# Arteriosclerosis, Thrombosis, and Vascular Biology

## CLINICAL AND POPULATION STUDIES

# Circulating Markers of Neutrophil Extracellular Traps Are of Prognostic Value in Patients With COVID-19

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**OBJECTIVE:** The full spectrum of coronavirus disease 2019 (COVID-19) infection ranges from asymptomatic to acute respiratory distress syndrome, characterized by hyperinflammation and thrombotic microangiopathy. The pathogenic mechanisms are poorly understood, but emerging evidence suggest that excessive neutrophil extracellular trap (NET) formation plays a key role in COVID-19 disease progression. Here, we evaluate if circulating markers of NETs are associated with COVID-19 disease severity and clinical outcome, as well as to markers of inflammation and in vivo coagulation and fibrinolysis.

**APPROACH AND RESULTS:** One hundred six patients with COVID-19 with moderate to severe disease were enrolled shortly after hospital admission and followed for 4 months. Acute and convalescent plasma samples as well as plasma samples from 30 healthy individuals were assessed for markers of NET formation: citrullinated histone H3, cell-free DNA, NE (neutrophil elastase). We found that all plasma levels of NET markers were elevated in patients with COVID-19 relative to healthy controls, that they were associated with respiratory support requirement and short-term mortality, and declined to those found in healthy individuals 4 months post-infection. The levels of the NET markers also correlated with white blood cells, neutrophils, inflammatory cytokines, and C-reactive protein, as well as to markers of in vivo coagulation, fibrinolysis, and endothelial damage.

**CONCLUSIONS:** Our findings suggest a role of NETs in COVID-19 disease progression, implicating their contribution to an immunothrombotic state. Further, we observed an association between circulating markers of NET formation and clinical outcome, demonstrating a potential role of NET markers in clinical decision-making, as well as for NETs as targets for novel therapeutic interventions in COVID-19.

**GRAPHIC ABSTRACT:** A graphic abstract is available for this article.

**Key Words:** cytokines ■ extracellular traps ■ fibrinolysis ■ histone ■ immunothrombosis ■ Sars-Cov2 ■ thrombosis

T he novel coronavirus (SARS-CoV-2; severe acute respiratory syndrome coronavirus 2) infection, causing the coronavirus disease 2019 (COVID-19), is rapidly spreading and has taken pandemic proportions. Although the majority of infected individuals display mild symptoms, the full spectrum of the disease ranges from asymptomatic to severe acute respiratory distress syndrome requiring hospitalization, and is in many cases fatal. Patients with COVID-19-associated acute respiratory distress syndrome exhibit increased pulmonary inflammation, extensive lung damage, and microthrombosis driven by an exacerbated and poorly understood host response.[1,2] In response to pathogens, neutrophils can form neutrophil extracellular

## Nonstandard Abbreviations and Acronyms

| | |
|---|---|
| **ADAMTS13** | a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13 |
| **cfDNA** | cell-free DNA |
| **COVID-19** | coronavirus disease 2019 |
| **H3Cit-DNA** | nucleosomal citrullinated histone H3 |
| **IL-6** | interleukin-6 |
| **NE** | neutrophil elastase |
| **NET** | neutrophil extracellular trap |
| **PAP** | plasmin-antiplasmin |
| **RS** | respiratory support |
| **TAT** | thrombin-antithrombin complex |
| **TNFα** | tumor necrosis factor alpha |
| **VWF** | von Willebrand factor |

## Highlights

- Elevated circulating markers of neutrophil extracellular traps (NETs) are prognostic indicators for respiratory support requirements and survival in patients with coronavirus disease 2019 (COVID-19).
- Circulating NET markers correlate with markers of inflammation and endothelial damage in COVID-19.
- Targeting NETosis to reduce the immunothrombosis in COVID-19 pathogenesis forms a promising therapeutic strategy.

traps (NETs) by releasing decondensed chromosomal DNA decorated by granular proteins. Although first described to combat invading microorganisms,[3] excessive NET formation has been shown to induce an immunothrombotic state observed in sepsis, acute respiratory distress syndrome, and cancer.[4] Emerging data now suggest a role of NETs also in COVID-19 pathophysiology: (1) neutrophilia and neutrophil-to-lymphocyte ratio are linked to COVID-19 disease severity,[5,6] (2) NETs have been demonstrated in pulmonary microvessels of patients with COVID-19,[7–11] (3) neutrophils from patients with COVID-19 are prone to spontaneously form NETs,[7,8,10] (4) plasma from patients with COVID-19 induce the release of NETs by healthy neutrophils,[7,12,13] and (5) circulating markers of NET formation are correlated to COVID-19 disease severity.[7,8,13,14] Here, we show that circulating markers of NET formation are prognostic in a well-phenotyped, prospective study of 106 patients, and that they are associated with an inflammatory and prothrombotic state, suggesting the implication of NETs in COVID-19-related immunothrombosis.

## METHODS

### Study Population

One hundred six patients with COVID-19 admitted to Danderyd Hospital, Stockholm, Sweden, between April 15, 2020 and June 8, 2020 were included in the study. Inclusion criterion was COVID-19 diagnosis based on reverse-transcriptase polymerase chain reaction viral RNA detection of nasopharyngeal or oropharyngeal swabs or clinical presentation. The only exclusion criterium was age <18 years. Blood samples were collected within 7 days of admission. Demographics, medications, routine laboratory (C-reactive protein, white blood cell count, lymphocyte count, and neutrophil count) and clinical data including respiratory support (RS) were obtained from medical records. All patients were invited for a follow-up within the study protocol, and follow-up blood samples 4 months (median 122 [109–132] days) postinfection onset were obtained from 55 patients. Patients who did not come to follow-up were either deceased or did not answer on repeated invitations. There was no difference in RS during hospital stay between survivors that came for follow-up and survivors that did not come for follow-up (*P*=0.196). Plasma samples were prepared by centrifugation at 2000*g* for 20 minutes in room temperature and stored at −80 °C. Plasma samples from 30 healthy individuals were obtained from a previous study (2015/1533-31/1). These samples were prepared by the same research nurse as in the current study, following the same protocol. The study was approved by the Swedish Ethical Review Authority (2020-01653), and informed consent was obtained from all study participants or, in the case of incapacity, their next-of-kin.

### Laboratory Analyses

Plasma levels of nucleosomal citrullinated histone H3 (H3Cit-DNA) were quantified using an in-house and validated ELISA as previously described.[15] Briefly, a monoclonal anti-histone H3 (citrulline R8) antibody (Abcam) was used for capture and a monoclonal anti-DNA antibody (Cell Death ELISAPLUS, Roche) was used for detection. Semisynthetic nucleosomes containing citrulline in place of arginine at histone H3, arginine residues 2, 8, and 17 (H3R2,8,17Cit) (EpiCypher) were used as calibration standards. Cell-free DNA (cfDNA), NE (neutrophil elastase), TNFα (tumor necrosis factor alpha), and IL-6 (interleukin-6) were quantified using commercially available kits according to manufacturer (Quant-iT PicoGreen dsDNA assay, Invitrogen, Human PMN Elastase ELISA Kit, Abcam, Human TNF-alpha DuoSet and IL-6 DuoSet ELISA Kits, R&D Systems, respectively). More details are provided in the supplementary material. Plasma levels of markers of in vivo coagulation and fibrinolysis (TAT [thrombin-antithrombin complex], D-dimer, and PAP [plasmin-antiplasmin]), and endothelial activation/damage (VWF [von Willebrand factor], ADAMTS13 [a disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13] activity) were obtained from a separate study investigating their relation to COVID-19 disease severity/outcome,[16] and quantified as previously described.[17]

## Statistical Analyses

Patients were divided into 2 groups: low level of RS; ≤5 L oxygen on cannula, and high level of RS; >5 L oxygen on cannula, noninvasive respiratory support or intubation. Short-term mortality was defined as mortality <30 days from admission. Comparisons of continuous variables were performed using Mann-Whitney *U* test and categorical variables with Fisher exact test. Comparisons of >2 groups were performed using Kruskal-Wallis 1-way ANOVA followed by Dunn multiple comparison test. Paired samples were compared using Wilcoxon test. The relationship between NET markers and markers of inflammation, coagulation, fibrinolysis, and endothelial activation/injury was evaluated using the Spearman rho. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc, San Diego, CA). All tests were 2-sided, and *P*<0.05 was considered statistically significant.

**Table 1.** Demographic Data, Clinical Characteristics, and Laboratory Data of Study Participants

| | Healthy (n=30) | COVID-19 (n=106) | Low RS* (n=87) | High RS* (n=19) | Survived (n=95) | Deceased (n=11) | Convalescent† (n=55) |
|---|---|---|---|---|---|---|---|
| Age, y; median (IQR) | 62 (27–71) | 60 (50–69) | 59 (49–68) | 62 (53–70) | 59 (49–68) | 69 (53–74)‡ | 59 (49–65) |
| Male sex, No. (%) | 20 (67) | 68 (64) | 54 (62) | 14 (74) | 58 (61) | 10 (91) | 39 (71) |
| Obesity, No. (%) | 1 (3.3)§ | 32 (30) | 25 (29) | 7 (37) | 31 (32) | 1 (9.1) | 19 (35) |
| Chronic kidney disease, No. (%) | 0 (0) | 10 (9.4) | 10 (11) | 0 (0) | 8 (8.4) | 2 (18) | 5 (9) |
| Hypertension, No. (%) | 2 (6.7)§ | 42 (40) | 36 (41) | 6 (32) | 39 (41) | 3 (27) | 21 (38) |
| Diabetes, No. (%) | 1 (3.3)§ | 26 (25) | 24 (28) | 2 (11) | 22 (32) | 4 (36) | 11 (20) |
| Cardiovascular disease, No. (%) | 2 (6.7) | 19 (18) | 13 (15) | 6 (32) | 14 (15) | 5 (45)‡ | 9 (16) |
| Active cancer, No. (%) | 0 (0) | 2 (1.9) | 2 (2.3) | 0 (0) | 1 (1.1) | 1 (9.1) | 0 (0) |
| Heart failure, No. (%) | 0 (0) | 7 (6.6) | 6 (6.9) | 1 (5.3) | 5 (5.2) | 2 (18) | 1 (1.8) |
| COPD, No. (%) | 0 (0) | 5 (4.7) | 5 (5.7) | 0 (0) | 4 (4.2) | 1 (9.1) | 1 (1.8) |
| Clinical data at sampling | | | | | | | |
| Received supplemental oxygen, No. (%) | | 66 (62) | 47 (54) | 19 (100)‖ | 56 (59) | 10 (91)‡ | |
| Invasive mechanical ventilation, No. (%) | | 2 (1.9) | 0 (0) | 2 (11)‖ | 0 (0) | 2 (18)‡ | |
| Medication | | | | | | | |
| LMWH preventive dose,¶ No. (%) | | 60 (57) | 55 (63) | 5 (26)‖ | 56 (59) | 4 (36) | |
| LMWH double preventive dose, No. (%) | | 25 (24) | 13 (15) | 12 (63)‖ | 22 (32) | 3 (27) | |
| LMWH treatment dose, No. (%) | | 1 (0.94) | 0 (0) | 1 (5.3) | 0 (0) | 1 (9.1) | |
| OAC, No. (%) | | 4 (3.8) | 3 (3.4) | 1 (5.3) | 2 (2.1) | 2 (18) | |
| Any anticoagulant, No. (%) | | 90 (85) | 71 (82) | 19 (100) | 80 (84) | 10 (91) | |
| Antibiotics, No. (%) | | 25 (24) | 17 (20) | 8 (42) | 20 (21) | 5 (45) | |
| Corticosteroids, No. (%) | | 14 (13) | 10 (11) | 4 (21) | 10 (11) | 4 (36)‡ | |
| Laboratory data at sampling | | | | | | | |
| H3Cit-DNA, ng/mL; median (IQR) | 84 (64–101) | 191 (136–294)# | 171 (129–251) | 331 (196–496)‖ | 176 (134–268) | 331 (200–575)‡ | 95 (66–122)§ |
| cfDNA, ng/mL; median (IQR) | 345 (319–486) | 551 (476–661)# | 527 (465–625) | 829 (575–931)‖ | 533 (470–639) | 900 (643–994)‡ | 334 (308–355)§ |
| NE, ng/mL; median (IQR) | 16 (12–23) | 144 (84–248)# | 129 (73–224) | 248 (192–557)‖ | 129 (82–226) | 336 (221–557)‡ | 22 (18–28)§ |

cfDNA indicates cell-free DNA; COPD, chronic obstructive pulmonary disease; COVID-19, coronavirus disease 2019; H3Cit-DNA, nucleosomal citrullinated histone H3; IQR, interquartile range; IU, international units; LMWH, low molecular weight heparin; NE, neutrophil elastase; OAC, oral anticoagulant; and RS, respiratory support.

*RS was divided into low (≤5 L oxygen on cannula) and high (>5 L oxygen on cannula, noninvasive respiratory support, or intubation).

†Patients sampled a median of 4-mo post-infection.

‡Significantly different from the survived group.

§Significantly different from the COVID-19 group.

‖Significantly different from the low RS group.

¶Five thousand IU of dalteparin or 4500 IU of tinzaparin.

#Significantly different from the healthy group.

# RESULTS

## Patient Characteristics

Demographics and clinical characteristics for the patients with COVID-19 (n=106) and healthy controls (n=30) are presented in Table 1. The median patient age was 60 (50–69) years (64.2% male) and did not differ from that in the healthy control group of 62 (27–71) years (66.7% male). No significant differences were observed in age, sex, or comorbidities between patients with low and high levels of RS. Anticoagulant treatment was given to 90/106 patients, and corticosteroids was given to 14/106 patients.

## Measurement of Circulating Markers of Neutrophil Activation and NETs in Patients With COVID-19

Plasma levels of H3Cit-DNA, cfDNA, and NE were all elevated in patients with COVID-19 compared with healthy individuals (Figure 1A). As expected, plasma levels of H3Cit-DNA correlated with cfDNA and NE (Table 2), indicating NET formation. Notably, 4 months postinfection onset all markers declined to levels found in healthy individuals (Figure 1B).

When the patient group was analyzed based on their need for respiratory support, it was clear that H3Cit-DNA, cfDNA, and NE all increased with increasing RS (Figure 2A). In addition, high levels at the time of admission were associated with poor clinical outcome defined as admission to ICU and/or short-term mortality ($P$=0.0002, $P$<0.0001, $P$=0.0058, respectively), as well as with short-term mortality alone (Figure 2B). This indicates a prognostic value of these markers in COVID-19.

## Circulating Markers of NET Formation Are Associated With Markers of Inflammation, a Prothrombotic State, and Endothelial Activation/Damage

H3Cit-DNA, cfDNA, and NE all correlated with white blood cell count, neutrophil count, neutrophil-to-lymphocyte ratio, and CRP. The link between NETs and an inflammatory state was further corroborated by correlations between all NET markers and TNFα, and between H3Cit-DNA, NE, and IL-6. All NET markers also correlated with D-dimer and PAP, and cfDNA and NE correlated with TAT, indicating a NET-associated prothrombotic state. Moreover, H3Cit-DNA, cfDNA, and NE correlated
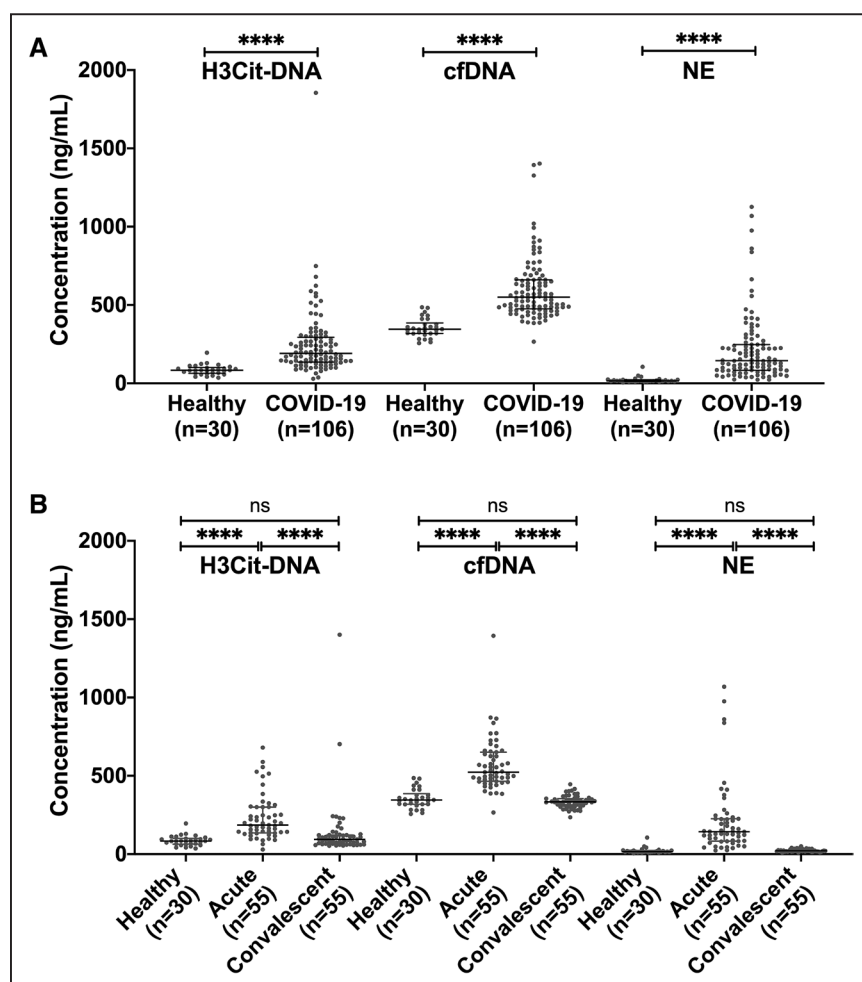


**Figure 1. Coronavirus disease 2019 (COVID-19) disease causes alteration in the circulating markers of neutrophil extracellular trap formation.**
**A**, Plasma levels of nucleosomal citrullinated histone H3 (H3Cit-DNA), cell-free DNA (cfDNA), and NE (neutrophil elastase) in patients with COVID-19 are increased compared with healthy individuals. **B**, Four months postinfection onset, plasma levels of H3Cit-DNA, cfDNA, and NE declined to those found in healthy individuals. Data are presented as median with interquartile range.

**Table 2. Circulating Markers of NET Formation Correlate With Markers of Inflammation, Dysregulated Hemostasis, and Endothelial Injury**

| | H3Cit-DNA | | cfDNA | | NE | |
|---|---|---|---|---|---|---|
| | $r_s$ | P value | $r_s$ | P value | $r_s$ | P value |
| H3Cit-DNA | 1.00 | … | 0.44 | ≤0.0001 | 0.33 | ≤0.001 |
| cfDNA | 0.44 | ≤0.0001 | 1.00 | … | 0.56 | ≤0.0001 |
| NE | 0.33 | ≤0.001 | 0.56 | ≤0.0001 | 1.00 | … |
| WBC | 0.27 | ≤0.01 | 0.37 | ≤0.0001 | 0.57 | ≤0.0001 |
| Neutrophil count | 0.25 | ≤0.05 | 0.44 | ≤0.0001 | 0.56 | ≤0.0001 |
| NLR | 0.33 | ≤0.01 | 0.48 | ≤0.0001 | 0.50 | ≤0.0001 |
| CRP | 0.46 | ≤0.0001 | 0.52 | ≤0.0001 | 0.42 | ≤0.0001 |
| TNFα | 0.26 | ≤0.01 | 0.24 | ≤0.05 | 0.24 | ≤0.05 |
| IL-6 | 0.35 | ≤0.001 | 0.07 | 0.50 | 0.29 | ≤0.01 |
| D-Dimer | 0.41 | ≤0.0001 | 0.43 | ≤0.0001 | 0.38 | ≤0.0001 |
| TAT | 0.09 | 0.36 | 0.28 | ≤0.01 | 0.21 | ≤0.05 |
| PAP | 0.43 | ≤0.0001 | 0.42 | ≤0.0001 | 0.34 | ≤0.001 |
| VWF | 0.21 | ≤0.05 | 0.32 | ≤0.001 | 0.35 | ≤0.001 |
| ADAMTS13 | −0.29 | ≤0.05 | −0.16 | 0.095 | −0.22 | ≤0.05 |

ADAMTS13 indicates a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; cfDNA, cell-free DNA; CRP, C-reactive protein; H3Cit-DNA, nucleosomal citrullinated histone 3; IL-6, interleukin-6; NE, neutrophil elastase; NET, neutrophil extracellular trap; NLR, neutrophil-to-lymphocyte ratio; PAP, plasmin-antiplasmin complex; TAT, thrombin-antithrombin complex; TNFα, tumor necrosis factor alpha; VWF, von Willebrand factor; and WBC, white blood cell.

with the endothelial activation/damage marker VWF, while H3Cit-DNA and NE, but not cfDNA, correlated inversely with the VWF-cleaving protease ADAMTS13 (Table 2), suggesting a NET-associated endothelial activation and injury. Taken together, these findings support a link between COVID-19-associated NET formation and immunothrombosis.

## DISCUSSION

Our findings expand upon prior work by showing a link between NETs and immunothrombosis as well as an association between circulating markers of NETs and clinical outcome in a large and unselected cohort of patients with moderate to severe COVID-19.

NETs are released by neutrophils in response to a variety of stimuli, including respiratory viruses[18,19] and inflammatory cytokines.[20] Given the key feature of virus-induced cytokine storms in severe COVID-19,[21] emanating excessive NET formation seems plausible, and is supported by our findings of correlations between NET markers and inflammatory cytokines. COVID-19-associated NET formation may, however, involve other, and perhaps overlapping, mechanisms, such as a direct infection of neutrophils by the SARS-CoV-2 virus,[10] and further experimental models are needed to fully understand the link between COVID-19 and NET formation.

Importantly, and regardless of mechanism of formation, an excessive NET formation may pose therapeutic targets in the combat of COVID-19. NETs are key elements in immunothrombosis, where they provide a stimulus and

scaffold for thrombus formation, endothelial damage, and organ dysfunction.[4,22] Although we did not investigate whether NET formation is a driver or a consequence of the disease, we corroborate a link between NETs and immunothrombosis in COVID-19 by revealing an association between circulating NET markers and inflammation, dysregulated hemostasis, and endothelial injury. Endogenous deoxyribonucleases (DNases) dismantle NETs and regulate NET-driven thrombosis,[23] and removal of NETs via recombinant DNase infusion improve microvascular perfusion in mouse models of sepsis.[24] Notably, aerosolized DNases are currently being evaluated in trials in COVID-19 (URL: https://www.clinicaltrials.gov/; unique identifiers: NCT04402944, NCT04541979, NCT04445285, NCT04432987, NCT04402970). Further investigations on the mechanisms behind NET formation in COVID-19 may also pave the way for other treatment options, such as agents that neutralize extracellular histones[25,26] or inhibit NET formation.[27,28] It will, however, be crucial to avoid disrupting protective effects of NET formation and the release of potentially damaging NET degradation products in tailoring novel therapeutic interventions targeting NETs. Circulating markers of NETs may thereby not only be useful in clinical prognostics but also in monitoring response to treatment.

Taken together, our findings support a link between COVID-19-associated NET formation and immunothrombosis and show that circulating markers of NETs are related to both disease severity and clinical outcome in a large cohort of patients with COVID-19. How to further translate the role of NETs in COVID-19 into clinical practice should be a priority for future research. Notably, the
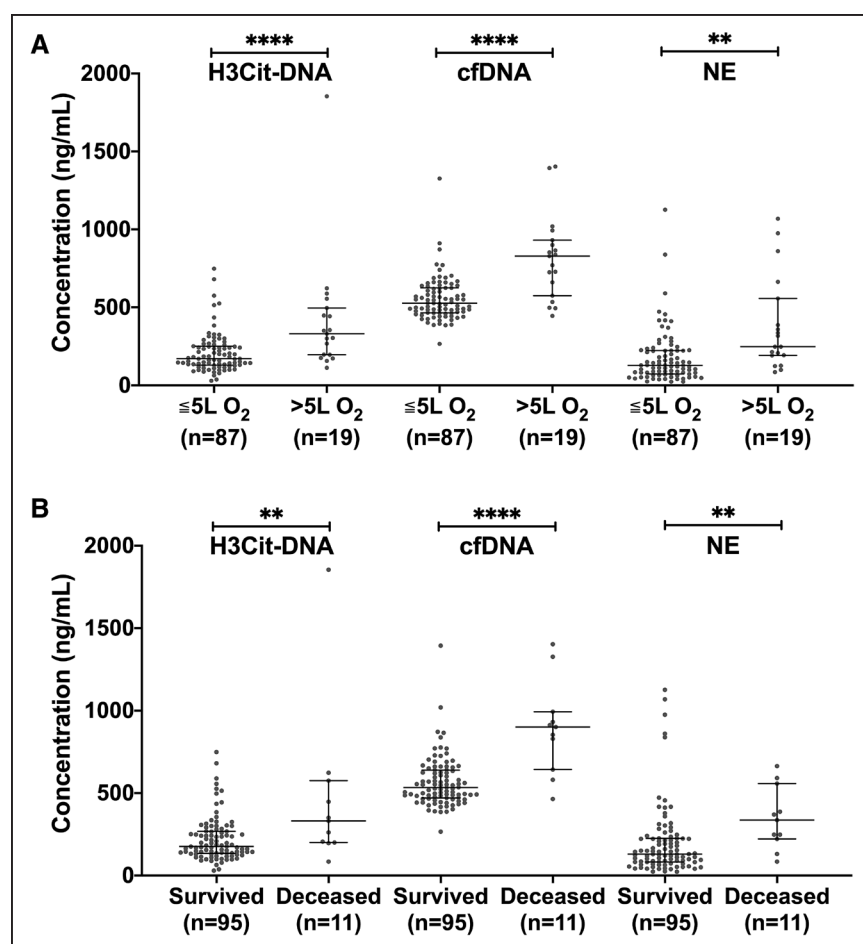
Figure 2. Circulating markers of neutrophil extracellular trap formation are associated to disease severity and clinical outcome in patients with coronavirus disease 2019 (COVID-19). A, Plasma levels of nucleosomal citrullinated histone H3 (H3Cit-DNA), cell-free DNA (cfDNA), and NE (neutrophil elastase) in patients with COVID-19 increase with increased need for respiratory support. B, Plasma levels of H3Cit-DNA, cfDNA, and NE were all lower in survivors compared with nonsurvivors. Data are presented as median with interquartile range.

assays quantifying circulating H3Cit-DNA, cfDNA, and NE are validated in human plasma samples and easily accessible, allowing for a potential clinical implementation. Our findings now argue for further studies determining if these markers are useful in clinical decision-making.

## ARTICLE INFORMATION

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### Disclosures

None.

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