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CLINICAL STUDY

Assessment of the role of neutrophil extracellular traps in SARS-CoV-2 pneumonia

CAKMAK Fatih[1], OZKAN Seda[1], KONUKOGLU Dildar[3], BIBEROGLU Serap[1], IPEKCI Afsin[1], AKDENIZ Yonca Senem[1], BOLAYIRLI Ibrahim Murat[2], BALKAN Ilker Inanc[3], DUMANLI Guleren Yartas[4], IKIZCELI Ibrahim[1]

Department of Emergency Medicine, Istanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine, Istanbul, Turkey. **sedacil@gmail.com**

ABSTRACT

BACKGROUND: Abnormal neutrophil extracellular traps are associated with lung diseases, thrombosis, increased mucosal secretion in the airways. The aim of this study is to evaluate the possible place of the most specific NETosis marker Cit-H3 protein in diagnostic algorithms by revealing its relationship with the severity, mortality and prognosis of SARS-CoV-2 pneumonia.

PATIENTS AND METHODS: Patients (n=78) who applied to the Emergency Department between March 11, 2020 and June 10, 2020, with positive SARS-CoV-2 polymerase chain reaction (PCR) test and lung involvement were included in the prospective study. Serum Cit-H3 levels and critical laboratory parameters were measured at baseline on the day of clinical deterioration and before recovery/discharge/death. Cit-C3 levels were determined by enzyme immunassay method.

RESULTS: Cit-H3 levels in patients with SARS-CoV-2 pneumonia during their first admission to the hospital were significantly higher compared to the healthy control group ($p<0.05$). Repeated measurements of Cit-H3 levels of the patients significantly correlated with D-dimer, procalcitonin, Neutrophil/ Lymphocyte ratio, lymphocyte, CRP, and oxygen saturation. Cit-H3 levels of the patients who died were significantly higher than that of those who survived ($p<0.05$). Cit-H3 levels were found to be statistically significantly higher in patients who developed acute respiratory distress syndrome, were admitted to the intensive care unit, and had mortality ($p<0.05$).

CONCLUSIONS: Cit-H3 plays a role in inflammatory processes in SARS-CoV-2 pneumonia, and changes in serum Cit-H3 levels of these patients can be used to determine prognosis and mortality *(Tab. 5, Fig. 1, Ref. 21)*. Text in PDF *www.elis.sk*

KEY WORDS: SARS-CoV-2, pneumonia, NETosis, citrullinated histone H3, biomarker, emergency medicine.

Introduction

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) primarily targets the respiratory system and lungs. It is characterized by hyperinflammation, and 10 % of patients progress to cytokine storm-induced acute respiratory distress syndrome (ARDS) (1, 2, 3). There are signs post autopsy that support the hypothesis that powerful function of neutrophils (neutrophil extracellular traps, the ability to create Neutrophil Extracellular Traps; NETs) contributes to organ failure and mortality in COVID-19 (1). As a part of innate immune defense, neutrophils have protective roles against bacterial and fungal infections, they kill fungi or bacteria through NET formation as well as phagocytosis (1, 2, 3). However, the role of neutrophils in COVID-19 infection is still unclear.

NETs are protein-structured chromatin networks that contain fibers coated with antimicrobial granules (DNA and histones), neutrophil elastase, and myeloperoxidase (MPO) enzymes (4). The production process of NETs called NETosis is a specific type of cell death unlike necrosis. It is a step by step cell death program. Enzymes released from granules enter the nucleus, facilitating the condensation of chromatin. This is followed by the breakdown of internal membranes, resulting in cytolysis and release of NETs. Approximately a decade ago, it was discovered that NETs released into the extracellular space by neutrophils capture and kill bacteria as an innate part of the immune system, and their relationship with various pathological conditions has been investigated. It has been shown that NETs disrupt fibrinolysis and induce tissue and organ

[1]Istanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine, Department of Emergency Medicine, Istanbul, Turkey, [2]Istanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine, Department of Medical Biochemistry, Istanbul, Turkey, [3]Istanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine, Department of Infectious Diseases and Clinical Microbiology, Istanbul, Turkey, and [4]Istanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine, Department of Intensive Care, Istanbul, Turkey

Address for correspondence: Seda OZKAN, Department of Emergency Medicine, Istanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine, Cerrahpaşa Mah. Kocamustafapaşa Cad. No: 34/E, Fatih, Istanbul, Turkey.
Phone: +90.2124143000-65208, Fax: +90.2126332987

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damage (5–7). In light of recent data, the use of new treatment agents that eliminate or alleviate these negative roles of NETs or prevent the formation of NET may benefit in the early period. In this regard, reliable and specific biomarkers of NETs can play a central role in predicting risk, prognosis, and therapeutic effects.

The most widely used biomarker to determine the formation of the NETs form is citrullinated histone H3 (Cit-H3). Histone H3 goes through many modifications that includes acetylation, methylation, phosphorylation, and citrullination for regulation of gene transcription. For post-translational modification of the Histon H3 citrullination is important. This modification leads to the development of NETs. Failure to remove citrullinated proteins and NET components after inflammation is associated with lots of human diseases (8, 9).

There is insufficient data on the role of NETs in SARS-CoV-2 like viral infections that can cause extremely fatal interstitial pneumonia in the medical literature (4). The aim of this study was to determine and compare the serum levels of Cit-H3 protein in the initial and advanced stages of infection as a marker that may be a determinant in the clinical course of SARS-CoV-2 pneumonia. There is no original, effective treatment currently available for NETosis in COVID-19 and viral pneumonias because its immunopathogenesis is not sufficiently understood. Further, the aim of this study is to evaluate the possible place of this biomarker in diagnostic algorithms by demonstrating its relationship with the severity, mortality and prognosis of SARS-CoV-2 pneumonia.

Patients and methods

This study was carried out in accordance with the principles of the Declaration of Helsinki of the World Medical Association and with the approval (no. 68357 dated June 5, 2020) of the Ethics Committee of our institution. Written informed consent was obtained from all patients participating in the study.

Patients (n = 78) who applied to the Emergency Department between March 11, 2020 and June 10, 2020, with positive SARS-CoV-2 polymerase chain reaction (PCR) test and lung involvement were included in the study. Age, gender, comorbidity, complaint during admission, clinical course (i.e., pneumonia severity, ARDS development, intensive care need), and the mortality data of the patients were recorded. The Berlin Criteria were used to diagnose ARDS.

Patients whose admission date was not within the pre-specified dates, those under 18 years of age, patients with a negative PCR test results, outpatient patients, pregnant women, and patients with inaccessible data were not included in the study. Outpatients with positive PCR test and no lung involvement were excluded from the study because their serum samples could not be followed up.

Age-matched, non-pregnant healthy adults (n=16) who did not have active infection were selected as the control group, and a single blood sample was collected from this group.

Serum Cit-H3 levels and critical laboratory parameters were measured in blood samples at the onset (day of hospitalization; sampling 1), at the stage of development of cytokine storm/ARDS clinical findings (if any) or the median day of hospitalization (sampling 2) and, before recovery or discharge or death (sampling 3).

Venous blood samples were collected into tubes containing EDTA (BD vacutainer, ref: 367836), gel tubes without anticoagulants (Vacuette, Greiner–bio, ref: 455071), tubes containing sodium citrate as anticoagulants and heparinized tubes. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Cr), lactate dehydrogenase (LDH), C-reactive protein (CRP), ferritin (FER), procalcitonin (PCT) assays were performed on Roche Cobas 8000 analyzer (Roche diagnostic, cobas 8000 modular analyzer). Sysmex CS2500 hemagglutinin analyzer was used for D-dimer and fibrinogen analysis. Ferritin and procalcitonin (PCT) analyses were performed by the electrochemiluminescence method using Roche Cobas 8000 systems (Roche diagnostic, cobas 8000 modular analyzer). Complete blood count and determination of leukocyte subgroups were performed on Beckman Coulter LH 780 analyzer. Analysis of blood gases was performed on Radiometer devices (Radimeter ABL90 Flex). All routine analyzes were carried out in maximum 2 hours, and blood gas analyzes were carried out within 20 minutes. Serum samples for Cit H3 analysis were frozen at −80 °C until analysis.

The human cit-H3 levels were assayed by sandwich enzyme immunassay methods (ELISA Kit, MyBioSource, Inc, Catalog number: MBS3804611, San Diego, USA). The assay was performed according to manufacturer's instructions. Briefly 50 µl of standards or samples are added to the micro ELISA plate wells. 100 µl Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated for 60 minutes at 37°C. After aspiration and washing 3 times, 50 µl of chromogen solution is added to each micro plate well and incubated for 15 minutes at 37°C. Then, the enzyme-substrate reaction is terminated by the addition of 50 µl of stop solution and the color turns yellow. The OD is measured spectrophotometrically at a wavelength of 450 nm (2 nm). The OD value is proportional to the concentration of human serum cit- H3 levels. Inter- and intra assay CV are 5.5 % and 7.5 %, respectively. Results were expressed as ng/mL. The sensitivity by this assay is 0.1 ng/mL. Detection range for the kit was 0.0–20 ng/mL.

Statistical analysis

Statistical analysis of the data obtained was performed with SPSS (Statistics Program for Social Scientists) 20 software. The Kolmogorov–Smirnov test was used in the normality analysis of the data. Student-t test was used for comparison of two independent groups with normal distribution, and the Mann-Whitney U test was used for those without normal distribution. $p < 0.05$ was considered statistically significant. Chi-square test was used to compare the frequency data of independent groups. $p < 0.05$ was considered statistically significant. In comparing three dependent variables, the Friedman test was used for those without normal distribution. $p < 0.05$ was considered statistically significant. Post-hoc analysis of statistically significant groups was performed using Wilcoxon test. $p < 0.017$ was considered statistically significant. The Spearman correlation test was used for correlation analysis of the data.

Results

Seventy-eight SARS-CoV-2 pneumonia patients who were hospitalized were included in our study. Demographic and clinical characteristics of patients are shown in Table 1. In our study, the most common comorbidity was hypertension (n = 30), and the incidence rate in patients who did not survive was significantly higher than that in patients who survived (p <0.005). Fourteen patients with malignant disease had the statistically highest mortality rate (p = 0.001). The most frequent complaints for admission to the hospital were dyspnea (n = 54) and myalgia in (n = 41).

There were significant differences in lymphocyte and platelet counts, serum levels of CRP, ferritin, fibrinogen, procalcitonin levels, and blood neutrophil / lymphocyte (N/L) ratio among the sampling days (Tab. 2). D-dimer, procalcitonin, lymphocyte, ferritin, CRP, LDH, creatinine, leukocyte levels, and N/L ratio were found to be significantly associated with mortality (Tab. 3).

Cit-H3 values of the patients with SARS-CoV-2 pneumonia (n = 78) were 4.67(± 1.02) ng/mL on the day of admission to the hospital, and of healthy controls (n = 16) were 3.25(± 0.59) ng/mL. Serum Cit-H3 values of patients with SARS-CoV-2 pneumonia during the initial hospital admission were significantly higher compared to the healthy control group (p < 0.05). Cit-H3 levels of the intensive care patients were significantly higher compared to ward patients (p < 0.05) (Fig. 1). These values were significantly different compared to the values of the patients who were not admitted to the intensive care unit (p = 0.003, p = 0.006, and p = 0.016, respectively).

Repeated measurements of Cit-H3 values of the patients significantly correlated with D-dimer, procalcitonin, N/L ratio, lymphocyte, CRP, and oxygen saturation (Tab. 4).

Cit-H3 values of the patients who developed ARDS were 5.05(± 1.22) ng/mL on the day of admission to the hospital, 5.03(± 1.21) ng/mL on the day of clinical worsening, and 4.85(± 0.97) ng/mL on the day of discharge or death. Cit-H3 values of the patients who did not develop ARDS were 4.4(± 0.75) ng/mL on the day of admission to the hospital, 4.33(± 0.8) ng/mL on the day of clinical worsening, and 4.31(± 0.68) ng/mL on the day of discharge or death. Cit-H3 levels of the patients who developed ARDS were statistically significantly higher compared to those of the patients who did not develop ARDS (p < 0.05) (Fig. 1). The difference was statistically significant compared to patients without ARDS (p = 0.005, p = 0.004, and p = 0.008, respectively).

Cit-H3 values of the patients who died were 4.94(± 0.78) ng/mL on the day of admission to the hospital, 4.99(± 0.97) ng/mL on the day of the start of clinical worsening, and 4.83(± 0.77) ng/mL on the day of death. Cit-H3 values of the patients who survived were 4.54(± 1.10) ng/mL on the day of admission to the hospital, 4.45(± 1.05) ng/mL on the day of clinical worsening, and 4.40(± 0.86) ng/mL on the day of discharge. Cit-H3 values of the patients who died were statistically significantly higher than that of those who survived (p < 0.05) (Fig. 1).

Tab. 1. Demographic and clinical characteristics of COVID-19 patients (n=78).

	All Patients (n=78)	Survivors (n=52)	Non-survivors (n=26)	p
Age, years	61.2±16.11	56.76±14.17	70.07±16.34	<0.001
Gender, n (%)				
Male	50 (64)	33 (63.)	17 (65.4)	
Female	28 (35.9)	19 (36.5)	9 (34,6)	
Fewer, °C mean	37.24±1.05	37.19±1.06	37.34±1.05	0.514
Comorbidities, n (%)				
COPD	5 (6.4)	1 (1.9)	4 (15.4)	0.022*
Type 2 Diabetes Mellitus	17 (21.8)	9 (17.3)	8 (30.8)	0.175
Congestive Heart Failure	13 (16.7)	5 (9.6)	8 (30.8)	0.018*
Hypertension	30 (38.5)	16 (30.8)	14 (53.8)	0.048*
Chronic Renal Failure	3 (3.8)	0 (0.0)	3 (11.5)	0.012*
Malignancy	14 (17.9)	4 (7.7)	10 (38.5)	0.001*
Immunosuppression	3 (3.8)	1 (1.9)	2 (7.7)	0.212
Coronary artery disease	9 (11.5)	4 (7.7)	5 (19.2)	0.151
Other	29 (37.2)	17 (32.7)	12 (46.1)	0.037*
Admission symptoms, n (%)				
Shortness of breath	54 (69.2)	32 (61.5)	22 (84.6)	0.037*
Cough	58 (74.4)	40 (76.9)	18 (69.2)	0.463
Headache	11 (14.1)	9 (17.3)	2 (7.7)	0.250
Myalgia	41 (52.6)	23 (44.2)	18 (69.2)	0.037*
Loss of smell/taste	13 (16.7)	7 (13.5)	6 (23.1)	0.283
Diarrhea	9 (11.5)	6 (11.5)	3 (11.5)	1.000

COPD – Chronic obstructive pulmonary disease; ARDS – Acute Respiratory Distress Syndrome

Tab. 2. Comparison of the blood samples of the COVID-19 patients on the first day (sampling1), the day of clinical worsening (sampling 2), and before recovery or discharge or death (sampling 3).

Laboratory parameters	Sampling 1	Sampling 2	Sampling 3	p
Leukocyte count/μL	8112±4948	9765±7261	10716±8086	0.05
Lymphocyte count/μL	1175±817[b,c]	1039±711[a,c]	1444±935[a,b]	<0.001
Neutrophil count/μL	6114±4898	7664±7095	8166±7629	0.188
N/L ratio	7.89±10.58[b]	13.6±22.53[a,c]	9.18±11.97[b]	<0.001
Platelet count/μL	207489± 83731[b,c]	232283± 103185[a,c]	291180± 136113[a,b]	<0.001
Serum CRP (mg/L)	93± 94[b,c]	145± 109[a,c]	59± 103[a,b]	<0.001
Serum Ferritin (ng/mL)	589± 649[b]	1038± 1077[a,c]	708± 729[b]	<0.001
Serum LDH (IU/L)	354± 207[b]	451± 260[a]	444± 361	0.001
Serum D-dimer (mg/L)	4.03± 12.95[b,c]	9.36± 16.92[a,c]	4.76± 7.42[a,b]	0.001
Serum Fibrinogen (mg/dL)	522± 178[c]	570± 256[c]	372± 218[a,b]	<0.001
Serum AST (IU/L)	51± 62[b]	77± 71[a,c]	77±136[b]	0.004
Serum ALT (IU/L)	39±40[c]	87±318[c]	82±108[a,b]	0.001
Serum Creatinine (mg/dL)	1.38±1.99	1.18±0.91	1.38±1.31	0.788
Serum Procalcitonin (ng/mL)	0.94± 4.56[b]	2.49± 11.56[a,c]	6.04± 22.7[b]	<0.001
Serum Cit-H3 (ng/mL)	4.67±1.02	4.63±1.05	4.54±0.85	0.390

[a] Shows statistically significant difference with the first blood sample, [b] Shows statistically significant difference with the second blood sample, [c] Shows statistically significant difference with the third blood sample, N/L – Neutrophil/Lymphocyte ratio

Tab. 3. Comparison of the blood parameters between survivors and non-survivors according to the clinical status.

Laboratory parameters	Survivors (n=52)	Non-survivors (n=26)	P*
Leukocyte – 1 (/μL)	7267±3706	9803±6552	0.040
Leukocyte – 2 (/μL))	7482±2767	14330±10688	0.002
Leukocyte – 3 ((/μL)	7551±3878	17046±10402	<0.001
Lymphocyte – 1 (/μL)	1348±918	830±390	0.002
Lymphocyte – 2 (μL)	1225±763	669±390	<0.001
Lymphocyte – 3 (/μL)	1744±892	846±715	<0.001
Neutrophil – 1 (/μL)	5190±3431	7961±6674	0.039
Neutrophil – 2 (/μL)	5363±2821	12265±10286	0.001
Neutrophil – 3 (/μL)	5094±3834	14311±9507	<0.001
N/L ratio – 1	5.56±5.65	12.55±15.67	0.005
N/L ratio – 2	6.42±7.07	27.96±33.77	<0.001
N/L ratio – 3	3.94±4.8	19.67±14.92	<0.001
Platelet – 1 (/μL)	210896±81264	200676±89718	0.615
Platelet – 2 (/μL)	247517±82207	201815 ±32510	0.065
Platelet – 3 (/μL)	353157 ±99817	167226 ±112996	<0.001
CRP – 1 (mg/L)	75±89	129±95	0.004
CRP – 2 (mg/L)	124±103	188±111	0.013
CRP – 3 (mg/L)	19±40	139±140	<0.001
Ferritin – 1 (ng/mL)	412±351	944±921	0.005
Ferritin – 2 (ng/mL)	691±626	1731±1424	0.001
Ferritin – 3 (ng/mL)	409±371	1304±895	<0.001
LDH – 1 (U/L)	299±148	466±260	0.004
LDH – 2 (U/L)	368±197	618±293	<0.001
LDH – 3 (U/L)	294±100	744±491	<0.001
D-dimer – 1 (ng/dL)	1.71±3.87	8.59±21.16	<0.001
D-dimer – 2 (ng/dL)	5.25±13.05	17.42±20.68	<0.001
D-dimer – 3 (ng/dL)	1.52±1.49	11.13±9.97	<0.001
Fibrinogen – 1 (mg/dL)	504±176	559±182	0.111
Fibrinogen – 2 (mg/dL)	566±233	578±301	0.869
Fibrinogen – 3 (mg/dL)	368±190	380±270	0.966
AST – 1 (U/L)	42±38	71±93	0.25
AST – 2 (U/L)	65±51	101±96	0.71
AST – 3 (U/L)	42±30	148±218	<0.001
ALT – 1 (U/L)	38±33	41±51	0.201
ALT – 2 (U/L)	54±50	152±548	0.779
ALT – 3 (U/L)	80±68	84±163	0.241
Creatinine – 1 (mg/dL)	1.21±2.1	1.72±1.75	0.017
Creatinine – 2 (mg/dL)	0.9±0.3	1.74±1.38	<0.001
Creatinine – 3 (mg/dL)	0.87±0.21	2.4±1.91	<0.001
Procalcitonin –1 (ng/dL)	0.94±5.53	0.92±1.35	<0.001
Procalcitonin –2 (ng/dL)	0.59±2.23	6.27±19.46	<0.001
Procalcitonin –3 (ng/dL)	0.08±0.16	17.95±36.95	<0.001
Cit-H3 – 1 (ng/mL)	4.54±1.1	4.94±0.78	0.016
Cit-H3 – 2 (ng/mL)	4.45±1.05	4.99±0.97	0.02
Cit-H3 – 3 (ng/mL)	4.4±0.86	4.83±0.77	0.038

N/L ratio – Neutrophil/Lymphocyte ratio, LDH – Lactate dehydrogenase, AST – Aspartate aminotransferase, ALT – Alanine aminotransferase, CRP – C-Reactive protein, Cit-H3 – Citrullinated histone H3, p* statistical difference between Survivors and Non-survivors group

1 – first day sampling, 2 – samples from the day of clinical worsening, 3 – sampling from before recovery or discharge or death, $p<0.05$ – statistically significance

These values were significantly different compared to patients who survived ($p = 0.016$, $p = 0.02$, and $p = 0.038$, respectively).

Multivariate logistic regression analysis was performed for mortality. Since the number of CRF and immunosuppressive patients was three, they could not be evaluated statistically. In the multivariate logistic regression analysis, age, malignancy and Cit-H3 were found to be significant independent risk factors for mortality ($p < 0.05$) (Tab. 5).

Discussion

There are studies that indicate that histones are formed in cases of ARDS and acute lung damage, and this is responsible for lung damage and inflammation (5). The purpose of our study was to investigate the relationship between mortality and severity of disease and NETosis in hospitalized SARS-CoV-2 patients. This study compared serum Cit-H3 levels with other laboratory and clinical parameters on the first day of admission to the hospital, on the day of clinical worsening, and on the day of discharge or death.

In our study, the serum Cit-H3 values of patients with SARS-CoV-2 pneumonia were significantly higher on the first day of admission to the hospital compared to the healthy control group. Similar to our result, Godement et al found the Cit-H3 levels of patients admitted to the intensive care unit with SARS-Cov-2 pneumonia to be higher compared to the healthy control group (10). Zuo et al have also shown the relationship between the severity of the disease and the occurrence of NETs in patients with COVID-19 (11). Another study found higher levels of NET in the plasma of COVID-19 patients compared to healthy controls, and it was also shown that neutrophils in COVID-19 patients secreted a higher rate of NET than in normal patients (12). Because all patients included in our study were hospitalized and had lung involvement, this result suggests that patients with COVID-19 lung involvement are at high risk for NETosis and that lung damage in COVID-19 patients may be resulting from a virus-induced NET formation. Although there is no excessive increase in the number of neutrophils in patients, it may be associated with an increase in the secretory capacity of NETs, which may lead to an aggravation of the clinical course of the disease.

In our study, there was no significant difference between Cit-H3 values on the day of hospitalization, the day of clinical worsening, and the day of discharge or death in any patient. This result was similar to that of Huckriede et al's study on NETs markers in critical COVID-19 patients (13). In that study, there was no significant difference between early and late NETs markers of patients. The reason for this similarity can be explained by the fact that all patients in our study were hospitalized and the number of patients in intensive care was high. All the hospitalized patients in our hospital had typical COVID-19 signs based on thorax CT, and a certain period of time was required following discharge for the full recovery of the lung findings of the patients. We are of the opinion that the effects of NETs, which we think may be responsible for lung damage, continue in the post-discharge period, even if they decrease. There is significant difference in Cit-H3 levels of patients with ARDS and without ARDS findings. This is why we think that Cit-H3 levels are related with lung injury. Therefore, even though there was a significant elevation in Cit-H3 values compared to the healthy control group, there was no significant difference in the repetitive measurements of any patient. We think that using IL-6 antagonists and other drugs may affect the Cit-H3 values. %55 of our patients take IL-6 antagonists treatment. We suggest that antibiotic theraphy, antiviral theraphy, and Il-6 antagonists may affect the Cit-H3 levels of patients. This is why we

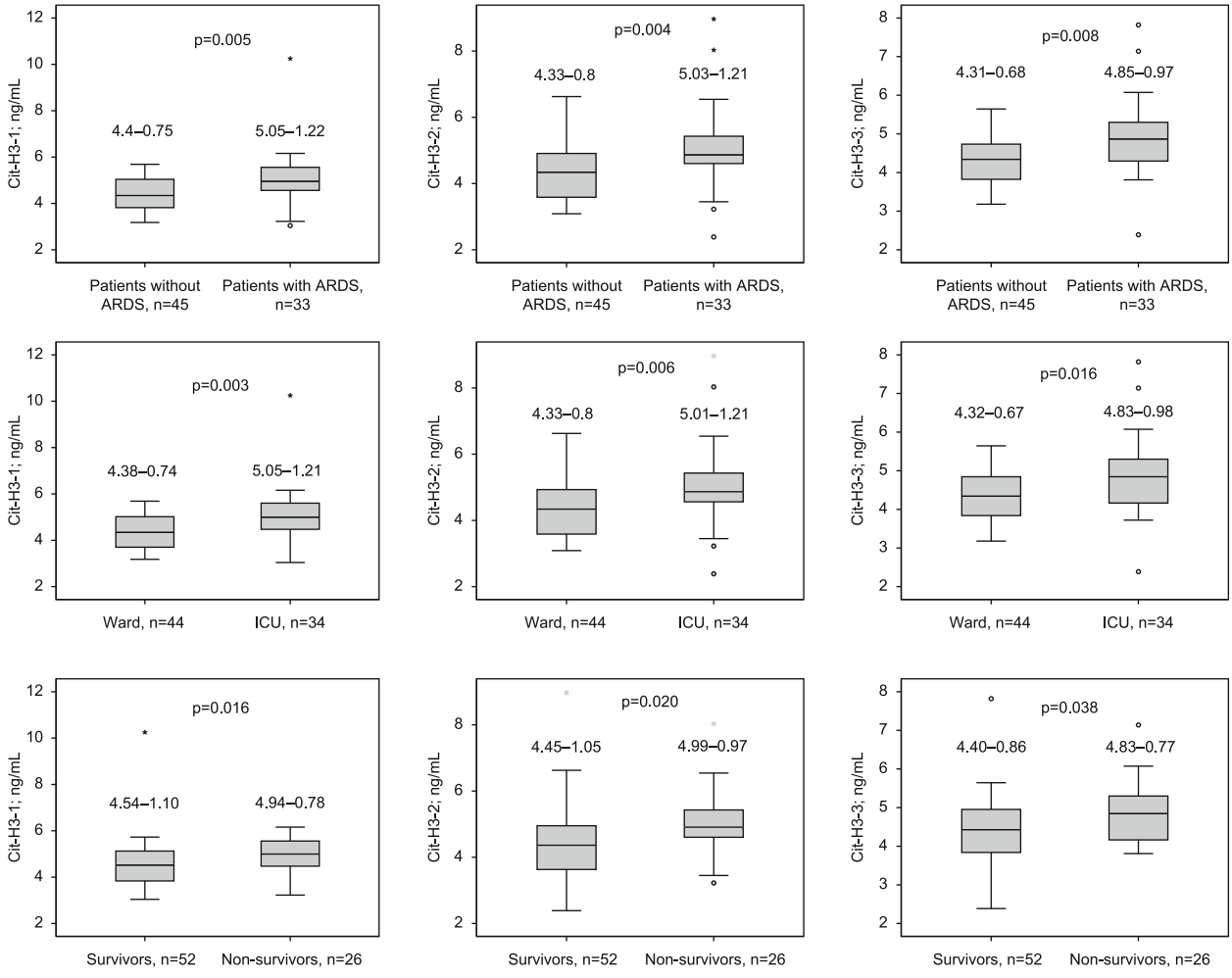

Fig. 1. Comparison of Cit-H3 values of the intensive care patients, patients who developed ARDS and non-survival patients. Cit-H3 levels of the patients who developed ARDS were statistically significantly higher compared to those of the patients who did not develop ARDS ($p < 0.05$). Cit-H3 levels of the intensive care patients were significantly higher compared to ward patients ($p < 0.05$). Cit-H3 values of the patients who died were statistically significantly higher than that of those who survived ($p < 0.05$).

measured the lowest levels on the last day. Also in non-survival patients because of the lung tissue destruction, the capacity of lung epithelium to produce NET is reduced and thus will result in low Cit-H3 levels on the last days.

The serum Cit-H3-1, Cit-H3-2 and Cit-H3-3 values of the patients who died were significantly higher compared to those who survived. Studies similar to our study also reported significantly higher plasma NET levels in COVID-19 survivors compared to survivors (1, 11–15). In our study, repetitive measurements of the Cit-H3 values of the patients who survived demonstrated a declining trend. Based on the repetitive measurements of the Cit-H3 values of the patients who died, the second measurements were higher compared to the day of hospitalization and the day of death. The lowest Cit-H3 measurements of the patients who died were found on the day of death. Similarly, in the study of Godement et al, it was observed that Cit-H3 levels decreased in repeated measurements (10). In this study, blood levels of NETs on day 1 were not associated with mortality. However, they showed that the reduction in blood level of NETs between day 1 and day 3 was strongly associated with survival up to day 28 (10). We believe that this is because interleukin-6 antagonists were included in the treatment in the majority of patients who died and interleukin-6 antagonists had an inhibitory effect on possible NET formation. However, treatment strategies should focus on treatments with higher NETs specificity. Further, antiviral and anticoagulant therapies, administered by increasing the dose after clinical worsening, have suppressed the formation of virus-related NETs. Researchers reported that NET formation was more common and closely correlated with mortality in severe patients infected with COVID-19 and also suggested to focus on NET formation to determine clinical course and possible treatment objectives (10, 11, 13, 14).

In our study, serum Cit-H3-1, Cit-H3-2, and Cit-H3-3 values of patients who developed ARDS were significantly higher than those who did not develop ARDS. This result was similar to the re-

Tab. 4. Spearman correlation analysis between Serum Citrullinated Histone 3 (Cit-H3) levels and critical laboratory parameters in COVID-19 patients.

Laboratory parameters	Correlation coefficient (r)	p
D-dimer 1 – Cit-H3-1	0.211	0.065
D-dimer 2 – Cit-H3-2	0.445	<0.001*
D-dimer 3 – Cit-H3-3	0.479	<0.001*
Ferritin 1 – Cit-H3-1	0.131	0.251
Ferritin 2 – Cit-H3-2	0.166	0.146
Ferritin 3 – Cit-H3-3	0.172	0.131
Procalcitonin 1 – Cit-H3-1	0.262	0.02*
Procalcitonin 2 – Cit-H3-2	0.331	0.003*
Procalcitonin 3 – Cit-H3-3	0.338	0.002*
Fibrinogen 1 – Cit-H3-1	0.102	0.375
Fibrinogen 2 – Cit-H3-2	0.2	0.078
Fibrinogen 3 – Cit-H3-3	0.037	0.748
C-Reactive protein 1 – Cit-H3-1	0.152	0.184
C-Reactive protein 2 – Cit-H3-2	0.27	0.017*
C-Reactive protein 3 – Cit-H3-3	0.223	0.049*
Neutrophil 1 – Cit-H3-1	0.09	0.433
Neutrophil 2 – Cit-H3-2	0.172	0.131
Neutrophil 3 – Cit-H3-3	0.174	0.129
Lymphocyte 1 – Cit-H3-1	–0.221	0.052
Lymphocyte 2 – Cit-H3-2	–0.261	0.021*
Lymphocyte 3 – Cit-H3-3	–0.173	0.13
Neutrophil/Lymphocyte ratio 1 – Cit-H3-1	0.184	0.106
Neutrophil/Lymphocyte ratio 2 – Cit-H3-2	0.363	0.001*
Neutrophil/Lymphocyte ratio 3 – Cit-H3-3	0.211	0.064
Platelet 1 – Cit-H3-1	–0.141	0.219
Platelet 2 – Cit-H3-2	0.082	0.473
Platelet 3 – Cit-H3-3	–0.14	0.223
Oxygen saturation 1 – Cit-H3-1	–0.167	0.143
Oxygen saturation 2 – Cit-H3-2	–0.113	0.326
Oxygen saturation 3 – Cit-H3-3	–0.306	0.006*

1 – first day sampling, 2 – samples from the day of clinical worsening, 3 – sampling from for recovery or discharge or death, $p < 0.05$ – statistically significance

Tab. 5. Multivariate Log regression analysis for mortality.

Parameters	p	OR	95% CI	
			Lower	Upper
Age	0.02	1.093	1.014	1.177
COPD	0.91	1.507	0.002	1389.828
Congestive Heart Failure	0.43	3.718	0.138	100.333
Hypertension	0.22	0.281	0.037	2.138
Malignancy	0.04	0.049	0.003	0.867
Other	0.45	2.085	0.308	14.127
Cit-H3	0.04	2.507	1.025	6.133
Leukocyte	0.38	1.003	0.996	1.010
Neutrophil	0.29	0.846	0.619	1.156
Platelet	0.50	1.000	1.000	1.000
CRP	0.21	0.987	0.967	1.008
Ferritin	0.38	1.001	0.988	1.004
D-dimer	0.38	1.095	0.893	1.344
Creatinine	0.65	1.145	0.640	2.049
Procalcitonin	0.29	2.052	0.957	1.156

sults of the study by Xin et al, which showed a significant increase in extracellular histones in ARDS patients compared to controls (16). In another study, Narasaraju et al demonstrated NET formation in ARDS associated with influenza virus (5). The association of high levels of Cit-H3 with disease severity and mortality in ARDS shows that histones can be a biomarker for predicting the progression of the disease and the termination of patients. When we examine other laboratory values used to predict the prognosis of the disease, we see that Cit-H3 is significantly correlated with these values. We think that patients with high initial measurement values of Cit-H3 should be followed up more closely and care should be taken especially in terms of respiratory failure that may occur in this patient group.

Serum Cit-H3-1, Cit-H3-2, and Cit-H3-3 values of patients hospitalized at the intensive care unit were higher than that of those who did not need intensive care. The results of our study were similar to those of the study by Huckriede et al who reported higher Cit-H3 levels in COVID-19 patients hospitalized in intensive care compared to non-COVID-19 patients (13). Similar to our study, Cit-H3 levels were high between days 4 and 8, following the admission of patients in the study by Huckriede et al (13). Ng et al also found that the H3Cit-DNA levels of patients who required serious respiratory support (> 5 L oxygen on cannula, noninvasive respiratory support, or intubation) were significantly higher than those who required low respiratory support (1). In the study by Zuo et al in which samples collected from severe COVID-19 patients (patients requiring mechanical ventilation) were compared with mild or moderate COVID-19 patients (oxygen saturation in ambient air > 94 %), significantly high levels of cell-free DNA and MPO-DNA were detected in patients requiring mechanical ventilation but not Cit-H3 (11). Absolute neutrophil counts in patients connected to a ventilator were not significantly higher (11). However, based on the examination of these data, there is a possible correlation between serum NETs level and COVID-19 severity. In contrast to our study, Zuo et al (11) reported that Cit-H3 levels were normal in intensive care patients. The difference of our results may be that the criterion for admission to intensive care in our hospital is O2 saturation below 90 % despite oxygen therapy. High serum Cit-H3 levels in COVID-19 patients are associated with lung damage, intensive care hospitalization, and mechanical ventilation need. Cit-H3 level, a marker of NET formation, can guide physicians as a marker for alveoli damage and worsening of SARS-CoV-2 pneumonia.

Based on the correlation of repeated Cit-H3 measurements with other blood parameters checked on the same dates, it is observed that the second and third measurements significantly correlated with D-dimer and CRP, all measurements with procalcitonin, and the second measurements with lymphocytes and N/L ratio. It has been shown that D-dimer elevation in COVID-19 patients is associated with thrombi formation and poor prognosis. The results of our study may suggest that NETs supports the effect of microthrombus formation and coagulopathy. Similar to our results, Middleton et al have shown a correlation between NET markers and markers of thrombus formation (14). In their study, Nicolai et al report a significant correlation between COVID-19 disease severity and D-dimer value and suggest that NETs play a role in thrombus formation (17). In the study by Huckriede et al, there was a significant correlation between D-dimer values and markers of NETs in COVID-19 patients in intensive care (13). These results are similar to that of the present study. It has been demonstrated that patients with severe COVID-19 share common features with the development of ARDS in terms of the presence of thick mucus

secretions in the respiratory tract and the development of blood clots. These symptoms are similar to diseases thought to be caused by NET formation such as COPD and non-SARS-CoV-2 pneumonia. The pathological effect of NETs is not limited to only airway obstruction in lung damage but also plays a role in obstruction of arteries and veins in degenerative cardiovascular diseases (18).

CRP elevation is significantly associated with mortality in SARS-CoV-2 pneumonia. In patients who did not survive, CRP values were significantly higher than that of those who survived, and Cit-H3 values were correlated with CRP on the days when patients clinically worsened and died. Furthermore, CRP and PCT levels are indicators of poor prognosis for sepsis and pneumonia (19, 20). In many studies, it was concluded that elevated PCT level was effective in predicting mortality (19, 20). We believe that the elevation of PCT in SARS-CoV-2 pneumonia is associated with tissue damage in the lungs and increased inflammatory response. In our study, it was found that all three repeated measurements of Cit-H3 were significantly correlated with procalcitonin value. This is because of the presence of typical COVID-19 involvement and lung damage in thorax CT images of all patients included in our study. During clinical follow-up, patients often had clinical worsening or even intensive care hospitalization in the first 3–4 days. Our results are similar to that of Huckriede et al (13). In the study of Ng et al, the levels of NET markers, including Cit-H3, were found to be correlated with neutrophils, inflammatory cytokines and CRP (1). In our study, the second measurements of Cit-H3 were correlated with lymphocyte ratio and N/L ratio on the same day. However, low lymphocyte value is an effective laboratory finding for diagnosis and clinical follow-up in patients with SARS-CoV-2 pneumonia. The N/L ratio shows the severity of bacterial infections and is widely used to assess the prognosis in patients with pneumonia and malignancy, and a high N/L ratio is associated with poor clinical prognosis. It is argued that N/L ratio is the most important factor in determining the severity of the disease in COVID-19 patients (21).

In our study, serum Cit-H3 levels were found to be an independent risk factor for mortality in SARS-CoV-2 pneumonia. The association of high serum Cit-H3 levels with disease severity and mortality in SARS-CoV-2 pneumonia and ARDS indicates that histones can be a biomarker in predicting disease progression and patient outcome.

As a result; we think that Cit-H3 plays a role in the inflammatory processes in SARS-CoV-2 pneumonia and that changes in the serum Cit-H3 values of these patients will be important in determining prognosis and mortality.

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