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Cytokine 141 (2021) 155455

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Prognostic bioindicators in severe COVID-19 patients

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ARTICLE INFO

Keywords:
IL-6
KL-6
Biomarkers
COVID-19

ABSTRACT

Background: Severe acute respiratory syndrome caused by novel coronavirus 2 (SARS-CoV-2) emerged in Wuhan (China) in December 2019. Here we evaluated a panel of biomarkers to phenotype patients and to define the role of immuno-inflammatory mediators as biomarkers of severity.

Materials and methods: Serum samples were obtained from 24 COVID-19 patients on admission to hospital, before any treatment or infusion of intravenous steroids or invasive ventilation. KL-6 IL-6 and C-peptide were measured by chemiluminescent enzyme immunoassay. IL-6 assay was validated for accuracy and precision. The validity of variables used to distinguish severe from mild-to-moderate patients was assessed by areas under curves (AUC) of the receiver operating characteristic (ROC) and logistic regression was performed to combine parameters of the two groups.

Results: In the severe group, IL-6, CRP and KL-6 concentrations were significantly higher than in mild-to-moderate patients. KL-6, IL-6 and CRP concentrations were directly correlated with each other. ROC curve analysis of the logistic regression model including IL-6, KL-6 and CRP showed the best performance with an AUC of 0.95.

Conclusions: Besides corroborating previous reports of over-expression of IL-6 in severe COVID-19 patients requiring mechanical ventilation, analytical determination of other mediators showed that IL-6 concentrations were correlated with those of KL-6 and CRP. The combination of these three prognostic bioindicators made it possible to distinguish severe COVID-19 patients with poor prognosis from mild-to-moderate patients.

1. Introduction

Severe acute respiratory syndrome caused by new coronavirus 2 (SARS-CoV-2) emerged in Wuhan (China) in December 2019 [1,2]. In a few months, the etiological agent spread worldwide and was declared pandemic by the World Health Organization (WHO) in March 2020 [3,4]. The severe pneumonia-associated respiratory syndrome caused by the new coronavirus shows different clinical phenotypes, varying from mild-moderate to severe and critical with acute respiratory distress syndrome, requiring hospitalization and mechanical ventilation [5,6,44]. In general, male patients over 65 years with chronic lung disease and/or metabolic disorders such as arterial hypertension, obesity and diabetes show higher risk of severe disease [7–9,49]. Although the physiopathology of this extremely contagious disease is

Abbreviations: IL, interleukin; KL-6, Krebs von den Lungen; CRP, c-reactive protein; LDH, lactate dehydrogenase.

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https://doi.org/10.1016/j.cyto.2021.155455
Received 6 July 2020; Received in revised form 17 January 2021; Accepted 18 January 2021
Available online 28 January 2021

not fully understood, sepsis is considered a critical illness prototype for coronavirus 2 (COVID-19) pathogenesis, since severe cases are associated with immune disorder, lymphopenia (mainly CD4 and B cells) and hyper-cytokinaemia, elevated serum levels of IL-6 and IL-10 being predictive of severe course [10–12]. A recent study reported high levels of IL-6, IL-8 and TNF in peripheral blood of COVID-19 patients, contributing to hyperinflammation [46,13].

Aberrant host immune responses and especially inflammatory cytokine storms contribute to alveolar exudation and lung damage [51,52]. Pleiotropic Th2 cytokines, including IL-6, mediate the transition from the acute to the chronic phase of infective processes [11,14] and their number in critical patients is about an order of magnitude greater than in mild COVID-19 patients [9,15,16]. Significant dysregulation of serum levels of coagulation factors and anti-fibrinolytic components have been observed in subjects with high levels of IL-6, suggesting a link to COVID-19 pathophysiology [17,18,50]. Thus, clinical trials of different monoclonal antibodies (such as JAK-STAT moAbs) that block many inflammatory cytokines (including IL6) were recently approved as an effective treatment to improve prognosis in severe COVID-19 patients [10,19]. Different commercial immunoassays are available to analyse serum concentrations of IL-6 for clinical and research purposes [20].

Metabolic disorders, such as diabetes, in COVID-19 patients, have been associated with higher mortality, especially in older people. These comorbidities can modify host immunity and disease progression [21]. Obesity has also been associated with a negative prognosis: adipocytokines have an impact on metabolic, oncological and rheumatological disorders [22] and it was recently suggested that adipocyte dysfunction may lead to a specific immune environment predisposing obese patients to respiratory failure during COVID-19 [23]. Different studies aiming to assist clinicians in early identification of severe and critical patients have tested combinations of immune-mediators, including inflammatory cytokines, against age, comorbidities and clinical features [24].

The aim of the present study was to evaluate a panel of biomarkers including IL-6, KL-6, C-peptide, CRP, LDH, glycemia and pancreatic amylase. These molecules were analysed to phenotype our population of COVID-19 patients and to define a possible role of immuno-inflammatory mediators as biomarkers of severity.

2. Methods

2.1. Study population

Forty-one patients with COVID-19 (27 males (65.8%), 64.6 ± 18.4 years), hospitalized at Siena University Hospital in March and April 2020, and 30 healthy controls (18 males (60%), 59 ± 9.8 years) were enrolled consecutively in the study. They underwent clinical and radiological evaluation and were divided into mild-to-moderate and severe groups (the latter requiring intubation and invasive mechanical ventilation). Signs, symptoms, radiological and immunological features and serum concentrations of inflammatory biomarkers were entered in a database.

Serum samples were obtained from 24 of the patients on admission to hospital, before any treatment or infusion of intravenous steroids or invasive ventilation, and from the 30 healthy controls. Serum aliquots were stored at −80 °C until assay. All patients gave their written informed consent to the study that was approved by our local ethics committee (BIOBANCA-MIU-2010).

2.2. KL-6 and C-peptide assay

Assay of serum Krebs von den Lungen-6 (sKL-6) is based on agglutination of sialylated carbohydrate antigen with KL-6 mAb reagent (Fujirebio Europe, UK). Concentrations (expressed in U/ml) were determined by measuring changes in absorbance as described in previous papers [25,26]. Test reagents for C-peptide were from Fujirebio Inc. (Tokyo, Japan). This biomolecule was measured by the Lumipulse G600 II system. The limit of detection and limit of quantification were 0.001 nmol/L for C-peptide. The manufacturer's reference interval was 0.06–0.24 ng/ml. The tests were conducted according to the manufacturer's instructions.

2.3. IL-6: Quality control of analytical determinations and comparison of results

Serum concentrations of IL-6 were determined with an automatic biochemical analyzer (Cobas 8000 e602, Roche, Mannheim, Germany) and detected by electro-chemiluminescent immunoassay (ECLIA). Serum samples were also analysed by Lumipulse G600 II (Fujirebio, Japan).

Precision was evaluated according to CLSI EP5-A3 guidelines [27] using two concentrations of reagent control serum assays: Precicontrol multimarkers-1 (PCMM1) (Roche, Germany) (median 40.8 (range 32.2–49.4) pg/mL and PCMM2 (median 247 (range 195–299) pg/mL. Control serum assays were performed in duplicate, twice a day for 20 consecutive days. Linearity was evaluated according to CLSI EP6-A guidelines [25]. Four concentrations of IL-6 calibrators from the IL-6 kit (Fujirebio Inc.) were used. To establish the regression equation, the measurements were repeated four times for each concentration. To compare methods, we measured 54 serum samples from COVID-19 patients and healthy subjects. Comparisons were performed according to CLSI EP9-A3 guidelines [26].

2.4. Other markers

C-reactive protein IV (CRP) was measured by immunoturbidimetric test; α-amylase pancreatic protein (AMY-P) and lipase colorimetric (LIPC) by a colorimetric test; lactate dehydrogenase (LDH), glucose HK 3 (GLU-3), alanine aminotransferase and aspartate (ALT and AST, respectively) by an UV test. Control and calibrator reagents were supplied by Roche/Hitachi, Mannheim, Germany and analysed with a Cobas 8000 e602-c702 automatic biochemical analyzer (Roche/Hitachi, Mannheim, Germany).

2.5. Statistical analysis

The results are reported as means ± SD or as medians and inter quartiles (25th and 75th percentiles) for continuous variables. The Shapiro-Wilk test showed that the data did not have a normal distribution. To evaluate precision, the coefficient of variation was calculated. To evaluate linearity, the coefficient of determination (R^2) was determined by logistic regression. Bias between systems was calculated by Bland-Altman analysis.

One-way ANOVA non parametric test (Kruskal-Wallis test) and Dunn test were therefore performed for multiple comparisons. The chi-squared test was applied to categorical variables. The validity of variables used to distinguish severe from mild-to-moderate patients was assessed by areas under (AUC) receiver operating characteristic (ROC) curves and a logistic regression was performed to compare parameters between the two groups. Sensitivity, specificity, and positive and negative predictive values (PPV and NPV, respectively) were calculated for cut-offs of the different variables. The Youden index (J = max [sensitivity + specificity − 1]) was used to establish the best cut-offs. Spearman's rank correlation coefficients were assessed for relationships between variables in each group. Statistical analysis and graphic representation of the data were performed by GraphPad Prism 8.0 software.

3. Results

3.1. Study population

The main characteristics of the COVID-19 population, including

demographic data and IL-6 levels, are reported in Table 1. Other markers, together with clinical and immunological findings are reported in Table 2. There was a prevalence of males in both groups: 78.5% and 80% in mild-moderate and severe patients, respectively. Bilateral diffuse pneumonia was detected in 60% of the severe group and 57.2% of mild-moderate patients. Regarding symptoms, 8 out of 14 (58%) mild-moderate patients and 6 out of 10 (60%) severe patients showed at least two symptoms at onset. Seven out of 24 patients were without comorbidities. In particular, the severe group included four patients with arterial hypertension, one with diabetes, one with heart failure, one with lung disease and four with cancer (one breast cancer, one melanoma, one leukemia and one adenoma). Five mild-to-moderate patients showed arterial hypertension, four dyslipidaemia, three heart failure, two lung diseases (one asthma and one COPD) and one stroke.

3.2. IL-6 assay and analytical validation

The precision of IL-6 analytical determinations (CV%) for PCMM1 and PCMM2 reagent concentrations was 1.49% for the lower and 0.52% for the higher, respectively. The results of the linearity evaluation for IL-6 are shown in Fig. 1a. The regression equation between the expected and the measured value was $Y = 1001 * X + 1738$. The coefficient of determination (R^2) (range: 0–1000 pg/ml) of the regression analysis was 0.999. IL-6 concentrations were higher when measured by Lumipulse G600 II than by the Roche Elecsys system in COVID-19 patients (62.4 (21.4–371) vs 16.13 (5.5–91.5) pg/ml, $p < 0.0001$). R^2 between Lumipulse G600 II and Roche Elecsys results for IL-6 was 0.98 ($p < 0.001$) (Fig. 1b).

Bland–Altman difference analysis revealed a mean bias of 122.5 ± 12.5 (95% limits of agreement 97.9–147.1) for IL-6 between the Lumipulse G600 II and the Roche Elecsys systems (Fig. 1c).

In the severe group, IL-6 concentrations were significantly higher than in mild-to-moderate patients ($p = 0.009$) and healthy controls ($p = 0.0004$) (Fig. 2a). Serum concentrations of IL-6 in mild to moderate patients were not significantly different from those of controls (median 32.4 and 19.2 pg/ml, $p = 0.76$).

3.3. Other serum biomarkers

Serum concentrations of KL-6 were correlated with IL-6 concentrations ($r = 0.43$; $p = 0.04$). They were higher in severe than in mild to moderate patients ($p = 0.035$) (Fig. 2b), and showed similar concentrations in healthy and mild-moderate patients (221, 226.3 and 903 U/ml for controls, mild-moderate and severe patients, respectively). CRP levels were significantly higher in severe than in mild to moderate patients ($p = 0.03$) (Fig. 2c). No significant differences in C-peptide concentrations were found between the two groups of COVID-19 patients ($p > 0.05$), although levels were above the normal range suggested by the manufacturer. Our population only included a single type 2 diabetes patient. Serum concentrations of CRP were correlated with KL-6 ($r = 0.51$; $p = 0.04$) and were significantly higher in severe than in mild-to-moderate patients ($p = 0.03$). In turn, CRP concentrations were very significantly correlated with LDH ($r = 0.71$; $p = 0.002$), without significant differences in LDH concentrations in the two groups ($p = 0.10$).

Table 1
Demographic data and IL-6 concentrations in healthy controls, Mild to moderate and severe groups.

	Healthy controls (n = 30)	Mild to Moderate (n = 14)	Severe (n = 10)	P value
Age (m ± SD)	59 ± 9.8	62.2 ± 15.6	65.2 ± 8	ns
Gender (M:F)	18/12	11/3	8/2	ns
Smoking Habits (never/current/former)	12/3/15	6/4/4	3/1/7	ns
IL-6 (pg/ml) (median (*IQR*))	19.2 (16.7–22.8)	32.4 (11.8–143.5)	333.9 (66.3–843.2)	0.0004

Table 2
Clinical, immunological and radiological data of COVID-19 groups.

	Mild to moderate group (n = 14)	Severe group (n = 10)	P value
Symptoms			
Fever	13	8	ns
Cough	4	4	ns
Vomit	1	0	ns
Weakness	1	1	ns
Dyspnoea	12	6	0.04
Comorbidities			
yes/no	10/4	7/3	ns
KL-6 (U/ml) (median (*IQR*))	320 (226.3–927.8)	903 (333.8–1956)	0.035
CRP (mg/l) (median (*IQR*))	3.5 (2.2–5)	5.4 (4.5–12.8)	0.03
LDH (U/l) (median (*IQR*))	256 (227.3–329)	342.5 (290–585)	ns
Glycemia (mg/dl) (median (*IQR*))	98.7 (75–113.5)	109 (103–125)	ns
Lipase (U/l) (median (*IQR*))	21 (18–25)	23 (15–29)	ns
Pancreatic Amylase (U/l) (median (*IQR*))	28.7 (15–41.5)	29.5 (17–35)	ns
AST (U/l)(median (*IQR*))	22.5 (14–27)	23.25 (12–49.5)	ns
ALT (U/l) (median (*IQR*))	14.2 (10–18)	20.5 (12–26.5)	ns
C-peptide (ng/ml) (median (*IQR*))	1.72 (1–1.93)	2.6 (1.73–2.8)	ns
Blood count			
RBC (cell/mm^3)	4.3 ± 0.9	4.5 ± 0.5	ns
WBC (cell/mm^3)	4.9 ± 1.6	7.6 ± 6	ns
PLT (cell/mm^3)	278 ± 156	183.7 ± 62	ns
Leucocytes counts			
Lymphocytes (×10^3/ml)	18.2 ± 11	13.4 ± 6.4	0.04
Neutrophils (×10^3/ml)	74 ± 13	77 ± 8	ns
Eosinophils (×10^3/ml)	0.05 ± 0.1	0.25 ± 0.7	ns
Monocytes (×10^3/ml)	7.4 ± 2.7	8.8 ± 9	ns
Basophils (×10^3/ml)	0.22 ± 0.2	0.15 ± 0.13	ns
Lymphocytes subsets			
CD4(%)	44.4 ± 7.8	44.6 ± 13	ns
CD8(%)	22 ± 8.7	31 ± 18	ns
CD19(%)	18.1 ± 7	12.6 ± 9.3	0.03
NK(%)	13 ± 8	10 ± 6.9	ns
Chest X ray (monolateral/bilateral/bilateral diffused)	2/4/8	0/4/6	ns

3.4. Immunological markers

Comparison of groups showed lower concentrations of peripheral lymphocytes in severe than in mild to moderate patients (13.4 ± 6.4 and 18.2 ± 11 respectively, $p = 0.04$). The same trend was found for CD19 percentages which were 18.1 ± 7 and 12.6 ± 9.3, respectively ($p = 0.03$). No other significant differences in immunological data were found. Interestingly, peripheral concentrations of platelets showed an indirect correlation with KL-6 and CD4 percentages ($r = -0.56$, $p = 0.04$ and $r = -0.63$, $p = 0.01$, respectively), in contrast with a direct correlation between platelet count and CD8 percentages ($r = 0.53$, $p = 0.03$).

3.5. Panel of biomarkers

The ROC analysis of IL-6 data distinguished COVID-19 patients from healthy subjects, with an area under the curve of 0.78 (95% CI 0.63–94, $p = 0.009$) and a best cut-off value of 27.3 pg/ml (75% sensitivity, 100% specificity). ROC analysis also distinguished mild to moderate from severe patients. Serum IL-6 showed the best performance with an area under the curve of 0.85 (95% CI 0.79–1, $p = 0.003$) and best cut-off value of 62.45 pg/ml (80% sensitivity, 71% specificity) (Fig. 3a). In

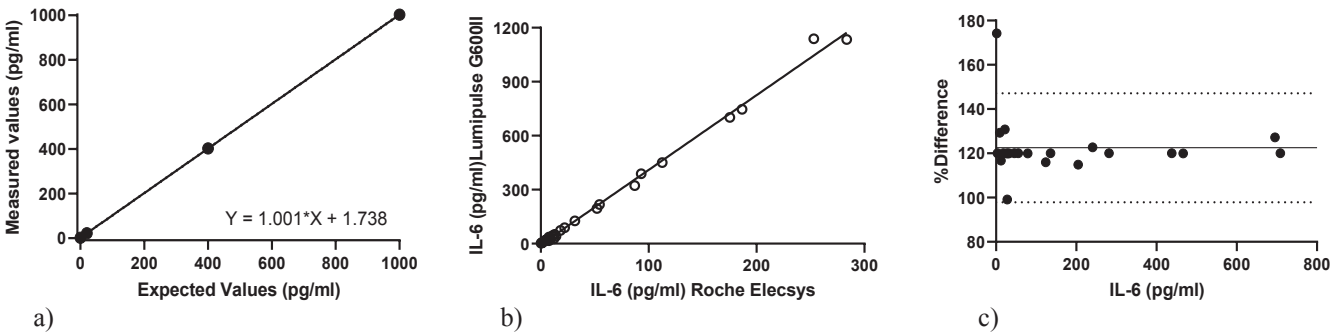

Fig. 1. . (a) Linearity of IL-6 in the Lumipulse G600II system. (b) Method comparison of the two technology. (c) Assay validation Bland-Altman plots. Dotted lined indicates 95% limits of agreement.

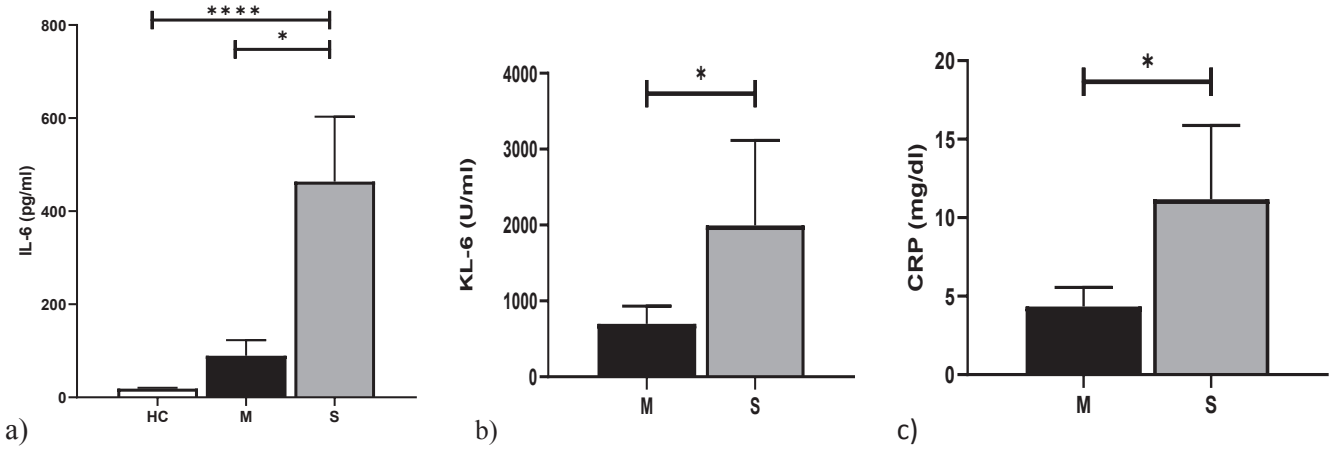

Fig. 2. . (a) comparison analysis of IL-6 between Healthy controls (HC), Mild-to-moderate and Severe Covid-19 Patients. (b–c) comparison analysis of KL-6 and CRP between Mild-to-moderate and Severe Covid-19 Patients. *p < 0.05, **p < 0.01, ***p < 0.001, *** p < 0.0001.

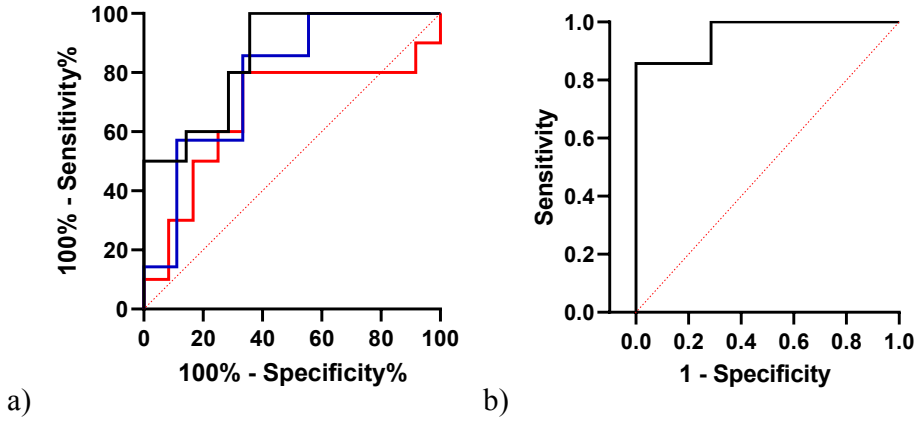

Fig. 3. . (a) ROC curve analysis of CRP (blue), KL-6 (Red) and IL-6 (black) between mild to moderate and severe Covid-19 patients. (b) Combination model of KL-6, IL-6 and CRP.

the logistic regression, severe patients were tested as dependent variable against KL-6, CRP, LDH, C-peptide and IL-6 as independent variables. ROC curve analysis combining IL-6, KL-6 and CRP proved to have the best performance, showing an AUC of 0.95 (95% CI 0.86–1; NPP(%) 85.7, PPP(%) 85.7, p = 0.004) (Fig. 3b).

4. Discussion

This monocentric retrospective study examined the clinical and laboratory characteristics of a population of patients with COVID-19 admitted to an Italian university hospital.

Combinations of biomarkers were compared in mild-to-moderate versus severe patients in search of a panel of biomarkers with prognostic value. We also evaluated the analytical performance of an IL-6 assay kit based on chemiluminescent enzyme immunoassay, which was confirmed to be good: cut-off values discriminating healthy subjects from COVID-19 patients were 7.0 and 27.3 pg/ml for Cobas 6000 and Lumipulse G600 II, respectively.

Analysis of IL-6 and other inflammatory biomarkers in serum of COVID-19 patients showed higher levels of IL-6, KL-6 and CRP in

patients admitted to the ICU and requiring intubation and mechanical ventilation due to diffuse interstitial pneumonia than in mild-to-moderate patients. IL-6 is involved in cytokine storms and huge increases in its concentrations have been reported in COVID-19 patients at high risk of death [28–30]. An increase in inflammatory mediators, such as CRP and IL-6, in the course of the disease may help identify patients with poor prognosis, requiring rapid clinical intervention [31,32]. Wang et al. reported high CRP concentrations in the early stages of SARS-CoV-2 infection and documented a positive correlation between CRP levels and the severity of lung damage detected by HRCT (i.e. CRP emerged as a prognostic biomarker) [33].

Interestingly, Zhu Z et al. compared clinical and immune-inflammatory parameters in non-severe and severe patients, reporting IL-6, CRP and hypertension as independent predictors of COVID-19 severity [34]. To our knowledge, ours is the first study investigating the combination of KL-6, IL-6 and CRP concentrations in a population of COVID patients. KL-6 is a mucin-like glycoprotein widely studied in interstitial lung diseases [35,36,47,48] and its concentrations in peripheral blood can reflect severe interstitial lung damage, epithelial lung alterations and regenerative processes secondary to SARS-CoV-2 infection [37]. Serum KL-6 concentrations were determined in a Japanese population of COVID-19 patients treated with ECMO due to severe lung involvement, and were in the same range as those of our cohort [38]. Other authors recently analysed serum concentrations of KL-6 in COVID-19 patients in relation to COVID-19-related acute respiratory distress syndrome, and suggested KL-6 as a differential prognostic biomarker [39,40,45].

Xue M et al. found KL-6 levels in severe patients significantly upregulated with respect to mild patients, supporting the idea that this mediator reflects the level of lung injury and inflammation [41]. In line with these findings, the same trend was confirmed in our albeit limited population. No data is currently available on C-peptide in COVID-19 patients. The findings of the present study suggest for the first time that inflammatory status induced by SARS-CoV-2 may be associated with insulin resistance.

Platelet count also showed a correlation with immunological data, in particular with CD4 and CD8 percentages and KL-6 concentrations. Recent papers report that platelet percentages are depressed in very severe patients [42,43]. In particular, Tien R et al. showed that critical patients had low lymphocyte and platelet counts and elevated CRP and IL-6 concentrations. Our data was perfectly in line with these findings and for the first time, also demonstrates a correlation between IL and 6 and KL-6 concentrations.

Although there is much data in the literature on over-expression of IL-6 in severe COVID-19 patients requiring mechanical ventilation, besides corroborating previous reports, our results suggest that a panel of three prognostic bioindicators can improve recognition of severe forms. The combination of KL-6, IL-6 and CRP made it possible to distinguish severe COVID-19 patients with poor prognosis from mild-to-moderate patients.

CRediT authorship contribution statement

L. Bergantini: Conceptualization, Methodology, Software. **E. Bargagli:** Conceptualization, Methodology, Software. **M. d'Alessandro:** Data curation, Writing - original draft. **R.M. Refini:** Data curation, Writing - original draft. **P. Cameli:** Data curation, Writing - original draft. **L. Galasso:** Visualization, Investigation. **C. Scapellato:** Visualization, Investigation. **F. Montagnani:** Supervision. **S. Scolletta:** Supervision. **F. Franchi:** Supervision. **S. Valente:** Supervision. **D. Bennett:** Software, Validation. **G. Sebastiani:** Software, Validation. **B. Frediani:** Writing - review & editing. **F. Dotta:** Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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