. With a cut-off value of 306.3 ng/ml, the sensitivity and specificity of cfDNA were 94.1% and 61.6%, respectively. Consistently, plasma histone-bound DNA had an AUC of 0.769 in predicting mortality. In addition to NETs, three other parameters that were closely related to a poor prognosis, the oxygen index, neutrophil proportion and plasma IL-8 level, were also included[26, 31, 32]. The AUCs of the oxygen index, neutrophil proportion and IL-8 were 0.679, 0.586 and 0.728, respectively, all of which were lower than the AUC of cfDNA or histone-bound DNA. Furthermore, a validation study with cohort 2009 showed that the AUC of plasma cfDNA (0.711) was comparable to that determined for cohort 2017. Taken together, these data suggest that the plasma levels of NETs on the day of admission could predict severe illness and a fatal outcome in H1N1 influenza.

Discussion

In this study, we investigated the clinical roles of NETs in H1N1 influenza and made three important observations. First, using samples from a separate patient cohort, we validated the previous findings that plasma levels of NETs are strongly associated with pulmonary dysfunction, systemic damage and mortality in H1N1 infection. Second, we made a novel finding that although the NET levels were significantly elevated in the lungs, they were not associated with disease severity or mortality in patients with severe H1N1 infection. Third, we demonstrated the predictive value of the plasma levels of cfDNA and histone-DNA complex on the day of admission for determining the disease prognosis.

Despite being diluted, cfDNA and the neutrophil protein-DNA complexes in the BALF were still detected at high levels in patients with severe H1N1 infection, suggesting the presence of extremely high levels of NETs in situ in the alveoli in patients with severe influenza. Consistent with previous studies of an H1N1-infected mouse model[25, 28], BALF NET samples from our cohort could cause serious damage to the extracellular matrix and alveolar epithelium, as indicated by in vitro functional analysis. However, high levels of circulating NETs rather than BALF NETs correlated with local pulmonary dysfunction, such as a reduced oxygen index, acute respiratory distress syndrome occurrence and the need for ECMO support as shown in additional figure (see Additional file 5), and disease outcome in patients with severe H1N1 infection. This surprising finding may be due to several reasons. First, 90.9% of the severely infected patients died from the development of MODS[32]. In our cohort, MODS was diagnosed in 88.2% (15/17) of the patients who died within 60 days. Thus, MODS rather than respiratory failure represented the major

cause of death in the patients with severe H1N1 infection. Correspondingly, circulating DNA levels are a better marker of MODS than those in BALF. Second, the bronchoscopy lavage was carried out between day 1 and day 7 post ICU admission according to the clinical requirement. The NET measurements were going to vary depending on what timepoint after admission the sampling occurred. Third, most of the patients with severe influenza had too much progression in the lungs so the NET level was maximized and no differences were detected. Forth, an increase in the plasma NET level might be due to overactivation of circulating neutrophils and/or destruction of the air-blood barrier. Both possibilities may reflect the deterioration of multiple organs caused by systemic inflammatory responses. This notion was supported by our recent study that showed that high levels of circulating cfDNA were associated with MODS occurrence and a poor prognosis in patients with severe fever and thrombocytopenia syndrome virus infection[30]. It is worth studying whether these plasma parameters related to NET formation are general parameters correlated with the outcomes of patients with MODS caused by other pathogens. Overall, the ability to exclude the use of BALF NETs to analyze the prognosis of severe influenza is still a novel and significant finding.

However, the results for NETs in the BALF must be interpreted with caution. One cannot arbitrarily ignore the therapeutic strategy of targeting NETs in the lungs because severely infected patients do have high levels of NETs in the BALF. Some treatments that block or degrade NET formation accompanied by antiviral treatment might be efficient strategies for treating H1N1 infection. Clinically, DNase has been successfully used to treat cystic fibrosis patients, and the beneficial effect may be due to the digestion of NETs[33, 34]. Thus, local administration of NET-targeting agents such as DNase may still be a feasible and optimal treatment strategy for reducing influenza-related severe pneumonia.

Previously, we found an association between plasma NET levels and disease severity in cohorts of H7N9- and H1N1-infected patients[25]. In the present study, we performed a necessary validation study and confirmed our previous findings in a separate H1N1 cohort. More importantly, we revealed that the plasma levels of cfDNA (> 286.6 ng/ml) or histone-bound DNA (> 9.4 ng/ml) had high accuracy in predicting severe influenza, and the predictive value of cfDNA was further verified in cohort 2009. According to the results of ROC analysis, we highly recommend using plasma histone-bound DNA to predict disease severity in clinical applications.

In the death risk prediction analysis, histone-bound DNA and cfDNA in the plasma showed higher AUC values than the oxygen index, neutrophil proportion and plasma IL-8 level, three parameters that are closely related to a poor prognosis in influenza virus-infected patients. Among these parameters, IL-8 is an activator of NET formation, while histone-bound DNA and cfDNA represent the amounts of NET production. Since cfDNA had a high sensitivity of 94.1% and IL-8 had a high specificity of 92.3%, we recommend a simple series analysis strategy using cfDNA first for a high-sensitivity prediction, followed by IL-8 for a high-specificity prediction to improve the ability to predict fatal outcomes.

It is worth noting that among the four markers we detected for NET quantification, only cfDNA and histone-DNA were tightly associated with the clinical manifestation and disease progression, other than

the more specific markers, MPO-DNA and LTF-DNA. In addition to neutrophils, cfDNA and histone-DNA could also be released by other leukocytes such as eosinophils and mast cells, as well as tissue parenchymal cells during apoptosis or necrosis. Thus, the extracellular DNA detected in the plasma and BALF of H1N1 patients might be derived from endothelial cells, epithelial cells and other leukocytes beyond neutrophils. These results consist with the prevalent notion that many factors contribute to influenza-related death. Despite the indistinct cellular source, the plasma levels of cfDNA and histone-DNA may reflect both inflammatory response and tissue damage, an advantage that may explain why cfDNA and histone-DNA can predict mortality more accurately than other parameters.

Conclusions

In the present study, we revealed that circulating NETs rather than alveolar NETs on the day of admission predict adverse outcomes in influenza. Detection of histone-bound DNA, cfDNA and IL-8 levels in the plasma may help physicians assess disease severity and prognosis, and this strategy represents a more feasible and less invasive method for sample collection than BAL. The discovery of the predictive value of circulating NETs for prognosis will help establish an early preventive intervention with great potential to reduce mortality. In addition to contributing to the development of clinical research, plasma NETs may be biomarkers for assessing therapeutic time points and curative effects in patients with influenza.

Declarations

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Author contributions

Liuluan Zhu, Hui Zeng and Qingyuan Zhan designed the experiments, interpreted the data and wrote the manuscript. Liuluan Zhu, Yue Zhang, Nannan Zhang and Xiaoyu Yang performed the experiments and analyzed the data. Qingyuan Zhan, Nannan Zhang, Shuyu Xiong, Linna Huang, Xu Huang, Chun Zhou, Rui Song, Zhihai Chen, Fei Xu, Yajie Wang and Ang Li collected patient samples and joined in interpreting the data. Gang Wan supervised the statistical analysis.

Conflict of interest

We declare that we have no conflicts of interest.

Ethics approval and consent to participate

Participating investigators obtained local institutional review board approval in accordance with local ethics regulations.

Availability of data and materials

Requests for data will be considered by the principal study investigators, based on the nature of the request and legal and ethical regulations.

Consent for publication

All authors have reviewed the manuscript and approved the publication.

Abbreviations

NETs: neutrophil extracellular traps

BALF: bronchoalveolar lavage fluid

cfDNA: cell-free DNA

MODS: multiple organ dysfunction syndrome

MPO: myeloperoxidase

NE: neutrophil elastase

LTF: lactotransferrin

ROC: receiver operating characteristic

CIs: confidence intervals

ECMO: extracorporeal membrane oxygenation

SOFA: sequential organ failure assessment

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Tables

Table 1. Characteristics of H1N1-infected patients.

Characteristics	H1N1 mild	H1N1 severe	*P* value
Patient amount	88	30	-
Age (year)	29.0 (24.0,42.0)	52.4±15.3	<0.001
Male sex, n (%)	46 (52.3)	17 (56.7)	0.677
Time from symptom onset to hospital admission (days)			
	1 (1,2)	8 (7, 9)	<0.001
Antiviral treatment, n (%)	88 (100.0)	30 (100.0)	-
Antibiotics treatment, n (%)	14 (15.9)	30 (100.0)	<0.001

Data are presented as means ± standard deviation (SD) when they were normally distributed or median (IQR) when they were not normally distributed. Comparisons were performed using

χ^2 test or Mann–Whitney U test.

Table 2. Characteristics of survival and death in patients with severe H1N1 infection.

Characteristics	H1N1 severe		
	Survival	Death	*P* value
Patient amount	13	17	-
Age (year)	50.9±9.7	53.5±18.7	0.643
Male sex, n (%)	7 (53.8)	10 (58.8)	0.785
Previous or pre-existing conditions, n (%)			
Respiratory diseases	0 (0.0)	2 (11.8)	0.201
Hypertension	4 (30.8)	5 (29.4)	0.936
Diabetes	7 (53.8)	7 (41.2)	0.491
Obesity (BMI > 28)	3 (23.1)	2 (11.8)	0.410
Heart diseases	0 (0.0)	2 (11.8)	0.201
Positive culture of pathogenic microorganism, n (%)			
Bacteria	8 (61.5)	11 (64.7)	0.858
Fungi	3 (23.1)	3 (17.6)	0.713
Bacteria and fungi	2 (15.4)	3 (17.6)	0.869
ICU Hospitalization period (days) range)			
	11 (4, 24)	17 (10, 32.5)	0.117
Time from symptom onset to ICU admission (days)			
	8.9±2.5	7.9±2.9	0.409
Neutrophil counts, (10^9/L)			
	7.9±6.2	8.6±4.9	0.742
Lymphocyte counts, (10^9/L)			
	0.8±0.4	0.7±0.4	0.408
N/L ratio	7.1 (4.6,11.9)	10.0 (6.2,27.4)	0.245
Oxygen index	154.7±56.5	124.4±60.5	0.174
Mechanical Ventilation, n (%)			
	7 (53.8)	15 (88.2)	**0.035**
ECMO, n (%)	3 (23.1)	11 (64.7)	**0.024**
ECMO during time (days)			
	13.0±8.7	11.5±8.3	0.623
MODS, n (%)	2 (15.4)	10 (58.8)	**0.016**
Apache II score	15.5±6.7	16.9±6.5	0.425
SOFA score	5.8±3.5	7.4±3.5	0.168

Data are presented as means ± standard deviation (SD) when they were normally distributed or median (IQR) when they were not normally distributed. Comparisons were performed using χ^2 test, Student's *t* test, or Mann–Whitney U test.

Abbreviations: BMI, Body Mass Index; ECMO, extracorporeal membrane oxygenation; MODS, multiple organ dysfunction syndrome; Apache II score, Acute Physiology and Chronic Health Evaluation II; SOFA, sequential organ failure assessment.

Table 3. Correlations between cfDNA and cytokine levels in the plasma and BALF from patients with severe H1N1 infection.

	Cytokines	*r* value	*P* value
Plasma cfDNA	IL-8	0.444	0.020
	IL-6	0.393	0.038
	MIP-1α	0.426	0.024
	MIP-1β	0.411	0.033
BALF cfDNA	IL-8	0.151	0.503
	IL-6	0.205	0.360
	MIP-1α	0.117	0.605
	MIP-1β	0.055	0.809

Correlations were calculated by Spearman correlation analysis (r). Correlation analysis was conducted between cfDNA and cytokine in the plasma (n = 27) and BALF (n = 22).

Abbreviations: cfDNA, cell-free DNA; IL, interleukin; MIP, Macrophage inflammatory protein; BALF, bronchoalveolar lavage fluid.

Table 4. Statistical analysis of ROC curve in fatal outcome prediction in cohort 2017 (n=30).

Variable	Cut-off value	AUC (95% CI)	Sensitivity (%)	Specificity (%)	*P* value
cfDNA	> 306.3 ng/mL	0.751 (0.564 to 0.938)	94.1 (71.3 to 99.9)	61.5 (31.6 to 86.1)	0.020
Histone-DNA	> 23.1 ng/mL	0.769 (0.594 to 0.944)	64.7 (38.3 to 85.8)	84.6 (54.6 to 98.1)	0.013
Oxygen Index	< 109 mmHg	0.679 (0.486 to 0.872)	58.8 (32.9 to 81.6)	76.9 (46.2 to 95.0)	0.098
Neutrophil%	> 90.4%	0.586 (0.373 to 0.799)	47.1 (23.0 to 72.2)	76.9 (46.2 to 95.0)	0.427
IL-8	> 40.4 pg/mL	0.728 (0.538 to 0.918)	60 (32.3 to 83.7)	92.3 (64.0 to 99.8)	0.040

Abbreviations: ROC curve, receiver operating characteristic curve; AUC, area under the curve; CI, confidence interval; cfDNA, cell-free DNA; IL, interleukin; MIP, Macrophage inflammatory protein; BALF, bronchoalveolar lavage fluid.

Figures

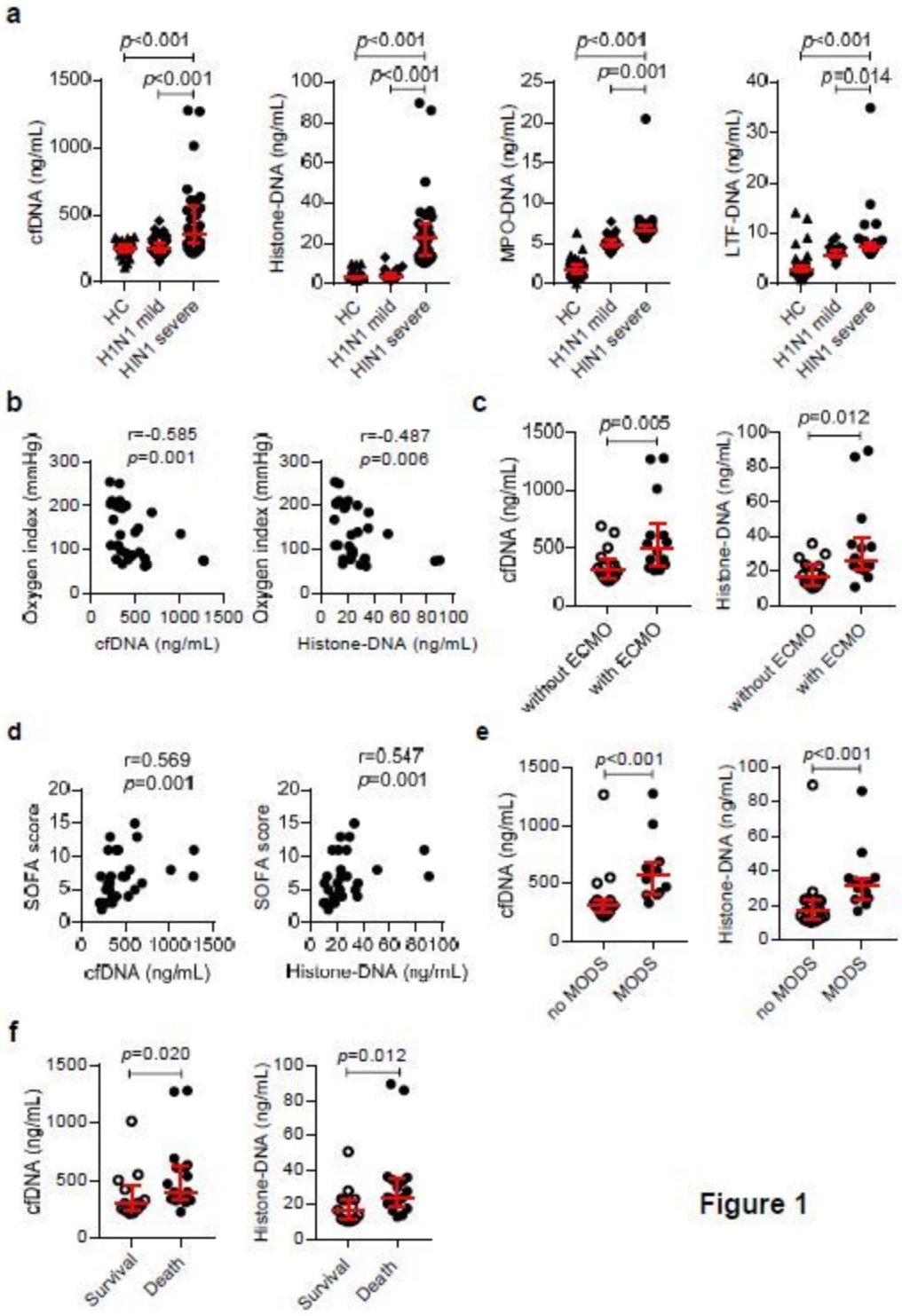


Figure 1

Figure 1

The plasma levels of NETs in patients with H1N1 infection. a Plasma levels of cfDNA, histone-DNA, MPO-DNA and LTF-DNA in the patients and healthy controls (HCs). HC, n = 40-55; H1N1 mild, n = 30-88; H1N1 severe, n = 30. b Correlations between oxygen index and cfDNA or histone-DNA levels in 30 severe H1N1-infected cases. c Plasma levels of cfDNA and histone-DNA in severe patients with and without ECMO. With ECMO group, n = 16; without ECMO group, n = 14. d Correlations between SOFA scores and cfDNA or histone-DNA levels in 30 severe H1N1-infected cases. e Plasma levels of cfDNA and histone-DNA in the severe patients with or without MODS. MODS group, n = 12, no MODS group, n = 18. f Plasma cfDNA and histone-DNA levels in survival and died patients with severe H1N1 infection. Survival group, n = 13; death group, n = 17. Data are presented as median (inter quartile range) or mean ± standard deviation. P values were obtained by Mann-Whitney U test or student t test between two groups. Correlations were calculated by Spearman correlation analysis.

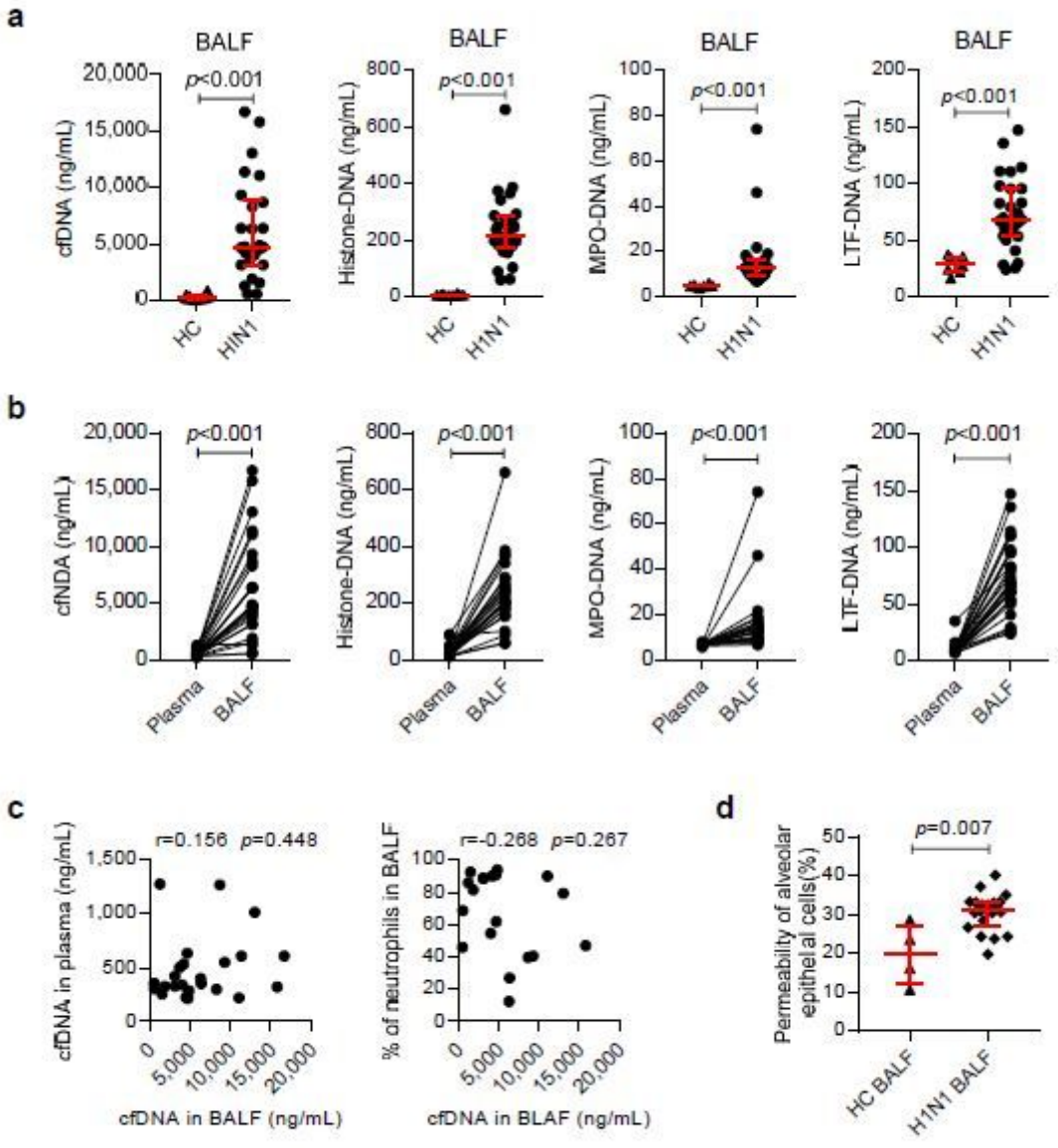


Figure 2

Figure 2

Increased levels of NETs in the BALF from patients with severe influenza. a The levels of cfDNA, histone-DNA, MPO-DNA and LTF-DNA in the BALF were elevated in the severe H1N1 patients. HC, n = 8-12; severe H1N1, n = 26. b The levels of cfDNA, histone-DNA, MPO-DNA and LTF-DNA in the plasma versus BALF from the same patients. Data are representative of at least three independent experiments. c Correlations between cfDNA in the BALF and that in the plasma (n = 26) and the percentage of neutrophils in the BALF (n = 19) in severe cases with H1N1 infection, respectively. d Permeability of alveolar epithelial cells co-incubated with BALF samples from severe H1N1 patients and HC controls. HC, n= 4; severe H1N1, n = 20. Data are presented as median (inter quartile range). P values were obtained by Mann-Whitney test or paired t test. Correlations were calculated by Spearman correlation analysis.

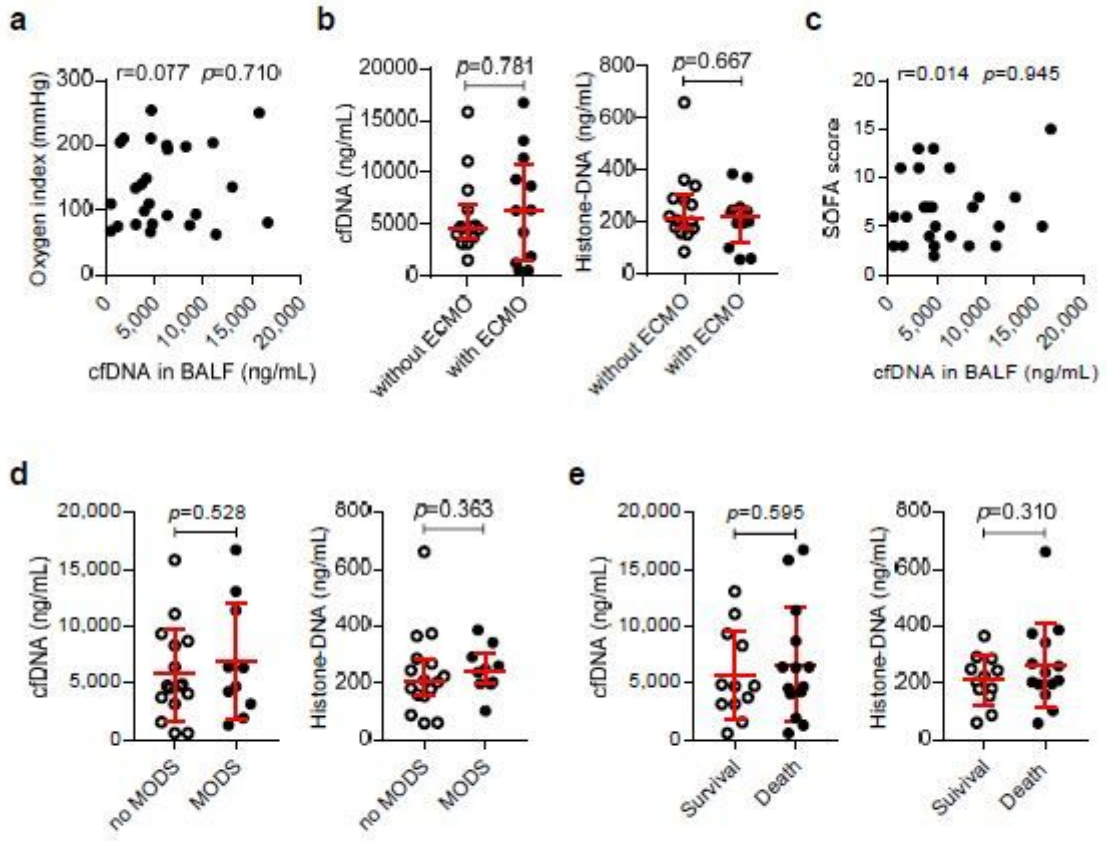


Figure 3

Figure 3

BALF cfDNA and histone-DNA were not associated with the local or systemic damage. a Correlations between cfDNA in the BALF and oxygen index in severe cases with H1N1 infection (n = 26). b The levels of cfDNA and histone-DNA in the BALF from severe patients with or without ECMO support. Without

ECMO group, n = 14; with ECMO group, n = 12. c Correlations between cfDNA in the BALF and SOFA scores (n = 26). d BALF cfDNA and histone-DNA levels in the MODS and no MODS patients with severe H1N1 infection. MODS group, n = 10; no MODS group, n = 16. e The levels of cfDNA and histone-DNA in the BALF from survival and died patients with severe H1N1 infection. Survival group, n = 12; death group, n = 14. Data are presented as median (inter quartile range) or mean ± standard deviation. P values were obtained by Mann-Whitney U test or student t test between groups. Correlations were calculated by Spearman correlation analysis.

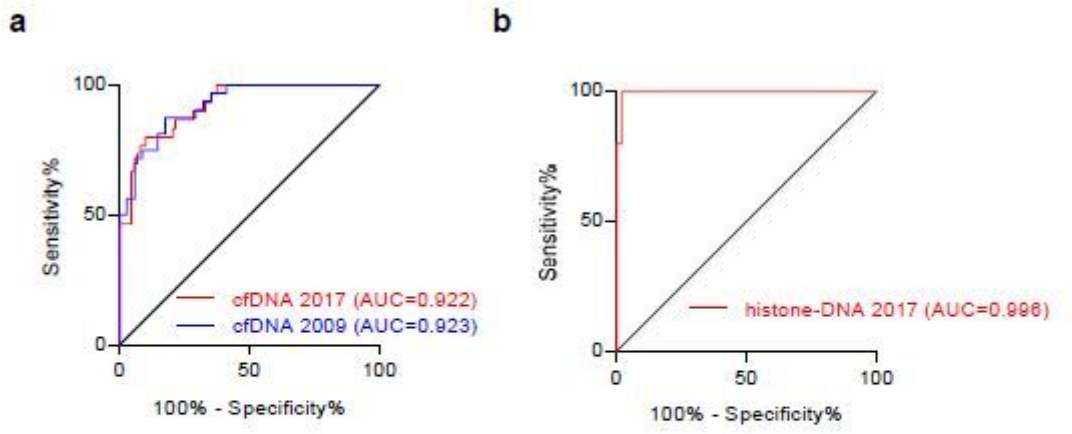


Figure 4

Figure 4

ROC curves of plasma cfDNA and histone-DNA in predicting severe illness. a ROC curves and AUCs of plasma cfDNA in predicting severe illness in cohort 2017 (red line; mild, n = 88; severe, n = 30) and cohort 2009 (blue line; mild, n = 34; severe, n = 32). b ROC curve and AUC of plasma histone-DNA in predicting severe illness in cohort 2017. Mild, n = 34; severe, n = 32. Data were collected at the time of initial diagnosis in each patient.

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