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RESEARCH ARTICLE

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Clinical utility of circulating calprotectin to assist prediction and monitoring of COVID-19 severity: An Italian study

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Abstract

Background: Calprotectin (S100A8/A9) has been identified as a biomarker that can aid in predicting the severity of disease in COVID-19 patients. This study aims to evaluate the correlation between levels of circulating calprotectin (cCP) and the severity of COVID-19.

Methods: Sera from 245 COVID-19 patients and 110 apparently healthy individuals were tested for calprotectin levels using a chemiluminescent immunoassay (Inova Diagnostics). Intensive care unit (ICU) admission and type of respiratory support administered were used as indicators of disease severity, and their correlation with calprotectin levels was assessed.

Results: Samples from patients in the ICU had a median calprotectin concentration of 11.6 μg/ml as compared to 3.5 μg/ml from COVID-19 patients who were not in the ICU. The median calprotectin concentration in a cohort of healthy individuals collected before the COVID-19 pandemic was 3.0 μg/ml (95% CI: 2.820–2.969 μg/ml). Patients requiring a Venturi mask, continuous positive airway pressure, or orotracheal intubation all had significantly higher values of calprotectin than controls, with the increase of cCP levels proportional to the increasing need of respiratory support.

Conclusion: Calprotectin levels in serum correlate well with disease severity and represent a promising serological biomarker for the risk assessment of COVID-19 patients.

KEYWORDS

circulating calprotectin, COVID-19, ICU, respiratory support, risk assessment

1 | INTRODUCTION

The pandemic of coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread throughout the world. While the majority of infections are mild or moderate and do not lead to severe illness and hospitalization, an estimated up to 15%[1] of patients have severe complications. Tools to estimate and predict of the risk of severe complications in COVID-19 would be of significant clinical value to direct limited resources toward those at highest risk and need of more intensive management. A large number of biomarkers have been evaluated for their efficacy to estimate and predict risk in patients with COVID-19.[2–5] Among these, calprotectin (CP) appears to represent an important candidate biomarker especially for severity assessment, risk stratification,[6–10] and to define an optimal strategy for the management of COVID-19 patients. CP, a calcium and zinc finger heterodimer of S100A8 and S100A9, is particularly abundant

in the cytoplasm of neutrophils and has both intracellular and extracellular functions. Inside the cells, it regulates calcium homeostasis, interacts with the cytoskeleton and microtubules and plays a role in intracellular trafficking of phagocytes. Its role for leukocyte transmigration has been recently shown in a mouse model.[11] When released, CP functions as a damage-associated molecular pattern (DAMP) or alarmin, promoting the inflammatory response, and its levels mirror the inflammation status. In fact, its serum concentration, may increase by 100 times during an inflammatory process in numerous conditions such as infection, inflammation, or cancer.[12] Recent studies have shown that circulating calprotectin (cCP) levels are increased in patients with severe COVID-19,[6,7] and positively correlate with neutrophil count, fibrinogen, and D-dimer levels.[6] Additionally, cCP levels strongly correlate with quick-Sequential Organ Failure score (qSOFA) and oxygen demand, discriminating intensive care unit (ICU) from non-ICU patients,[7,13] and supporting its value as biomarker for risk stratification (based on ICU requirement), multiorgan failure (MOF), and death in the early management of COVID-19 patients.[14] Moreover, one of the most interesting findings is cCP's role in predicting mechanical ventilation. Indeed, patients with a worsening clinical condition and need of invasive ventilation have demonstrated increasing levels of cCP compared to stable or improving patients who have no significant alterations in CP concentration.[8] In addition, cCP was significantly higher in patients who died versus survivors,[7,10] suggesting a possible prognostic role as mortality-associated biomarker in COVID-19 patients.[15] The aim of our study was to evaluate the clinical utility of measurement of cCP levels as an initial assessment, predictive, and monitoring tool for patients with COVID-19, with a focus on patients admitted for hospital care.

2 | MATERIALS AND METHODS

2.1 | Study population

Serum samples from a total of 245 patients with COVID-19, of which 125 had an active infection and 120 convalescent for at least 3 months, were collected during the period of March 2020 to June 2020 (wild-type Wuhan-Hu-1 strain) at San Giovanni Di Dio Hospital (Florence, Italy) and at ASST Papa Giovanni XXIII Hospital (Bergamo, Italy). Among these, 13 patients with COVID-19 had longitudinal samples ($N = 33$) collected during their hospitalization along with their associated respiratory requirements at each time point. In addition, sera from 110 apparently healthy individuals collected before the COVID-19 pandemic were tested as controls.

2.2 | Demographic and clinical characteristics of studied patients are summarized in Table 1

Of the 125 active COVID-19 patients, 41 were admitted to the ICU. Eight patients in the convalescent group were admitted to ICU at the time of hospitalization. Criteria for ICU were respiratory failure, acute respiratory distress syndrome (ARDS), or multiple-organ failure. The baseline respiratory support required for all patients was recorded according to the following categories: room air (RA), nasal cannula (NC), oxygen mask Venturi mask (VM, low FiO_2) or Mask (M60, FiO_2 60%), continuous positive flow airway pressure (CPAP), and orotracheal intubation (OT).

Within the scope of this study, the demographic and clinical data of the patients were recorded from patient follow-up files. Demographic/clinical data, laboratory parameters, and cCP were compared between groups.

The study was performed according to local ethical approval protocol no. 250/20. Informed consent was obtained from all subjects enrolled in the study. The study was in accordance with the Helsinki Declaration, as revised in 2013.

2.3 | Laboratory examinations

cCP was measured using a chemiluminescent assay (QUANTA Flash® Circulating Calprotectin assay, Inova Diagnostics, CE marked for in vitro diagnostic use in the European Union, investigational use only in the United States) on the BIO-FLASH® Instrument (Biokit SA). This assay enables the quantitative determination of CP in human serum and sodium citrate and potassium ethilendiaminotetracetycacid plasma. The analytical measuring range (AMR) of the assay extends from 0.18 to 22.76 µg/ml. For this study, a cut-off of 4.00 µg/ml was chosen based on cCP levels derived from of a reference population of 110 apparently healthy blood donors. The cut-off was established based on the 99th percentile of the results obtained on the reference subjects.

2.4 | Statistics

Descriptive statistics were presented as mean or median for continuous variables and number or percentage for categorical variables. Analyse-it for Microsoft Excel (version 5.90) and Graph Pad Prism (version 5.03) were used for statistical analysis and graphical presentation. Wilcoxon Mann–Whitney and analysis of variance (ANOVA) analysis were used to compare categorical variables, Mann–Whitney used to analyze differences between groups. $p < 0.05$ were considered statistically significant and 95% confidence intervals were calculated. No outliers were excluded from the calculations

3 | RESULTS

3.1 | COVID-19 patients show higher median levels of cCP in comparison to healthy controls

Patients were stratified into three clinical groups (COVID-19 active, COVID-19 convalescent, and healthy). cCP levels significantly differed across the various clinical groups (ANOVA $p < 0.0001$) as shown in

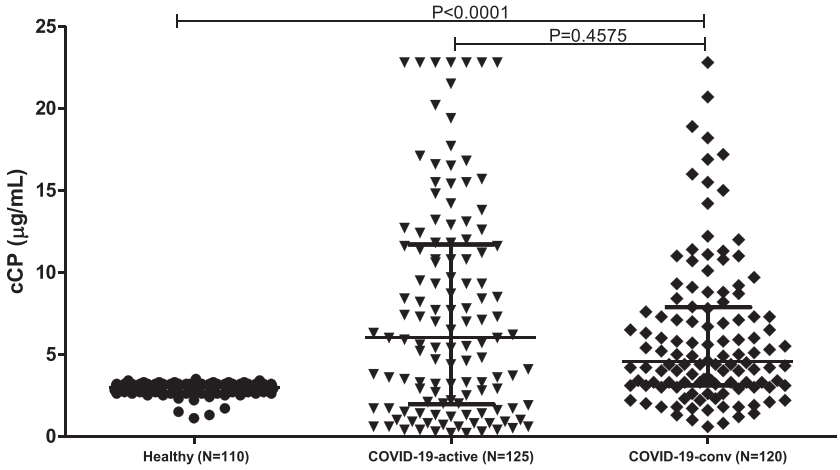

FIGURE 1 cCP levels in COVID-19 patients and control groups. Median levels of cCP were significantly lower in healthy controls than in COVID-19 patients with active or convalescent disease (COVID-19 conv). Comparison between groups evaluated by *t* test (Mann–Whitney) and across all groups by 1-way ANOVA Kruskal–Wallis test. ANOVA, analysis of variance; cCP, circulating calprotectin.

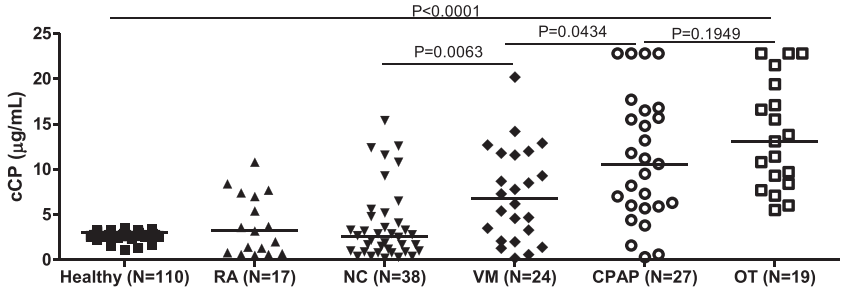

FIGURE 2 Respiratory support and cCP levels of patients at baseline. Patients were classified into six groups based on the respiratory support required at time of baseline specimen collection and cCP measurement. Median levels in each respiratory group are indicated. Comparison between groups evaluated by *t* test (Mann–Whitney) and across all groups by one-way ANOVA Kruskal–Wallis test. ANOVA, analysis of variance; cCP, circulating calprotectin. ANOVA, analysis of variance; cCP, circulating calprotectin; CPAP, continuous positive pressure flow airway pressure; NC, nasal cannula; OT, orotracheal intubation; RA, room air; VM, Venturi mask.

Figure 1. The lowest median cCP level among the three groups was observed in the healthy donor group (3.0 µg/ml). The median level of cCP in patients with active COVID-19 was twice as high as the healthy controls (6.0 vs. 3.0 µg/ml). While the median level of cCP in convalescent COVID-19 (COVID-19 conv) patients was lower than that in patients with active COVID-19 (4.6 vs. 6.0 ug/ml), the difference was not statistically significant.

3.2 | cCP levels correlate with impaired respiratory status

Based on the respiratory support required at time of baseline specimen collection and cCP measurement, we divided patients into six groups (in detail: control, RA, NC, VM, CPAP, OT). Baseline cCP levels and corresponding respiratory status were reported for 235 patients, including 125 patients with active COVID-19 and 110 controls. We compared the median levels of cCP among the clinical groups. Median levels of cCP were increased in all hospitalized COVID-19 patient groups (RA, NC, VM, CPAP, and OT), including whose required no additional respiratory support (RA), compared to the healthy control group (Figure 2). Interestingly, a very clear and significant rise in cCP levels was observed with an increasing need of respiratory support (VM, CPAP, and OT) (ANOVA $p < 0.0001$). In particular, the median level of cCP in patients with OT was over 5.0× (13.1 vs. 2.6 µg/ml) the level of patients on NC support.

3.3 | cCP levels are higher in patients admitted to the ICU

Of the 125 baseline specimens collected from COVID-19 patients with active infection, information on ICU admission was reported for 124 patients. Forty-nine patients were admitted to the ICU at hospital admission. These patients had median cCP levels more than three times higher (11.6 vs. 3.5 µg/ml, $p < 0.0001$) than the 83 patients who were not admitted to the ICU (Figure 3).

3.4 | cCP levels correlate with OT in patients with active and convalescent COVID-19

Nineteen of the 125 patients hospitalized with active COVID-19 were intubated. Additionally, 8 of the 120 convalescent patients (specimens collected 3 months after discharge) had previously been in the ICU and intubated. The median level of cCP was

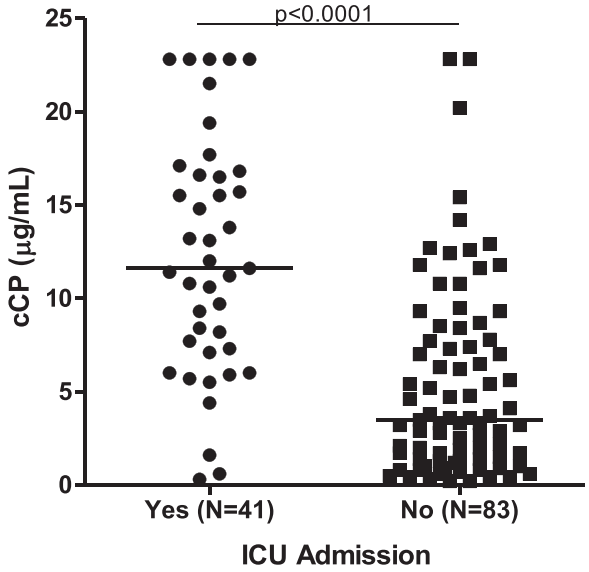

FIGURE 3 Baseline cCP levels in patients admitted to ICU (yes) compared to patients not admitted to ICU (no). Comparison between groups evaluated by *t* test (Mann–Whitney). cCP, circulating calprotectin; ICU, intensive care unit.

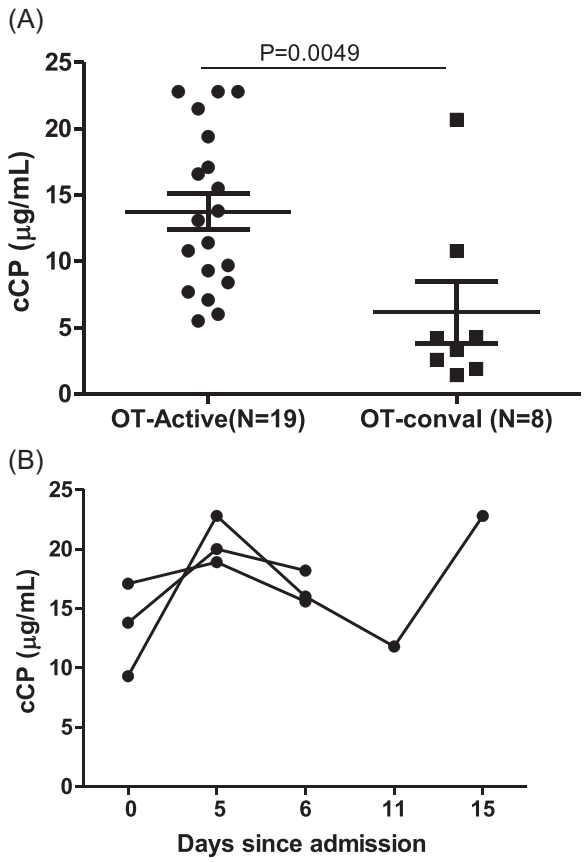

FIGURE 4 cCP levels in intubated patients (OT) (A) Baseline cCP in patients with active infection and in convalescent patients (>3 months after discharge) who were intubated during previous hospitalization. Comparison between groups evaluated by *t* test (Mann–Whitney); (B) cCP values over course of hospitalization for three intubated patients. cCP, circulating calprotectin; OT, orotracheal intubation.

13.1 µg/ml in the baseline specimens of intubated patients with active COVID-19 (Figure 4A). The median cCP value of convalescent specimens was 3.8 µg/ml. In 4/8 convalescent patients with a history of OT (OT-conval), the cCP levels were less than 4 µg/ml, in two sample levels were just over the study cut-off at 4.2 and 4.3 µg/ml, and in two specimen levels were moderate to strong positive (10.8 and 20.7 µg/ml, respectively). Three of the intubated patients with active infection had longitudinal follow-up samples available (see Figure 4B). Levels of cCP remained high throughout their hospitalization while intubated.

3.5 | Correlation of cCP levels and respiratory requirements in longitudinally followed patients

Twelve patients had longitudinal specimens (range two to six specimens) collected during their hospitalization. Seven patients had severe disease and changing respiratory requirements that were generally reflected in corresponding changes in cCP levels. As shown in Figure 5A, decreasing cCP levels were associated with decreasing respiratory support. One patient (patient 8) who eventually died, was admitted to the ICU, intubated, and remained there until death 17 days later (Figure 5B). During this time, cCP rose over the first 5 days to a very high level (~23 µg/ml, decreased over the next 6 days to ~12 µg/ml (still a very high level), and then rose back to 23 µg/ml for the next 5 days until death. At Day 4 of hospitalization, the patient was receiving fluimucil, omeprazol, paracetamol, potassium chloride, dexmedetomidine, insulin, sufentanil, fondaparinux, bisoprolol, clopidrogel, darunavir/cobicistat, and plaquenil (no steroids given during hospitalization). The remaining four patients required only a low level of respiratory support (RA or NC), not admitted to the ICU during hospitalization, and, therefore, are not included in Figure 5).

4 | DISCUSSION

SARS-CoV-2 can induce different clinical situations such as pneumonia, ARDS, disseminated intravascular coagulation, respiratory failure, shock, cytokine storm, and multiorgan dysfunction,[16–18] Patients in these clinical settings usually require ICU follow-up and treatment.[18,19] Moreover, severe disease also raises the rates of morbidity and mortality.[19]

Recently, several biomarkers have shown value to distinguish mild/moderate disease from severe disease in COVID-19 individuals at an early stage.[20–22] This is especially important for the variants of virus more aggressive, highly transmissible, vaccine-resistant, and able to cause more severe disease. For example, in a recent meta-analysis study, WBC, lymphocyte and platelet count, interleukin-6, and serum ferritin showed correlation with critical disease

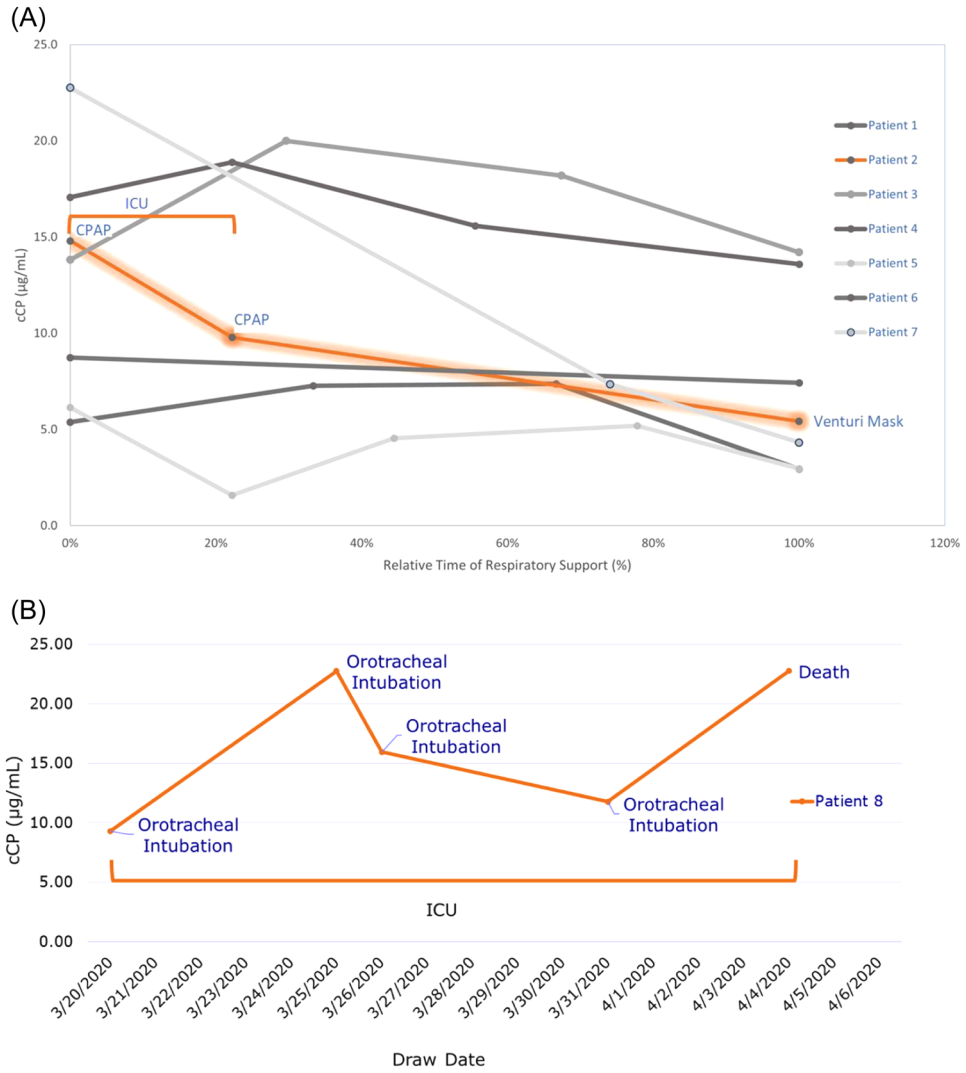

FIGURE 5 Longitudinal cCP levels and Respiratory Supplementation in hospitalized patients. (A) Highlighted patient (orange), initially in ICU receiving CPAP (high FiO_2) improved to venturi mask (low FiO_2) with corresponding decrease cCP levels. (B) Orotracheal intubated ICU patient who showed varying levels of cCP which steadily increased over the final 6 days of hospitalization before death. cCP, circulating calprotectin; CPAP, continuous positive pressure flow airway pressure; ICU, intensive care unit.

progression.[22] Circulating CP, released primarily by neutrophils, has recently been identified as a potential biomarker of inflammation that can be used to monitor the activity of a variety of inflammatory illnesses such as ANA associated rheumatic diseases (AARD), cardiovascular disease, sepsis, and other conditions. Regarding AARD, several studies have demonstrated the potential utility of cCP as a biomarker for monitoring rheumatic disease activity in rheumatoid arthritis,[23–25] psoriatic arthritis,[26] and systemic lupus erythematosus.[23,27] Importantly, cCP does not need de novo synthesis, thus offering a decisive kinetic advantage as a biomarker detecting the first sign of severe inflammation, in contrast to other routinely measured serum biomarkers such as C-reactive protein (CRP) or procalcitonin (PCT).

The literature on the connection between cCP and COVID-19 severity has been evolving,[5–8,10,13,14,28] In our large cohort of patients recruited at two Italian sites (Florence–Tuscany and Bergamo–Lombardy) and at different clinical stages, we assessed the clinical performance of cCP in COVID-19 patients as an initial evaluation, predictive, and monitoring parameter with special attention focused on patients admitted for hospital care. Furthermore, we compared the obtained cCP levels with those of healthy

TABLE 1 Demographic and clinical characteristics of study patients

Characteristics	COVID-19 active	COVID-19 convalescent (>3 months)	Healthy controls
Patients, *N* = 355	125	120	110
Age, years	23–94	23–93	20–64
Mean (SD)	68 (15)	71 (13)	42.85
Median (IQR)	71 (22)	73 (13)	43 (12)
Sex % m/f	51.2%/48.8%	64.8%/35.2%	80%/20%

and other inflammatory disease controls, notably AARD and HyperG patients.

In line with previous results, we observed that the cCP median level in patients with COVID-19 was higher than the controls (both healthy and disease controls), confirming the significant association between the high values of cCP and the presence of the disease. Similar to our results, in one of the most comprehensive studies on cCP in COVID-19, Silvin et al. demonstrated that cCP levels can achieve excellent discrimination between COVID cases and controls: area under the curve = 0.959 derived from receiver operating charateristic analysis.[6] Moreover, the authors defined signatures that were associated with disease severity in COVID-19 patients,[6] suggesting a predictive value that deserves prospective evaluation. They also observed that cCP concentrations correlated with the neutrophil count, plasma fibrinogen, and D-Dimer.[6] Similar data were reported by Shi et al. demonstrating that cCP levels were significantly higher in those individuals who required mechanical ventilation at any point during their hospitalization.[8] They also reported that cCP levels among those hospitalized was able to identify patients who needed mechanical ventilation as opposed to those who did not need intubation. In accordance with Shi et al., we also observed a significant correlation between cCP levels and respiratory status. In particular, all hospitalized COVID-19 patient groups, including those who required no additional respiratory support, showed increased median levels of cCP. However, a very well-defined and significant grading of cCP levels related to the increasing need of respiratory support was observed. Notably, patients on VM showed mean cCP level over 3.6× the level of patients on NC support. This result is also in line with the work of Chen et al. who demonstrated that increased serum cCP level correlated with need for oxygen support and overall poor outcome in COVID-19 patients.[7] Remarkably, regarding OT patients, we observed for the first time that median cCP levels were significantly higher in patients who remained intubated compared to baseline level. In contrast, cCP levels decreased in convalescent patients, suggesting the additional potential role of cCP as a recovery marker.

Moreover, we observed that patients admitted to the ICU, both at admission and during convalescence, displayed over 2.5× higher median levels of cCP than patients not admitted to the ICU. This result is in agreement with Chen et al. who reported significantly elevated levels of cCP in COVID-19 patients admitted to the ICU compared with non-ICU admitted patients, and further, that patients with fatal outcomes had significantly higher levels of cCP than those who survived.[7] In particular, the authors highlighted that patients with higher serum cCP had a 13-fold risk of death at 60 days from hospital admission.[7] Comparable results were reported by Bauer et al., observing that cCP had the best discriminative ability to predict ICU admission and MOF within 72 h if compared to other commonly employed biomarkers (i.e., lactate, CRP, PCT).[14] Additionally, in a recent case series, De Guardiana-Romualdo et al. reported that hospitalized COVID-19 patients who did not survive the infection had two-fold higher median values of cCP than those who survived.[10]

Since CP is an abundant normal constituent of neutrophil and related cells, considerable efforts have examined pre-analytical variables that could influence the accuracy of cCP measurement. Differences in blood collection matrices impact the stability and accuracy of cCP levels and this has led to concern over the practical measurement and reliability of cCP values. Several studies have now demonstrated that prompt processing of serum or plasma can minimize problems of artifactually increased cCP because of cellular degradation. With prompt processing of either serum or plasma within 2–6 h,[29,30] cCP can be reproducibly and accurately determined

Our study includes several strengths, such as the comparison between patients with active COVID-19, convalescent COVID-19 patients, and healthy controls collected before the COVID-19 pandemic. Furthermore, our cohort is derived from two different hospitals from two different cities (Florence and Bergamo) to minimize hospital-specific biases in patient populations and management differences. Circulating CP measurements at both hospitals were completed utilizing the same assay and instruments to minimize interlaboratory differences. A limitation of our study was the limited availability of data on other laboratory biomarkers during the collection period, as well as detailed information that would allow correlation of changes in cCP levels and respiratory requirements with drug administration.

In conclusion, inflammatory biomarkers, such as cCP, can be useful tools in early triage and risk stratification of patients presenting with COVID-19. Unfortunately, the evidence of the cCP role in COVID-19 is only in its infancy; however, an increasing number of studies suggest that cCP is a potentially reliable biomarker able to discriminate severe or critical COVID-19 cases versus controls, to assess the risk of disease severity, and to predict the need for ICU admission and mechanical ventilation. The high performance of cCP strongly suggests it may be a valuable biomarker in the development of personalized strategies for risk assessment and precision medicine management of patients.[31] Nevertheless, more studies are required to further define and validate the functionalities of cCP in COVID-19 patients, as well as in those with non- COVID-19 acute inflammatory conditions.

AUTHOR CONTRIBUTIONS

Data curation: Maria Infantino, Maria Grazia Alessio, Giulia Previtali, Valentina Grossi, Antonio Faraone, Alberto Fortini, Elisa Grifoni, Luca Masotti, Edda Russo, Emily FitzGerald, Gary L. Norman, and Roger Albesa. *Methodology*: Maurizio Benucci, Edda Russo, Amedeo Amedei, and Gary L. Norman. *Formal Analysis*: Gary L. Norman, Emily FitzGerald, and Roger Albesa. *Investigation*: Maria Infantino and Maria Grazia Alessio. *Writing–original draft preparation*: Maria Infantino, Maria Grazia Alessio, Giulia Previtali, Valentina Grossi, Maurizio Benucci, Antonio Faraone, Alberto Fortini, Elisa Grifoni, Luca Masotti, Edda Russo, Emily FitzGerald, Roger Albesa. *Writing—review and editing*: Maria Infantino, Edda Russo, Amedeo Amedei, Emily FitzGerald, Gary L. Norman, and Michael Mahler. *Visualization*: Maria Infantino, Gary L. Norman. *Roger Albesa*: *Supervision*: Maria Infantino,

Mariangela Manfredi, Amedeo Amedei, Gary L. Norman, and Michael Mahler. *Conceptualization*: Maria Infantino, Mariangela Manfredi, Emily FitzGerald, Gary L. Norman, and Roger Albesa.

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CONFLICTS OF INTEREST

Emily FitzGerald, Roger Albesa, Gary L. Norman, and Michael Mahler are employees of Werfen at the Headquarters & Technology Center Autoimmunity (Inova Diagnostics), Werfen, San Diego, CA, USA. The remaining authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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