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ARTICLE

Neutrophil calprotectin identifies severe pulmonary disease in COVID-19

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Abstract

Severe cases of coronavirus disease 2019 (COVID-19) are regularly complicated by respiratory failure. Although it has been suggested that elevated levels of blood neutrophils associate with worsening oxygenation in COVID-19, it is unknown whether neutrophils are drivers of the thrombo-inflammatory storm or simple bystanders. To better understand the potential role of neutrophils in COVID-19, we measured levels of the neutrophil activation marker S100A8/A9 (calprotectin) in hospitalized patients and determined its relationship to severity of illness and respiratory status. Patients with COVID-19 ($n = 172$) had markedly elevated levels of calprotectin in their blood. Calprotectin tracked with other acute phase reactants including C-reactive protein, ferritin, lactate dehydrogenase, and absolute neutrophil count, but was superior in identifying patients requiring mechanical ventilation. In longitudinal samples, calprotectin rose as oxygenation worsened. When tested on day 1 or 2 of hospitalization ($n = 94$ patients), calprotectin levels were significantly higher in patients who progressed to severe COVID-19 requiring mechanical ventilation (8039 ± 7031 ng/ml, $n = 32$) as compared to those who remained free of intubation (3365 ± 3146, $P < 0.0001$). In summary, serum calprotectin levels track closely with current and future COVID-19 severity, implicating neutrophils as potential perpetuators of inflammation and respiratory compromise in COVID-19.

KEYWORDS
calprotectin, COVID-19, neutrophils, neutrophil extracellular traps, SARS-CoV-2

1 | INTRODUCTION

Since December 2019, the outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 has spread to hundreds of countries and territories and has been declared a global pandemic. Severe COVID-19 results in death due to progressive hypoxemia, acute respiratory distress syndrome (ARDS), and multiorgan failure.[1] The

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role of the host response in this progression remains to be fully defined.

S100A8 (myeloid-related protein 8/MRP8) and S100A9 (MRP14) are calcium-binding proteins that belong to the S100 family. They exist mainly together as a biologically functional heterodimer known as S100A8/A9 or calprotectin. Calprotectin is found in abundance in neutrophils, where it can account for almost two-thirds of soluble protein in the cytosol. Calprotectin may also be detected at low levels in monocytes, macrophages, platelets, and squamous epithelial cells.[2] Upon neutrophil activation or death, calprotectin is released extracellularly where it has microbicidal functions (via heavy-metal chelation) and also serves as a proinflammatory ligand for innate receptors such as receptor for advanced glycation end products (RAGE) and TLR4.[3] Given its small size, easy diffusion between tissue and blood, and resistance to enzymatic degradation, calprotectin is a sensitive and dynamic marker of neutrophil activation anywhere in the body.[4,5] High levels of calprotectin have been found in many types of infectious and inflammatory diseases—including sepsis, myocardial infarction, inflammatory bowel disease, lupus, and adult-onset Still's disease—where it tracks closely with disease severity.[6–10]

Although work to date exploring COVID-19 pathophysiology has focused especially on macrophages and their products such as IL-6 and IL-1β, it has also been observed that elevated levels of blood neutrophils associate with worsening oxygenation in COVID-19.[11–13] Furthermore, our group and others have also revealed a potentially pathogenic role for neutrophil-derived extracellular traps (NETs) in COVID-19.[14,15] There remains though a paucity of information about neutrophil catalysts, checkpoints, and effector mechanisms in COVID-19—all of which could add actionable context to our understanding of the COVID-19 thrombo-inflammatory storm. Here, to better understand the potential role of neutrophils in COVID-19, we measured calprotectin in the blood of patients hospitalized with COVID-19 and determined its relationship to severity of illness and respiratory status.

2 | RESULTS AND DISCUSSION

Serum samples were obtained from 172 patients hospitalized with COVID-19 at a large academic hospital (Supplemental Table S1). In some cases, sera were stored at 4°C in the clinical laboratory for up to 48 h before being frozen. Interestingly, we found that calprotectin levels are stable in both serum and plasma for up to 6 d at 4°C (Supporting Information Fig. S1), which is in line with past research on the topic.[16] As compared with serum samples from 47 healthy controls, the COVID-19 samples showed markedly higher levels of calprotectin (Fig. 1A). For 36 patients, longitudinal sera were available. Nine of those patients showed a clinically meaningful change in oxygenation status during the period of collection (six worsening and three improving). Notably, calprotectin levels trended upward in the six patients for whom oxygenation worsened (Fig. 1B). We next asked how calprotectin compared to commonly available clinical measurements. Specifically, we assessed potential correlations with C-reactive protein, ferritin, lactate dehydrogenase, absolute neutrophil count, absolute lymphocyte count, hemoglobin level, and platelet count. Calprotectin demonstrated a positive correlation with C-reactive protein (Fig. 1C), ferritin ($r = 0.31$, $P = 0.0002$), lactate dehydrogenase ($r = 0.52$, $P < 0.0001$), absolute neutrophil count (Fig. 1D), and platelet count ($r = 0.39$, $P < 0.0001$). There was no correlation with absolute lymphocyte count (Fig. 1E), and a negative correlation with hemoglobin level ($r = -0.34$, $P < 0.0001$). In summary, calprotectin is markedly elevated in the sera of patients with COVID-19 and may rise as clinical status deteriorates.

We next determined each patient's clinical respiratory status at the time calprotectin was measured. As compared with patients breathing room air, patients requiring mechanical ventilation had significantly higher levels of calprotectin (Fig. 2A). Interestingly, differences were also appreciated between patients requiring noninvasive oxygen support (such as nasal-cannula oxygen) and mechanical ventilation (Fig. 2A). In contrast, C-reactive protein did not discriminate between patients requiring noninvasive oxygen support and those requiring mechanical ventilation (Fig. 2B). To further evaluate the potential clinical utility of calprotectin, we performed receiver operating characteristic curve analysis based on requirement for mechanical ventilation. As compared with C-reactive protein, ferritin, and lactate dehydrogenase, calprotectin had a superior area under the curve (Fig. 2C). Beyond clinical respiratory status, oxygenation efficiency can also be measured by comparing pulse oximetry (SpO_2) to the fraction of inspired oxygen (FiO_2). We tested the correlation between calprotectin and SpO_2/FiO_2 ratio, and found a striking negative association (Fig. 2D). A less robust association was also appreciated for C-reactive protein (Fig. 2E). In summary, calprotectin levels strongly associate with severe respiratory disease requiring mechanical ventilation.

To confirm these findings, we also obtained plasma from 119 of the 172 patients. As compared with plasma samples from 50 healthy controls, the COVID-19 samples showed markedly higher levels of calprotectin (Supporting Information Fig. S2A). Furthermore, for the COVID-19 patients, plasma calprotectin demonstrated a distinct negative correlation with SpO_2/FiO_2 ratio (Supporting Information Fig. S2B), and positive correlations with C-reactive protein (Supporting Information Fig. S2C) and absolute neutrophil count (Supporting Information Fig. S2D). We also found positive correlations between calprotectin and markers of NET release including cell-free DNA (Supporting Information Fig. S2E) and myeloperoxidase-DNA complexes (Supporting Information Fig. S2F).

Finally, of the 172 patients with serum samples evaluated here, 94 had sera available from the first two days of their hospitalization. When some of the aforementioned correlation analyses were reanalyzed with just these 94 samples (Supporting Information Fig. S3), we found a strong negative correlation with SpO_2/FiO_2, and strong positive correlations with C-reactive protein and absolute neutrophil count. Most importantly, calprotectin levels were significantly higher in those individuals who required mechanical ventilation at any point during their hospitalization ($n = 32$), as compared with those who did not ($P < 0.0001$, Fig. 2F). C-reactive protein was also analyzed (when available on the same day as the calprotectin measurement) and was found

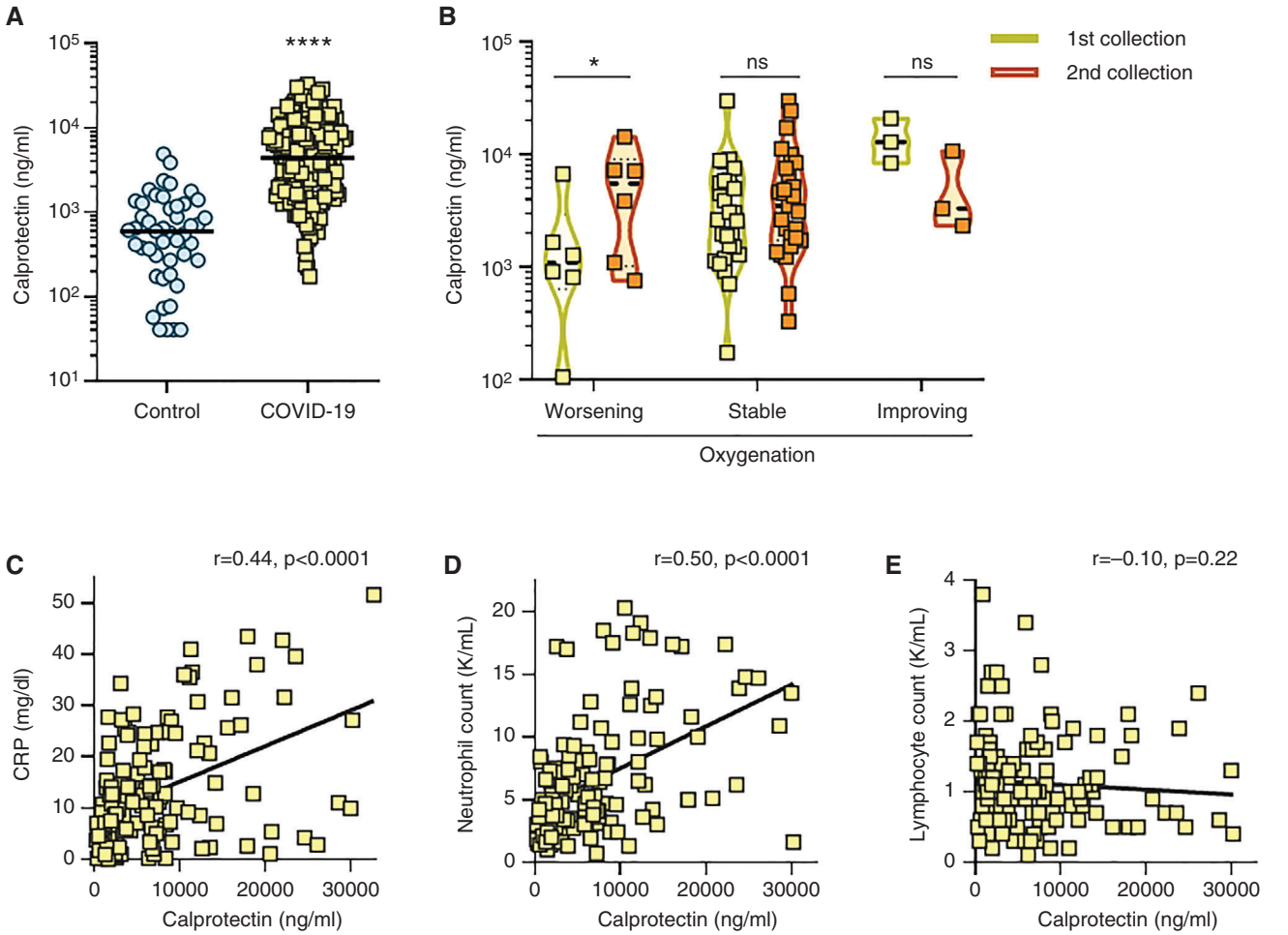

FIGURE 1 **Calprotectin in sera of COVID-19 patients and its association with clinical studies.** (**A**) Sera from COVID-19 patients ($n = 172$) and healthy controls ($n = 47$) were assessed for calprotectin (note log scale). COVID-19 samples were compared to controls by Mann-Whitney test; $^{****}P < 0.0001$. (**B**) For 36 patients, serum samples from two time points were available. Patients were grouped by whether their oxygenation was worsening, stable, or improving; $^{*}P < 0.05$ by paired Wilcoxon test. (**C**–**E**) Calprotectin levels were compared to clinical laboratory results (when available on the same day as the research sample). Spearman's correlation coefficients were calculated for C-reactive protein ($n = 138$), absolute neutrophil count ($n = 139$), and absolute lymphocyte count ($n = 139$)

to be predictive of any mechanical ventilation with a *P*-value of 0.0054 (Fig. 2G). Taken together, these data suggest a compelling relationship between neutrophil activation, as defined by serum calprotectin levels, and severe respiratory disease in COVID-19.

In summary, we report markedly elevated levels of serum and plasma calprotectin in the majority of patients hospitalized with COVID-19. Furthermore, we found that high levels of serum calprotectin on day 1 or 2 of hospitalization tracked with a requirement for mechanical ventilation at any point during the admission, a finding that should be assessed in larger prospective cohorts. These data provide strong evidence in support of neutrophils as potential players in moderate-to-severe cases of COVID-19.

Our study raises the need to investigate the specific form of neutrophil activation and/or cell death that floods COVID-19 blood with excess calprotectin. Tissue damage and neutrophil necrosis are potential sources of passive calprotectin release.[17] Active calprotectin secretion has been documented upon stimulation of neutrophils with complement C5 and fMLP.[18] Engagement of neutrophil P-selectin glycoprotein ligand-1 by E-selectin can also trigger neutrophils to actively release calprotectin in calcium ion-dependent fashion (TLR4 and its downstream MyD88 and Rap1-GTP trigger release via this same mechanism).[19] A newer consideration regarding active calprotectin release is NETosis.[14,20] NETs are extracellular webs of DNA, histones, and microbicidal proteins that appear to perpetuate many types of lung disease including smoking-related disease, cystic fibrosis, and ARDS. NETs leverage calprotectin as an antimicrobial strategy against Candida[21] and Aspergillus,[22] but, when left unchecked, NETs are also an important source of macrophage activation and microvascular occlusion. Whether passive calprotectin release in necrotic lung tissue or active release via NETosis (or another mechanism) is most important to COVID-19 pathophysiology awaits further research.

In addition to being an inflammatory marker, calprotectin may also have a direct role in the self-amplifying thrombo-inflammatory storm of COVID-19 via engagement and activation of innate immune sensors such as RAGE[23,24] and TLR4.[3,25] Depending on the system, calprotectin has also been detected both upstream[26] and downstream[27] of

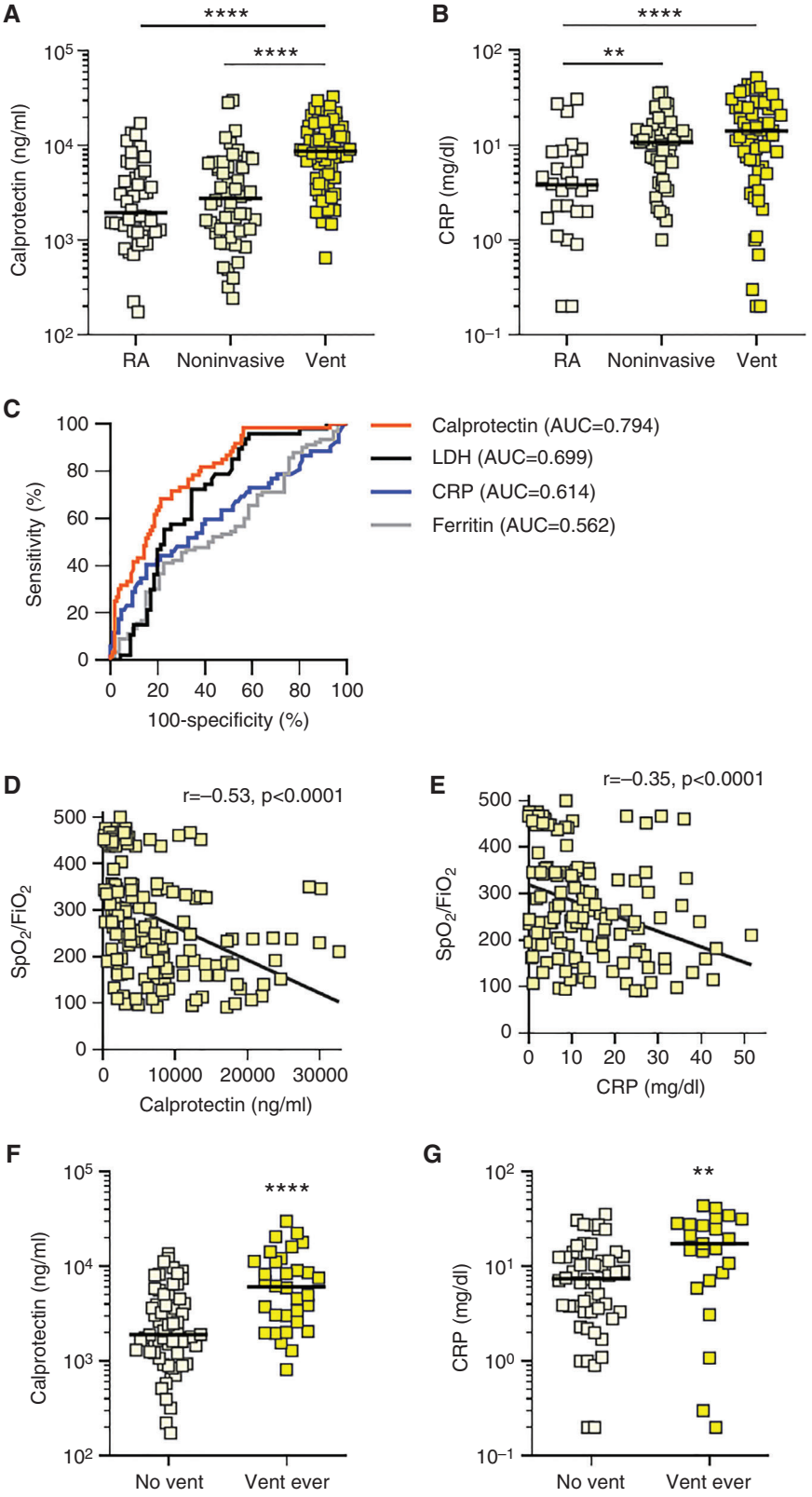

FIGURE 2 Levels of calprotectin track closely with oxygenation status. (**A**, **B**) Patients (n = 172) were grouped by clinical status: room air (n = 41), noninvasive supplemental oxygen (n = 71), or mechanical ventilation (n = 60). Levels of calprotectin and C-reactive protein were compared by Kruskal-Wallis test corrected by Dunn's test for multiple comparisons; $^{*}P < 0.05$ and $^{****}P < 0.0001$. (**C**) Receiver operating characteristic curves based on requirement for mechanical ventilation. (**D**, **E**) Calprotectin (n = 172) and C-reactive protein (n = 137) were compared to SpO_2/FiO_2 ratio for each patient, and correlations were determined by Spearman's test. (**F**, **G**) For 94 patients, a calprotectin level was available from hospital day 1 or 2. For 75 of the 94 patients, a CRP level was also available. Patients were then grouped by whether they at any point required mechanical ventilation (vent ever, n = 32) during their hospitalization. Groups were compared by Mann-Whitney test; $^{**}P < 0.01$ and $^{****}P < 0.0001$

IL-6, which has emerged as a possible therapeutic target in COVID-19. As a key alarmin molecule of the immune system, calprotectin modulates the inflammatory response by recruiting leukocytes and stimulating cytokine secretion.[28] Calprotectin also induces reactive nitrogen and oxygen species[29,30] and triggers microvascular endothelial cells to take on thrombogenic and proinflammatory phenotypes characterized by increased vascular permeability and synthesis of cytokines, chemokines, and adhesion molecules.[28] Furthermore, calprotectin is a potent stimulator of neutrophils themselves, promoting degranulation and phagocytosis,[31,32] as well as NETosis.[33] Intriguingly, crosstalk

between neutrophils, platelets, and calprotectin appears to play a role in both arterial and venous thrombosis,[33,34] which are being increasingly identified as complications of COVID-19.[35,36]

As we await definitive antiviral and immunologic solutions to the current pandemic, we posit that antineutrophil therapies[37–39] may be part of a personalized strategy for some individuals affected by COVID-19 who are at risk for progression to respiratory failure. In this context, calprotectin is well positioned to be an early indicator of patients with COVID-19 likely to progress to respiratory failure and who therefore require immunomodulatory treatment.

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DISCLOSURES

The authors declare no conflicts of interest.

AUTHORSHIP

H.S., Y.Z., S.Y., K.G., M.Z., J.A.M., C.B., W.W., and S.P.L. conducted experiments and analyzed data. H.S., Y.Z., N.L.L., R.J.W., C.L., J.S.K., and Y.K. conceived the study and analyzed data. All authors participated in writing the manuscript and gave approval before submission.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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