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ORIGINAL RESEARCH

Neutrophil–lymphocyte ratio as an early new marker in AIV-H7N9-infected patients: a retrospective study

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Background: Avian AIV-H7N9 influenza progresses rapidly and has a high fatality rate. However, it lacks an early effective biomarker to predict disease severity and fatal outcomes successfully. Our study aimed to explore whether the neutrophil-to-lymphocyte ratio (NLR) taken within 24 h after admission can predict disease severity and fatality in AIV-H7N9-infected patients.

Methods: We retrospectively studied 237 AIV-H7N9-infected patients from multiple centers from 2013 to 2015. We used univariate analysis and multivariate analysis to compare clinical variables between the survival and fatal groups to evaluate the prognostic value.

Results: The NLR taken within 24 h after admission in the fatal group was significantly higher than that in the survival group ($P<0.01$). Our study found that NLR was independently associated with fatality. The area under the curve (AUC) of the NLR was 0.70, and moreover, when the NLR =19.94, the specificity was 100%, and the sensitivity was 28.4%. The fatality in the NLR ≥19.94 group was significantly increased relative to the patients with an NLR <19.94 ($P<0.05$).

Conclusion: The NLR is potentially a predictive prognostic biomarker in patients infected with the AIV-H7N9 influenza virus.

Keywords: NLR (neutrophil-to-lymphocyte ratio), influenza A, AIV-H7N9, biomarker

Background

The novel avian influenza virus (AIV) H7N9 subtype (AIV-H7N9) is characterized by rapid progression and with a high fatality rate of 39%.[1] Fatal outcome of AIV-H7N9 infections is often related to acute lung injury and acute respiratory distress syndrome (ARDS). Early biomarkers for predicting the progression of the disease and fatal outcomes are needed. Previous studies have shown that the level of angiotensin II and certain cytokines are biomarkers for the outcome of AIV-H7N9-infected patients.[2,3] However, there is still a lack of clinically useful biomarkers to predict the disease progression and clinical outcome of a patient.

Recently, the neutrophil-to-lymphocyte ratio (NLR) was shown to be a useful indicator of systemic inflammation.[4] Studies have shown that the NLR was positively associated with serious diseases, such as acute pancreatitis, liver disease, certain rheumatic diseases, and ARDS.[5–14] Moreover, the NLR was found to be associated with hospital fatality in critically ill patients.[15] Neutrophils and lymphocytes, as important components of the innate immune system, have vital functions in the development and recovery of influenza. The neutrophil count reflects mostly

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innate immune cell function.[16] Neutrophils release large amounts of cytokines and chemokines to promote the formation of cytokine storms. It has able been shown that neutrophils can serve as antigen-presenting cells to antiviral CD8+ T-cells.[17] Low levels of lymphocytes have been observed in severe AIV-H7N9 patients.[18] In our study, we monitored the proportion of cell types in the peripheral blood of patients infected with AIV-H7N9 viruses. Herein, we performed a retrospective study to evaluate the NLR within 24 h after admission in patients infected with AIV-H7N9. Our aim was to determine how changes in the differential leukocyte count in AIV-H7N9-infected patients correlated with the virulence of AIV.

Methods

Ethics statement

This study was approved by the First Affiliated Hospital of Zhejiang University ethics board.

Patient enrollment

There were initially a total of 282 patients included in the study, but 45 of them were excluded due to incomplete information. Thus, a total of 237 confirmed AIV-H7N9-infected patients were retrospectively enrolled from February 26, 2013 to June 30, 2015. These patients came from different hospitals in Zhejiang (110 patients), Guangzhou (47 patients), Shanghai (22 patients), Hunan (17 patients), Jiangsu (16 patients), Fujian (9 patients), Henan (4 patients), Anhui (3 patients), Beijing (2 patients), Guangdong (2 patients), Shandong (2 patients), Guangxi (2 patients), Guizhou (1 patient), and Yunnan (1 patient) provinces. Among them, 74 cases were from the First Affiliated Hospital of Zhejiang University Medical College. Informed written consents were obtained from all participants before enrollment in the study. Confirmed AIV-H7N9 patients were defined as patients with a positive laboratory diagnosis.[19]

Data collection

Medical charts and electronic databases were reviewed, and standardized forms were used to gather information retrospectively. Clinical and laboratory information, including demographics, baseline clinical information, and follow-up clinical information, was collected systematically from admission to discharge for every patient. The laboratory examinations were all collected within 48 h after admission, and the routine blood tests were collected within 24 h after admission. The NLR was calculated by dividing the number of neutrophils (N) by the number of lymphocytes (L) from the blood test. The PaO_2/FiO_2 ratio was the partial pressure of arterial oxygen to the fraction of inspired oxygen. Secondary infection was defined as any bacterial or fungal infection having corresponding clinical manifestations, with one or more positive cultures. The cultures were obtained from blood, valid sputum, lower respiratory tract (endotracheal or bronchoalveolar lavage) samples, or other normally sterile fluids.

Statistical analysis

Categorical variables were calculated by frequency analysis. The numerical variables of normal distributions were represented by means ± standard deviations, and abnormal distributions were represented by medians (interquartile, IQR). The Student's *t*-test and chi-square test were used to conduct univariate analysis. Multivariate analysis was performed by logistic regression. Receiver operator curves (ROC) were used to assess the accuracy of variables. Kaplan–Meier survival analysis was performed to compare the difference in the overall survival between different groups. A P-value <0.05 was considered significant. All analyses were conducted using SPSS for Windows (version 16.0, SPSS Inc., Chicago, IL, USA).

Results

Study population characteristics

The baseline characteristics of all of the 237 patients are shown in Table 1. The mean age of the 237 patients was 56.42 ±15.84 years, and 70.89% (168 patients) were male. There was a history of smoking in 23.20% (55 patients) of the patients, and 56.12% (133 patients) of the patients had one or more coexisting conditions, with hypertension, diabetes, and coronary heart disease as the most common coexisting conditions. The median value of the NLR taken within 24 h after admission was 10.29±12.16 for the entire study population. The overall fatality rate was 31.22% (Table 1).

NLR as an independent risk factor of fatality in AIV-H7N9-infected patients

The 237 patients were divided into two groups based on the survival of the patient. There were 163 patients in the survival group and 74 patients in the fatal group. The baseline characteristics of the survival and fatal group are listed in Table 1. There was a significant difference between the two groups in terms of age (P=0.00), PaO_2/FiO_2 (P=0.00),

Table 1 Univariate analyses of variables during the early stage of disease between the survival group and the fatal group.

Variables	Total population (N=237)	Survival group (N=163)	Fatal group (N=74)	*P*-value
Age (years)	56.42 (15.84)	54.03 (15.32)	61.69 (15.79)	0.00
Male-n (%)	168 (70.89%)	119 (73.00%)	49 (66.22%)	0.29
Coexisting disease-n (%)	133 (56.12%)	89 (54.60%)	44 (59.46%)	0.57
Hypertension	97 (40.93%)	64 (39.02%)	33 (44.59%)	0.48
Diabetes	40 (16.88%)	24 (14.72%)	16 (21.62%)	0.20
Coronary heart disease	27 (11.39%)	17 (10.43%)	10 (13.51%)	0.51
Chronic obstructive pulmonary disease	11 (4.64%)	5 (3.07%)	6 (8.11%)	0.10
Chronic renal disease	9 (3.8%)	6 (3.68%)	3 (4.05%)	1.00
Cancers*	6 (2.53%)	4 (2.45%)	2 (2.70%)	1.00
Chronic liver disease	2 (0.84%)	2 (1.22%)	0 (0.00%)	1.00
Cerebrovascular disease	2 (0.84%)	2 (1.22%)	0 (0.00%)	1.00
Current smoker-n (%)	55 (23.20%)	38 (23.17%)	17 (22.97%)	1.00
Time from symptom onset to NAIs administration (days): median (IQR)	6.00 (4.00, 8.00)	6.00 (4.00,8.00)	6.00 (5.00,10.00)	0. 11
Time from symptom onset to diagnosis (days): median (IQR)	7.00 (5.00, 9.00)	7.00 (5.00,9.00)	7.00 (5.00,10.00)	0.25
PaO2/FiO2 (mmHg)	150.71 (103.24)	172.10 (109.66)	104.96 (68.93)	0.00
CRP (mg/L)	88.16 (65.21)	80.22 (62.60)	105.65 (67.84)	0.01
WBC (109/L)	5.46 (4.44)	4.91 (2.80)	6.65 (6.66)	0.03
N (109/L)	4.46 (4.02)	3.85 (2.51)	5.78 (5.97)	0.01
L (109/L)	0.61 (0.57)	0.67 (0.59)	0.50 (0.51)	0.03
NLR	10.29 (12.16)	7.36 (4.98)	16.74 (19.02)	0.00
Hb (g/L)	129.67 (21.13)	131.27 (18.15)	126.11 (26.41)	0.13
PLT (109/L)	132.60 (65.27)	135.77 (67.79)	125.53 (59.09)	0.27
Cr (μmol/L)	98.49 (123.26)	88.46 (115. 49)	120.47 (137.03)	0.08
Shock-n (%)	87 (36.71%)	30 (18.40%)	57 (77.02%)	0.00
Treatment				
Administration of oseltamivir or peramivir-n (%)	237 (100%)	163 (100%)	74 (100%)	0.31
Mechanical ventilation-n (%)	118 (49.79%)	49 (30.06%)	69 (93.24%)	0.00
Intravenous immune globulin therapy-n (%)	82 (34.75%)	49 (30.06%)	33 (44.59%)	0.04
Continuous renal replacement therapy-n (%)	46 (19.41%)	14 (8.59%)	32 (43.24%)	0.00
Extracorporeal membrane oxygenation-n (%)	28 (11.81%)	12 (7.36%)	16 (21.62%)	0.00
Li's non-bioartificial liver-n (%)	23 (9.70%)	14 (8.59%)	8 (10.81%)	0.63
Secondary infection-n (%)	131 (55.27%)	85 (52.15%)	46 (62.16%)	0.20

Note: *Cancers included leukemia and lymphoma.
Abbreviations: CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; SE, standard error; WBC, white blood cell.

CRP (C-reactive protein; *P*=0.01), NLR (*P*=0.00), shock (*P*=0.00), mechanical ventilation (MV; *P*=0.00), intravenous immune globulin therapy (IVIG; *P*=0.04), continuous renal replacement therapy (CRRT; *P*=0.00), and extracorporeal membrane oxygenation (ECOM; *P*=0.00).

We used logistic regression to analyze the factors that had a *P*-value <0.05 in Table 1. In the multivariate regression analysis, age, NLR, shock, EV, and CRRT were statistically significant (Table 2). Moreover, the NLR, age, shock, EV, and CRRT were risk factors for the fatality of AIV-H7N9 infection. We constructed a ROC curve of the NLR to examine the predictive values of the NLR in AIV-H7N9-infected patients (Figure 1). The area under the curve (AUC) of the NLR was 0.70. Our data suggest that the NLR value measured within 24 h after admission could potentially predict the fatal outcome of AIV-H7N9 infection. According to the ROC curve, the NLR was 1.54, with a sensitivity of 98.6% and specificity of 2.5%. An NLR of 19.94 showed 100% specificity and a sensitivity of 28.4%. We then used the NLR =19.94 as the cutoff value to divide the 237 patients into two groups (NLR ≥19.94 and NLR <19.94). There were 21 patients with an NLR ≥19.94, whereas 216 had an NLR

Table 2 Logistic regression analysis of independent outcomes predictor in AIV-H7N9-infected patients.

Index	B	SE	Wald	Sig.	Exp(B)	95.0% CI for Exp(B)	
						Lower	Upper
Age	0.027	0.014	3.942	0.047	1.028	1.000	1.056
PaO_2/FiO_2	−0.002	0.003	0.247	0.619	0.998	0.992	1.005
CRP	−0.002	0.003	0.290	0.591	0.998	0.992	1.005
Shock	1.611	0.461	12.206	0.000	5.006	2.028	12.357
NLR	0.079	0.029	7.408	0.006	1.082	1.022	1.145
Mechanical ventilation	1.899	0.585	10.547	0.001	6.680	2.123	21.015
Extracorporeal membrane oxygenation	−0.061	0.573	0.011	0.915	0.941	0.306	2.894
Continuous renal replacement therapy	1.341	0.484	7.669	0.006	3.823	1.480	9.878
Intravenous immune globulin therapy	0.667	0.457	2.131	0.144	1.948	0.796	4.767
Constant	−5.465	1.199	20.766	0.000	0.004		

Abbreviations: CRP, C-reactive protein; NLR, neutrophil-to-lymphocyte ratio; SE, standard error.

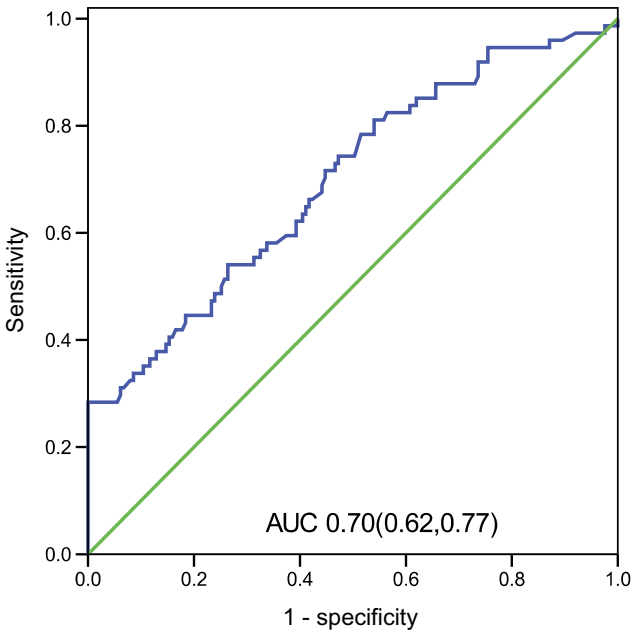


Figure 1 Receiver operating characteristic (ROC) curves of the NLR taken within 24 h and other biomarkers for 180-day fatality.
Abbreviation: AUC, area under the curve.

<19.94. Furthermore, there was a statistically significant difference in fatality between the two groups (P=0.02). The Kaplan–Meier curve also demonstrated that an NLR ≥19.94 was associated with the survival time of AIV-H7N9-infected patients (Figure 2).

Discussion

Our retrospective study showed that the NLR levels in patients with AIV-H7N9 infection taken within 24 h after admission were higher in non-survivors than in survivors. The NLR might play a valuable role in predicting the in-hospital fatality of patients with AIV-H7N9 infection. To the best of our knowledge, this study is the first to show that the NLR is an independent predictor of fatality in patients infected with the AIV-H7N9 influenza virus.

Previous studies have described the relationship between NLR and severe, acute inflammatory diseases, such as sepsis and ARDS. An observational cohort study included 5,056 unselected patients in intensive care units (ICU), and concluded that the NLR was associated with hospital mortality.[15] However, in the subgroup analysis, there was no association between the NLR and mortality in sepsis patients.[15] Another study in patients with septic shock demonstrated an association between the NLR and mortality.[20] Furthermore, a retrospective study conducted in severe sepsis and septic shock patients in emergency departments found that the NLR taken within 24 h was associated with a 28-day mortality rate.[21] Li et al found that a high NLR was associated with a poor outcome in critically ill patients with ARDS.[9] In addition, Wang et al showed that the NLR in ARDS survivors was significantly lower than non-survivors, and an NLR >14 was associated with a shorter overall survival.[12] A prior study found an increased mean initial neutrophil count and a lower mean initial lymphocyte count in the patients who died of AIV-H7N9 infection compared to patients who recovered.[22] However, there is currently no research about the relationship between the NLR and the prognosis of influenza A patients.

Our data showed that the leukocytes in the peripheral blood within 24 h after admission were in the normal range in both groups, but the proportion of cell types was different. The neutrophils of non-survivors were higher, and the lymphocytes were lower than the survivors, which contributed to the higher NLR level in the

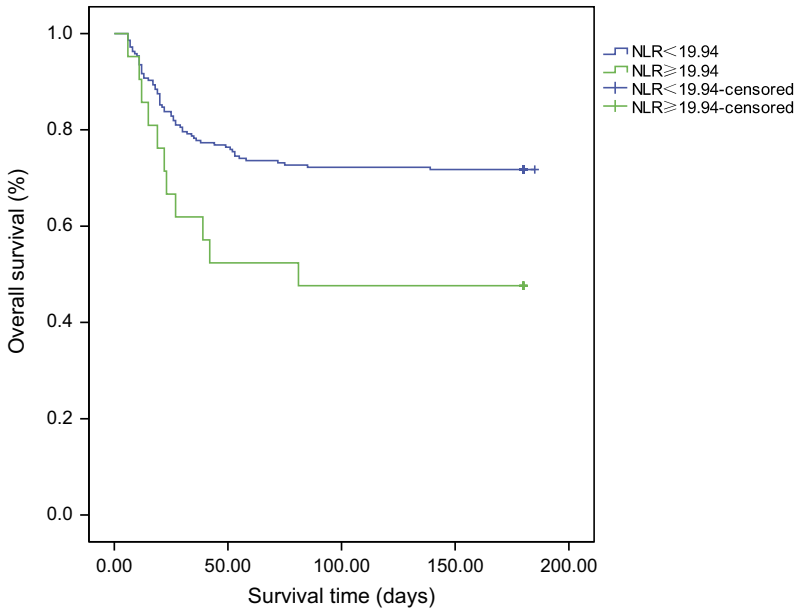


Figure 2 Kaplan–Meier analyses of the NLR ≥19.94 group and the NLR <19.94 group in patients infected with the AIV-H7N9 influenza virus.
Abbreviation: NLR, neutrophil-to-lymphocyte ratio.

non-survivors. The course of infection with the AIV-H7N9 virus varied according to the interaction between the virus and the immune system. In severe AIV-H7N9 infected patients, the immune system was overactivated, and substantial amounts of cytokines and chemokines were released.[3] Cytokine/chemokine storm and severe hyperinflammatory responses are associated with pulmonary injury and ARDS, which ultimately results in fatal outcomes. Although several studies have reported on the role of neutrophils in the pathogenesis of influenza, there are still many unanswered questions.[23] Neutrophils are an important component of the innate immune system. They secrete large amounts of cytokines and chemokines to regulate the immune response.[24] Cytokines and chemokines are able to influence many aspects of the immune response, including antiviral defense, hematopoiesis, angiogenesis, and fibrogenesis.[25] Neutrophils can also act as antigen-presenting cells to antiviral CD8+ T-cells.[17] A previously published study reported that neutrophils release chromatin-based structures called neutrophil extracellular traps (NETs) to kill microbes when stimulated by various antigens, including viruses, bacteria, toxic factors, and cytokines, such as IL-8 and TNF-α.[26] However, excessive NET formation was shown to cause tissue damage.[26] This study also indicated that NETs had the potential to be used as a biomarker for predicting the prognosis of patients infected with influenza A virus. Furthermore, a high level of NETs was shown to cause lung injury and were related to the severity of disease.[12] Research has indicated that neutrophils can be infected by the AIV, and may act as vehicles transporting the virus to other organs.[27] Past studies have reported that neutrophils were implicated both in the development of ARDS and in recovery from ARDS.[28–30] Some have even suggested that neutrophils should be a therapeutic target in severe influenza pneumonia.[31] In our study, the neutrophils of non-survivors were relatively higher than the survivors. In AIV-H7N9-infected patients, the neutrophils respond rapidly to inflammation, are continuously released from the bone marrow, and increase in numbers quickly.[3] Cytokines, especially serum IL-8 levels, were positively correlated with neutrophil counts in patients infected with the AIV-H7N9 virus.[12] The neutrophil count reflects mostly innate immune cell function, indicating systemic oxidative stress, inflammation, and tissue damage.[16] The level of neutrophils may represent the intensity of the immune response. A prior study found that severe influenza A virus infection caused an increase in the numbers of circulating neutrophils, as well as the serum IL-8 levels.[12] An early increase in the airway and circulating blood neutrophil levels is a feature of AIV infection in humans,[32,33] and is common in experimental AIV infection in mice.[34–36] In addition, the relatively higher neutrophil count in non-survivors may relate to secondary

bacterial infection. Neutrophils respond to a variety of inflammatory conditions, especially those associated with bacterial infection.[28–30] Studies have shown that one of the main causes of a fatal outcome when infected with the influenza virus is bacterial superinfection.[37] Our study showed that the proportion of patients with secondary infection was relatively high in non-survivors, which may be one of the reasons for the higher NLR in non-survivors.

Previous studies have shown that AIV-H7N9-infected patients have a low percentage of peripheral blood T lymphocytes.[3] Moreover, it was reported that T cell levels in patients that succumbed to AIV-H7N9 infection were much lower on the day of admission compared to the survivors of AIV-H7N9 infection.[3] Shen et al investigated 18 AIV-H7N9 patients and found that all the patients had lymphopenia (<1.5 x 10^9 lymphocytes/L).[18] Yu et al investigated six fatal cases of AIV-H7N9 infection and found that five had lymphopenia.[38] Lymphopenia was observed not only in AIV-H7N9 infection, but also other types of influenza A infections, such as H5N1 and H1N1.[18] Our study showed lower lymphocytes in non-survivors. Previous studies have shown that infection with several virulent influenza A viruses (such as H5N1) causes hypoplastic marrow with histiocytic hyperplasia, with these histiocytes showing hemophagocytosis.[39–42] An H5N1 murine infection model demonstrated that the number of $CD4^+$ and $CD8^+$ T cells decreased in mice infected with the H5N1 virus. It was also shown that after exposure to the influenza A virus, the lymphocytes underwent apoptosis through Fas–Fas ligand signaling.[43] Furthermore, a previous study found that in fatal cases of AIV-H7N9 infected patients, the number of cytokines and chemokines were high on the day of admission and even higher on the day of death, while the lymphocyte count was just the opposite.[3] Hence, cytokine-driven conditions may be associated with this lymphopenia.[42,44] A decrease in the levels of lymphocytes results in the absence of cellular immunity, delayed viral clearance, and an increased death rate. Lymphopenia has been reported as an early reliable biomarker of influenza A H1N1 infection.[43] This study also concluded that low lymphocyte counts (<0.5 x 10^9 cells/L) were a risk factor for an adverse clinical outcome of AIV-H7N9 infection.[18] Thus, T lymphocyte counts were shown to be a potential indicator that can be utilized to assess the severity of diseases, in addition to serving as an early predictor of the clinical outcome. Furthermore, since the lymphocytes decreased and the neutrophils increased in fatal cases, the NLR would be high, which indicates that the NLR may also serve as a potential indicator of disease progression and outcome.

Avian AIV-H7N9 influenza was characterized with high fatality. There were extensive amounts of cytokines and chemokines produced by the immune system of patients in attempts to clear the virus from the body; however, this also resulted in systemic inflammation and the progression of ARDS. More than 70% of AIV-H7N9-infected patients developed ARDS,[45] which was associated with high fatality in AIV-H7N9 patients.[46,47] Furthermore, two retrospective studies showed that the NLR was a predictive prognostic biomarker in ARDS patients.[9,12] Since ARDS was associated with the high fatality rate of AIV-H7N9-infected patients, and the NLR was a predictive prognostic biomarker of ARDS, then the NLR may also predict the clinical outcome of AIV-H7N9 patients.

Previous studies had found several indicators that predict the prognosis of AIV-H7N9 patients. Huang et al found that angiotensin II was a biomarker for lethality in influenza infections.[2] Guo et al reported that the levels of MIF, SCF, MCP-1, HGF, and SCGF-β were positively correlated with AIV-H7N9 disease severity, and the mediators MIF, SCF, MCP-1, HGF, SCGF-β, IP-10, IL-18, and IFN-γ were independent prognostic factors.[48] Additionally, Zhu et al found that high levels of NETs correlated with the poor prognosis of severe influenza A infection.[49] However, there is still a lack of biomarkers that are not only easily collected from the patient, but that can also be used to predict disease progression and fatal outcomes. The neutrophil and lymphocyte count is easily obtained, as routine blood testing is available and affordable for each patient; thus, the NLR may serve as a potential biomarker.

In our study, we took the median NLR (=19.94) as the cutoff point to divide the 237 patients into two groups, and we used 180-day fatality as an outcome. The Kaplan–Meier curve showed that patients with an NLR >19.94 had a higher fatality rate (P<0.01), indicating that patients with an NLR >19.94 have an increased chance of dying of AIV-H7N9 infection, and thus, a poor prognosis. Since AIV-H7N9 is characterized by rapid progression and high fatality, it is essential to identify the patients who may potentially have a poor prognosis. Therefore, when a confirmed AIV-H7N9 patient is admitted to the hospital, analysis of the NLR value may be useful for risk stratification. And it can help clinicians comprehensively evaluate the condition and progress of AIV-H7N9-infected patients to determine the initial treatment (including outpatient service, hospitalization, or ICU admittance) and select

the appropriate treatment. Furthermore, it helps predict the outcome of AIV-H7N9 patients. It may also be used to determine the timing of some special treatments and discharge time. However, further studies are needed in order to fully understand the NLR value and fatal outcomes in AIV-infected patients.

Our study also showed that compared with survivors, patients in the fatal group were older, exhibited increased CRP levels, had a worse PaO_2/FiO_2, suffered more occurrences of shock, and needed more MV, IVIG, CRRT, and ECOM therapy. Our findings agree with previous studies.[50–53] Elderly people had a worse prognosis, probably because they have an increased risk of coexisting illnesses and are more susceptible to severe forms of disease than younger persons.[54] CRP is not only a systemic marker of inflammation, but also a mediator of inflammatory factors.[55] Studies have found that high levels of CRP are corrected with a high fatality rate in AIV-H7N9-infected patients.[56,57]Another study showed that a peak CRP of 120 mg/L or higher was significantly associated with case fatality in AIV-H7N9 infection.[56] In fatal cases, more patients developed clinical complications, such as ARDS, heart failure, and septic shock,[51] and as such, they needed more MV, IVIG, CRRT, and ECOM therapy.

There are, however, several limitations to the current study. First, it was a retrospective observational study, and as such, subject selection bias was avoidable. Second, the NLR value used in this study was collected within 24 h after admission and not on the day of the onset of disease. Thus, the NLR value might be influenced by drugs already given to the patient and comorbidities. The dynamic changes of the NLR at different time points during disease have not been explored in this paper; therefore, the best time for the NLR to predict death has not been determined. Third, although our study included 237 patients from several different regions, a prospective study with larger sample size is required in order to definitively confirm the NLR as a biomarker of AIV-H7N9 disease progression and clinical outcome.

In conclusion, our study demonstrates that the NLR is a simple and useful biomarker for predicting in-hospital fatality in patients infected with the AIV-H7N9 influenza virus. A high NLR value within 24 h after admission was associated with an overall poor prognosis. Furthermore, since the NLR value is calculated from the neutrophil and lymphocyte counts, which are easily obtained from a routine blood draw, this value could be widely in used as a clinical biomarker for disease progression and clinical outcomes in patients infected with the AIV-H7N9 influenza virus.

Availability of data and materials

The dataset used and analyzed during this study is available from the corresponding author upon request.

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Author contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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