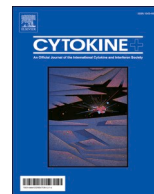




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# Cytokine profiles in the detection of severe lung involvement in hospitalized patients with COVID-19: The IL-8/IL-32 axis

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## ARTICLE INFO

**Keywords:**  
Cytokines  
Interleukins  
Profiles  
COVID19

## ABSTRACT

Coronavirus disease 2019 (COVID-19) is an infectious respiratory disorder caused by a new coronavirus called SARS-CoV-2. The pathophysiology of severe COVID-19 is associated with a “cytokine storm”. IL-32 is a key modulator in the pathogenesis of various clinical conditions and is mostly induced by IL-8. IL-32 modulates important inflammatory pathways (including TNF- $\alpha$ , IL-6 and IL-1b), contributing to the pathogenesis of inflammatory diseases. IL-32 was never evaluated before in COVID-19 patients stratifying as mild-moderate and severe patients. A total of 64 COVID-19 patients, 27 healthy controls were consecutively enrolled in the study. Serum concentrations of biomarkers including IL-1 $\beta$ , IL-10, IFN- $\gamma$ , TNF- $\alpha$  and IL-6 were quantified by bead-based multiplex analysis and Serum concentration of IL-8 and IL-32 were determined by enzyme-linked immunosorbent assay (ELISA) kits. Interestingly, among the blood parameters, neutrophil and lymphocyte counts were significantly lower in severe COVID-19 patients than in the other, on the contrary, CRP was significantly higher in severe patients than in other groups. The cytokines that best distinguished controls from COVID-19 patients were IL-8 and IL-32, while IL-6 resulted the better variables for discriminate severe group.

The best model performance for severe group was obtained by the combination of IL-32, IL-6, IFN- $\gamma$ , and CRP serum concentration showing an AUC = 0.83. A cut off of 15 pg/ml of IL-6 greatly discriminate survivor from death patients. New insights related to the cytokine storm in COVID-19 patients, highlighting different severity of disease infection.

## 1. Introduction

Coronavirus disease 2019 (COVID-19) is an infectious respiratory disorder caused by a new coronavirus called SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), that broke out in 2019 and quickly became pandemic [1,34]. SARS-CoV-2 infection may cause a respiratory syndrome associated with pneumonia, which has different clinical phenotypes, varying from mild to critical. Severe forms are characterized by acute respiratory distress, requiring hospitalization and mechanical ventilation [2–4,35]. The individual clinical course and prognosis of SARS-CoV-2 infection remain unpredictable [5]. The pathophysiology of severe COVID-19 is associated with a “cytokine storm”, characterized by anomalous release of various pro-inflammatory cytokines [6,33] which contribute to alveolar exudation and lung

damage [7]. However, it is unclear whether all patients show the same type of cytokine release [8,36]. Efforts have recently been made to predict which patients will develop severe COVID-19.

Clinical and biochemical indices and cytokines have been widely investigated. Low lymphocyte and neutrophil percentages and elevated C-reactive protein (CRP) are the most common abnormalities related to SARS-CoV-2 infection and correlated with acute lung injury [9,37]. Regarding cytokine production, many cytokines and chemokines have been evaluated to improve patient management and enable a targeted personalized approach. Overproduction of the proinflammatory cytokines IL-1, IL-6, IL-8 and TNF- $\alpha$  and low of expression IFN- $\gamma$  have been found in severe COVID-19 patients [9,10].

Another proinflammatory cytokine, IL-32, activates signalling pathways of mitogen-activated protein kinases (MAPKs) and of nuclear

**Abbreviations:** IL, interleukins; FVC, forced vital capacity; FEV1, forced expiratory volume in the first second; DLCO, diffusing capacity of the lung for carbon monoxide.

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<https://doi.org/10.1016/j.cyto.2022.155804>

Received 20 October 2021; Received in revised form 13 December 2021; Accepted 4 January 2022

Available online 18 January 2022

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factor (NF)- $\kappa$ B. It is produced by various epithelial and immune cells such as T lymphocytes, NK cells and monocytes. The IL-32 gene is transcribed as six alternative splice variants [11,12]. The effect of IL-32 ranging from host immune responses to cell differentiation. Nine different IL-32 isoforms have been identified and characterized for their effects on cells, but their roles remain not completely understood [13].

The mechanism by which IL-32 exerts its signalling properties is unclear [14]: IL-32 is a key modulator in the pathogenesis of various clinical conditions and is mostly induced by IL-8 [15]. IL-32 modulates important inflammatory pathways (including TNF- $\alpha$ , IL-6 and IL-1b), [15] contributing to the pathogenesis of inflammatory diseases. Moreover, elevated IL-32 levels triggered by influenza virus infection appear to hamper viral replication [16–18].

Although the role of IL-32 in several viral infections has been investigated in recent years, no evidence is yet available for COVID-19 [15]. In the present study, we analysed cytokine production profiles in serum of patients with COVID-19 at the time of hospitalization. These profiles were compared with those of healthy controls. A stratification of COVID-19 patients was also performed based on severity progression of the disease. IL-1 $\beta$ , IL-10, IFN- $\gamma$ , TNF- $\alpha$  and IL-6 were analysed through bead-based assay and IL-8 and IL-32 through an ELISA kit. Moreover IL-32 were for the first time evaluated in COVID-19 patients.

A panel of cytokines emerged as predictive of disease severity. The IL-8 – IL-32 axis could be involved in the pathogenesis of COVID-19 and may be linked to acute lung damage.

## 2. Materials and methods

### 2.1. Patients and samples

This prospective study was performed at Siena University Hospital between October 2020 and April 2021. Patients with positive nasopharyngeal swab for SARS-CoV2 and requiring hospitalisation for clinical manifestations related to COVID-19 were enrolled upon admission to hospital. Patients needing hospitalization for other than COVID-19 issues with positive nasopharyngeal swab were not included in the study, as well as all those patients not able or refusing to provide informed consent for this study. Other exclusion criteria included SARS-Cov2 vaccination and/or previous home treatment with monoclonal antibodies specific for COVID-19.

A total of 64 COVID-19 patients (46 male, 65 (59–67) years) were consecutively enrolled in the study. Upon hospitalization, patients were divided into three groups according to the severity of lung involvement: i) “mild” for patients treated with or without conventional oxygen support, ii) “moderate” for patients requiring non-invasive mechanical ventilation and/or high-flows oxygen therapy and iii) “severe” for patients requiring orotracheal intubation. Signs, symptoms, radiological data, immunological features and serum concentrations of inflammatory biomarkers were entered in a database.

Twenty-seven healthy controls (9 male, 58 (36–78) years) were also included in the study.

Serum samples were all collected on the day of hospital admission, before any treatment or infusion of intravenous steroids or invasive ventilation. Serum aliquots were stored at  $-80^{\circ}\text{C}$  until assay.

Clinical, demographical and laboratories data were collected for all patients.

All patients gave their written informed consent to the study that was approved by our local ethics committee (BIOBANCA-MIU-2010).

### 2.2. Immunoassays

Serum concentrations of biomarkers including IL-1 $\beta$ , IL-10, IFN- $\gamma$ , TNF- $\alpha$  and IL-6 were quantified by bead-based multiplex LEGENDplex™ analysis (LEGENDplex™ Custom Human Assay, Biolegend) according to the manufacturer's instructions. Reactions were run in duplicate with a BD FACSCantoII flow cytometer (BD-Biosciences San Jose, CA, USA).

The data was processed with Legendplex V8.0 software (Biolegend) and concentrations were expressed in pg/ml.

### 2.3. ELISA kit

Serum concentration of IL-8 and IL-32 were determined by enzyme-linked immunosorbent assay (ELISA) kits by Invitrogen and Mybio-source, USA, following the manufacturers' instructions. The concentrations were read at 450 nm with a Perkin Elmer Victor X4 fluorimeter and expressed in pg/ml. The detection limit of IL-8 ranging from 15.625 to 1000 pg/ml, while for IL-32 The detection limit of IL-8 ranging from 15.63 to 1000 pg/mL pg/ml.

### 2.4. Statistical analysis

The results were expressed as means and standard deviations (SD) or medians and 25th and 75th percentiles as appropriate. One-way ANOVA non-parametric test (Kruskal-Wallis test) and Dunn test were performed for multiple comparisons among HC group and mild, moderate and severe COVID-19 patients. The Chi-squared test was used for categorical variables. A p value less than 0.05 was considered statistically significant. Principal component analysis (PCA) was used to determine the cytokine combinations associated with severity. We also assessed the validity of variables used to distinguish controls from COVID-19 patients through Heatmap, and COVID-19 severity groups, by areas under the receiver operating characteristic curve (AUC ROC). Sensitivity, specificity and positive and negative predictive values (PPV and NPV, respectively) were calculated for cut-offs of the different variables. The Youden index ( $J = \max [\text{sensitivity} + \text{specificity} - 1]$ ) was used to establish the best cut-offs for diagnosis. Statistical analysis and graphic representation of the data were performed by GraphPad Prism 4.0 software and BioVinci software (BioTuring Inc., San Diego, CA, USA).

## 3. Results

### 3.1. Patients features

Demographic data, blood cell count, clinical data and immunological findings are reported in Table 1. There was a prevalence of males in the three groups of patients: 73%, 70% and 73% in the mild, moderate and severe groups, respectively. All patients underwent chest X-ray within the first two days of hospitalization: bilateral diffuse pneumonia was detected in 65%, 57.2% and 52.6% of patients in the severe, mild and moderate groups, respectively. Regarding symptoms, 30 out of 32 severe patients, 10 out of 17 mild patients and 12 out of 15 moderate patients showed at least two symptoms at onset, with a prevalence of fever. In the total population of 64, only 17 patients were without specific medical or surgical comorbidities.

Interestingly, among the blood parameters, neutrophil and lymphocyte counts were significantly lower in severe COVID-19 patients than in the other groups ( $p = 0.002$  and  $p = 0.03$  respectively). On the contrary, CRP was significantly higher in severe patients than in other severity groups ( $p = 0.02$ ).

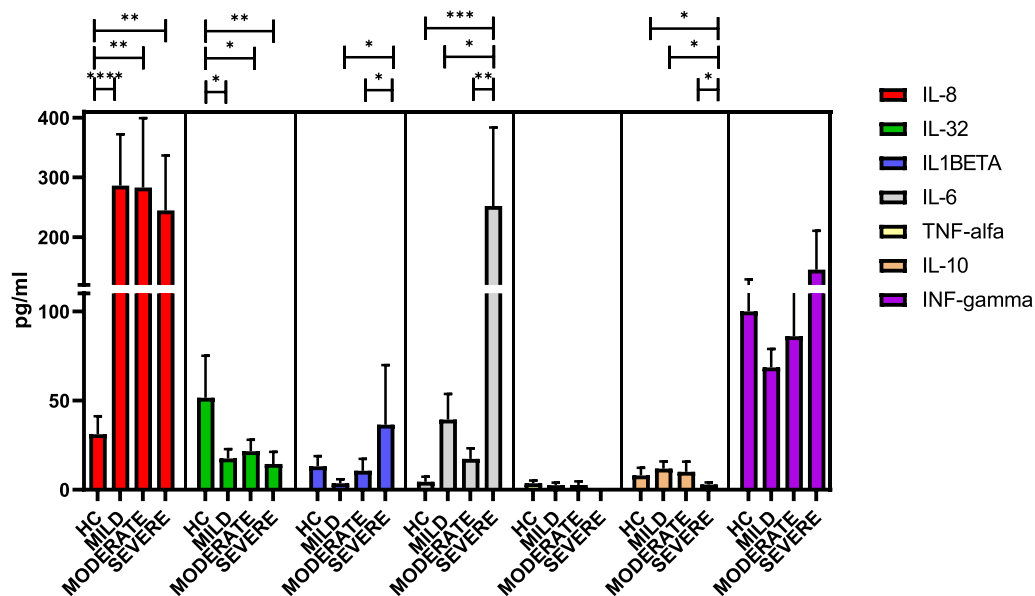
### 3.2. Cytokine levels in relation to severity of pulmonary involvement

Serum concentrations of the cytokines considered in our study are reported in Fig. 1 and supplementary Table 1. We observed that IL-8 concentrations were significantly higher in COVID-19 patients than in controls. On the contrary, IL-32 concentrations were significantly higher in controls than in COVID-19 patients ( $p = 0.02$ ). IL-6 concentrations were higher in severe COVID-19 patients than in the mild, moderate and control groups ( $p = 0.0002$ ). Higher concentrations of IL-1 $\beta$  were recorded in the severe group than in mild and moderate COVID-19 patients ( $p = 0.048$ ;  $p = 0.042$ ) Finally, lower concentrations of IL-10 were detected in severe COVID-19 patients than in other groups ( $p < 0.05$ ).

**Table 1**

Demographic data and blood parameters of analyzed cohort.

|                                         | HC (n = 27) | Mild (n = 32)    | Moderate (n = 17) | Severe (n = 15)  | P values |
|-----------------------------------------|-------------|------------------|-------------------|------------------|----------|
| Sex (m/f)                               | 12/15       | 23/9             | 12/5              | 11/4             | ns       |
| Age (years)                             | 58 (36–78)  | 69 (60–83)       | 62 (57–71)        | 66 (60–70)       | ns       |
| Past medical history – no. (%)          |             |                  |                   |                  |          |
| Cardiovascular Diseases                 | –           | 4 (12.5)         | 3 (17)            | 2 (13.3)         |          |
| Type 2 Diabetes                         | –           | 3 (9.3)          | 4 (23.5)          | 2 (13.3)         |          |
| Obesity (<30)                           | –           | 5 (15.6)         | 3 (17.6)          | 4 (26.6)         |          |
| Hypertension                            | –           | 10 (31)          | 6 (35)            | 4 (26.6)         |          |
| Other conditions*                       | –           | 9 (28)           | 4 (23.5)          | 7 (46)           |          |
| Blood Count                             | –           |                  |                   |                  |          |
| Neutrophils (%)                         | –           | 78 (67.5–84)     | 78.2 (70–85)      | 60.5 (51–73)     | 0.002    |
| (n.r: 55–70)                            |             |                  |                   |                  |          |
| Monocytes (%)                           | –           | 5.9 (4.7–9.8)    | 7.2 (5.3–8.4)     | 6.6 (5.3–8.4)    | ns       |
| (n.r: 1–13)                             |             |                  |                   |                  |          |
| Lymphocytes (%)                         | –           | 16.5 (10.1–24)   | 15.1 (9–19)       | 9.4 (6–13.4)     | 0.03     |
| (n.r: 25–48)                            |             |                  |                   |                  |          |
| Eosinophils (%)                         | –           | 0.1 (0–0.7)      | 0 (0–0.2)         | 0 (0–0.1)        | ns       |
| (n.r: 1–5)                              |             |                  |                   |                  |          |
| Basophils (%)                           | –           | 0.3 (0.2–0.4)    | 0.2 (0.1–0.25)    | 0.2 (0.1–0.2)    | ns       |
| (n.r: 0–1.5)                            |             |                  |                   |                  |          |
| RBC (10 <sup>6</sup> /mm <sup>3</sup> ) | –           | 4.7 (4.2–5)      | 4.7 (4–5)         | 4.6 (4.4–4.8)    | ns       |
| (n.r: 4.1–5.8)                          |             |                  |                   |                  |          |
| WBC (10 <sup>3</sup> /mm <sup>3</sup> ) | –           | 6 (4.9–6)        | 6 (4.3–7.3)       | 6.2 (4.7–7.6)    | ns       |
| (n.r: 4–10)                             |             |                  |                   |                  |          |
| Hb (g/dl)                               | –           | 13.8 (11.7–14.5) | 13.7 (12.7–14.5)  | 14.2 (13.3–14.5) | ns       |
| (n.r: 12.8–18)                          |             |                  |                   |                  |          |
| HCT (%)                                 | –           | 42.5 (35.5–43.4) | 41.5 (37.7–42.3)  | 42.2 (40.2–43)   | ns       |
| (n.r: 4–52)                             |             |                  |                   |                  |          |
| PLT (10 <sup>3</sup> /mm <sup>3</sup> ) | –           | 199 (167–242)    | 169 (124–210)     | 227 (178–351)    | ns       |
| (n.r: 150–400)                          |             |                  |                   |                  |          |
| CRP (mg/dl)                             | –           | 3.3 (1.8–6.2)    | 4 (2.2–12)        | 4.8 (3.9–10.9)   | 0.02     |
| (n.r: 0–0.5)                            |             |                  |                   |                  |          |



**Fig. 1.** Cytokines concentrations comparison among HC and mild, moderate and severe COVID-19 patients. The histograms report mean (center bar)  $\pm$  SEM (upper and lower bars). If not indicated, p value is not significant. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

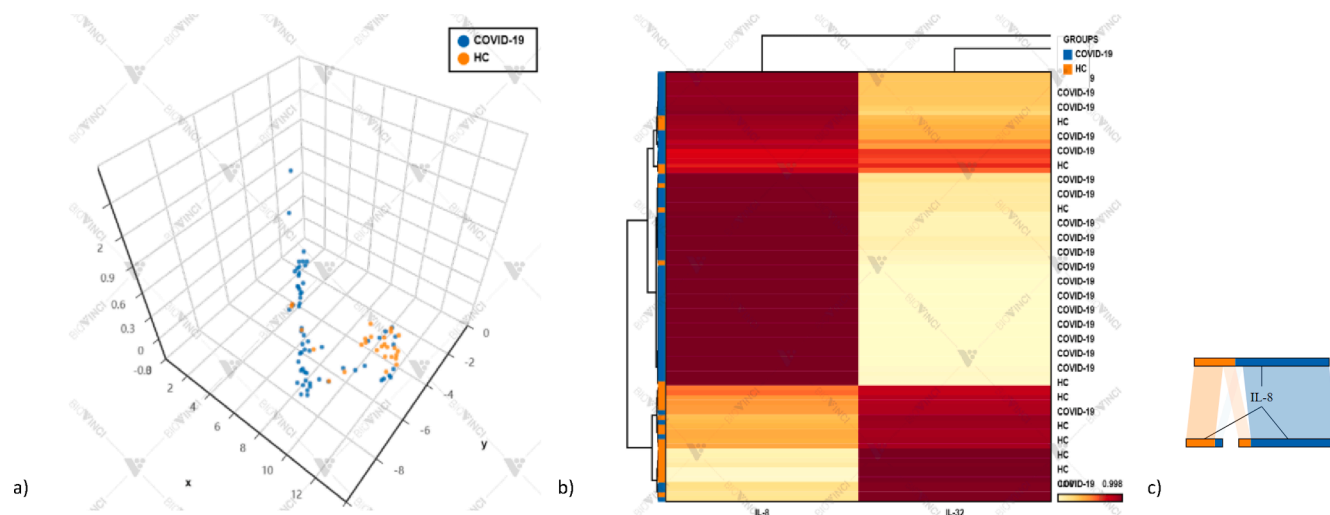
No differences in concentrations of TNF- $\alpha$  and INF- $\gamma$  were found between COVID severity groups and controls.

#### 4. Cytokine profiles in controls and COVID-19 patients

Concentrations of cytokines were measured in serum from COVID-19 patients on admission, and from healthy controls. As expected, PCA emphasized important overall cytokine profile differences between COVID-19 patients and healthy controls (Fig. 2). The first and second

principal components explained 95.8% and 4.2% of the total variance. The heatmap, based on hierarchical clustering applied to row and columns, is reported as Euclidean distance. Heatmap colours vary from white, indicating relative under-representation to red, indicating relative over-representation. The cytokines that best distinguished controls from COVID-19 patients were IL-8 and IL-32.

The cytokine profiles of controls and COVID-19 patients were used to create a decision tree to determine which variables clustered best by the Gini criterion. The tree indicated IL-8 concentrations.



**Fig. 2.** a) PCA analysis between HC (orange) and Covid19 patients (blue). b) Heatmap analysis between COVID-19 and HC: The heat map is based on hierarchical clustering applied for row and columns and reported as Euclidean distance. The color of the heat varies from white (indicating relative under-representation) to red (indicating relative over-representation). Clusters are sorted according to adjusted p values, so that the cluster at the top shows the most significant abundance changes between the two conditions (HC and COVID 19 Patients) (c) Decision tree model. The different cytokine levels of patients were employed to create a decision tree model for the detection of best clustering variables.

ROC curves were plotted to assess the discriminatory value of the parameters and to determine cut-off values with sufficient sensitivity and specificity. IL-8 and IL-32 were confirmed as the best discriminatory markers to distinguish controls from COVID-19 patients. IL-8 showed AUC = 0.88, 95 %CI: 0.81–0.96;  $p < 0.001$ , while IL-32 showed AUC = 0.71, 95 %CI: 0.64–0.77;  $p = 0.01$ . IL-6 also showed good discriminatory potential with AUC = 0.70, 95 %CI: 0.57–0.85;  $p = 0.007$ .

IL-8 values below a cut-off of 343.5 pg/ml had 66% sensitivity and 96% specificity in discriminating COVID-19 patients from controls. IL-32 below a cut-off of 54 pg/dl showed 59% sensitivity and 71% specificity in discriminating COVID-19 patients from controls. IL-6 below a cut-off of 5 pg/ml showed 59% sensitivity and 88% specificity in discriminating COVID-19 patients from controls.

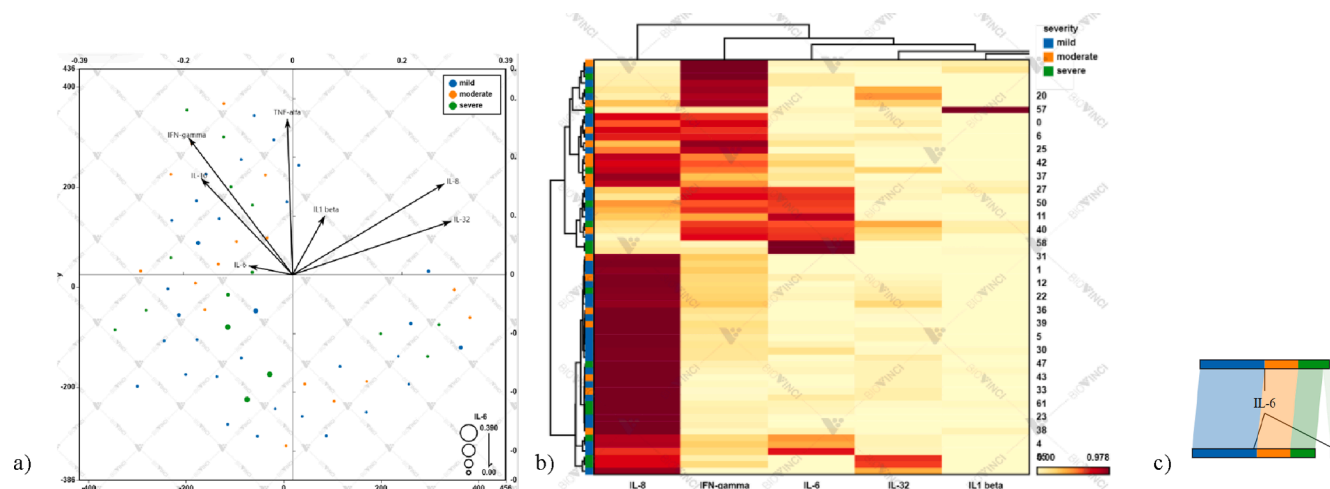
Interestingly, although the ROC curve did not show statistical significance for other cytokines, in the logistic regression, when COVID-19 patient was tested as dependent variable and all cytokines as independent variables, ROC curve analysis of model performance showed AUC

= 0.91 (95 %CI: 0.84–0.98; NPP(%): 92.5, PPP(%): 73.3;  $p < 0.0001$ ).

#### 4.1. Cytokine profiles in COVID-19 patients in relation to severity

PCA was performed to identify different cytokine profiles in COVID-19 patients with mild, moderate and severe lung involvement using the Barnes-Hut algorithm (Fig. 3). The first and second principal components explained 23.54% and 19.37% of the total variance. The heatmap showed good clustering of IL-8, INF- $\gamma$  and IL-6. Heatmap colouring was the same as above. The cytokine profiles of COVID-19 severity groups were used to create a decision tree to determine the variables that clustered best by the Gini criterion. The cytokine that best distinguished severity groups proved to be IL-6 (Fig. 3c).

ROC curves were plotted to assess the discriminatory value of the parameters and to determine cut-off values with sufficient sensitivity and specificity. IL-6 was confirmed to be the cytokine that best distinguished COVID-19 severity groups, showing an AUC = 0.69, 95 %CI:



**Fig. 3.** a) PCA analysis between Covid19 patients groups: severe, moderate and mild. b) Heatmap analysis between COVID-19 severity groups. The heat map is based on hierarchical clustering applied for row and columns and reported as Euclidean distance. The color of the heat varies from white (indicating relative under-representation) to red (indicating relative over-representation). Clusters are sorted according to adjusted p values, so that the cluster at the top shows the most significant abundance changes between the three conditions (mild, moderate and severe) c) Decision tree model. The different cytokine levels of patients were employed to create a decision tree model for the detection of best clustering variables.



0.53–0.86;  $p = 0.02$ .

IL-6 values below a cut-off of 15 pg/ml had 65% sensitivity and 67% specificity in discriminating severe from non-severe COVID-19.

The logistic regression model was applied in order to identify the variables that best distinguished the group of severe COVID-19 patients. The severe COVID-19 group was tested as dependent variable, and the cytokines as independent variables. The best model performance was obtained by the combination of IL-32, IL-6 and IFN- $\gamma$ , which showed  $AUC = 0.80$  ( $p = 0.0015$  (95 %CI: 0.67–0.92; NPP(%): 81.2, PPP(%): 60;  $p = 0.0015$ ). Interestingly, when we also added blood parameters to this model, only serum concentrations of CRP increased model performance, showing  $AUC = 0.83$  (95 %CI: 0.68–0.97; NPP(%): 81.2, PPP(%): 60;  $p = 0.0029$ ) (Fig. 4).

#### 4.2. Survival curve analysis

Fig. 5 shows the results of the log-rank (Mantel-Cox) test for COVID-19 patients, stratified by IL-6 concentrations. For IL-6 median values and a cut-off of 15 pg/ml, the severity groups of COVID-19 patients showed a significant difference in survival rate ( $p = 0.008$ , hazard ratio: 0.25 (95 %CI 0.0003–27)).

### 5. Discussion

This monocentric study examined blood parameters and cytokine levels of a population of patients admitted to Siena University Hospital with COVID-19. We evaluated serum concentrations of IL-6, TNF- $\alpha$ , INF- $\gamma$ , IL-10 and IL-1 $\beta$ , the best-known pro-inflammatory markers, and IL-8, known to play a key role in the development of this infection. For the first time, we also evaluated serum concentration of IL-32 in a cohort of COVID-19 patients.

In line with previously reported data, we found that IL-8 was significantly elevated in COVID-19 patients and was the marker that best discriminated patients from healthy controls [19,20]. On the other hand, we observed lower concentrations of IL-32 in COVID-19 patients than controls, irrespective of disease severity. These two cytokines therefore showed opposite trends in terms of concentration. What do other studies in the literature have to say? Interestingly, Imaeda et al. reported that a new isoform of IL-32 suppresses IL-8 expression [21]. Heinhuis et al. [22] reported that overexpression of some isoforms of IL-32 result in enhanced expression of IL-8 and in line with our results, Ouhara et al. reported that IL-32 plays a role in the downregulation of inflammatory responses, such as IL-8 production [23]. Our findings are evidence of converse effects of IL-8 and IL-32, suggesting that an imbalance in the expression of these two cytokines (favouring IL-8) may

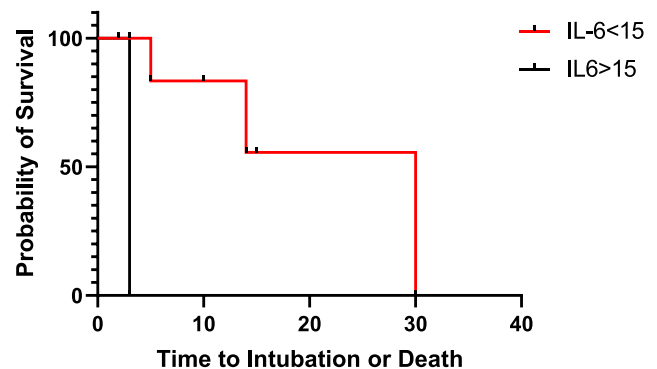


Fig. 5. Significant IL-6 values in survival analysis of COVID-19 patients. Kaplan-Meier survival analysis dividing Death Covid-19 patients according to IL-6 (cut-off 15 pg/ml).

be associated with the need for hospitalization of COVID-19 patients. Rasool et al. demonstrated that elevated levels of IL-32 during HIV infection may block viral replication [24]. Another paper reported that IL-32 participates in a negative feedback loop that inhibits sIL-6R, while upregulating IL-6 expression during influenza A virus infection [25].

Among blood parameters, low neutrophil and lymphocyte counts together with an increase in CRP already proved to be associated with severity on admission to hospital, as previously demonstrated by others [26–28]. On the other hand, among the cytokines analysed, IL-6 and IL-10 seemed to be the best for discriminating severe COVID-19 from mild and moderate groups. These observations were in line with the literature, where IL-6 has been demonstrated to be associated with severity and survival in patients with COVID-19 [29,30].

Low concentrations of IL10 were associated with severe lung involvement in our COVID 19 patients. These results are in line with the properties of IL-10, a cytokine with powerful anti-inflammatory properties that plays a central role in limiting host immune response to pathogens and maintaining normal tissue homeostasis [31].

In our study, we also analysed a panel of biomarkers to pinpoint the best combination of cytokines for stratification of COVID-19 severity. First of all, the combination of all the cytokines considered showed excellent discrimination between COVID-19 patients and controls, together with remarkable sensitivity and specificity. The panel of IL-6, IL-32, INF- $\gamma$  and CRP proved to be the best combination, showing high accuracy in discriminating severe forms of COVID-19. In our previous study, we evaluated the best combination markers in a small cohort of patients, confirming IL-6 and CRP as reliable biomarkers of disease

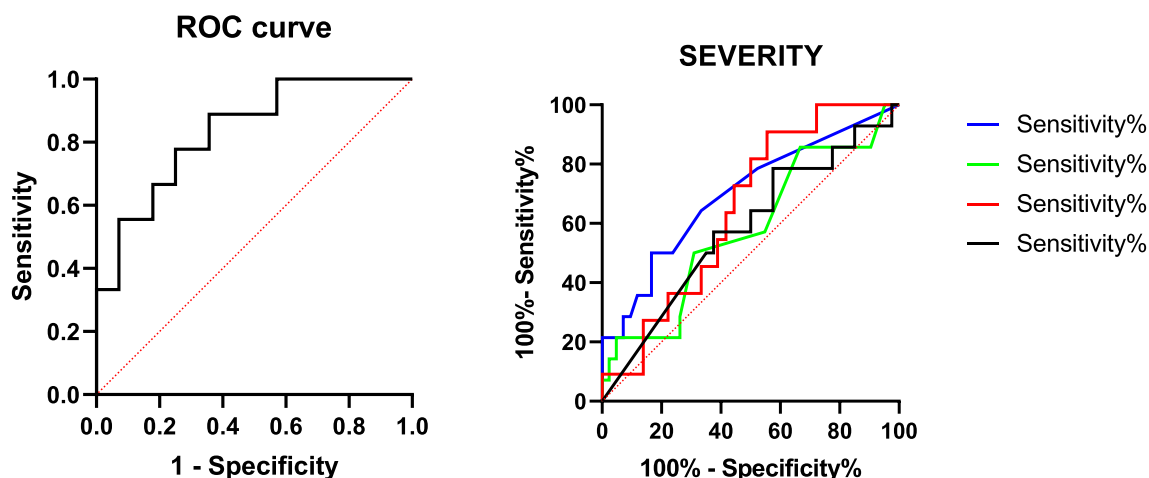


Fig. 4. a). ROC curve analysis of IL-6 (blue), CRP (Red) and INF- $\gamma$  (green) and IL-32 (black) represent the better combination in discriminate COVID-19 severity group.

severity [32].

In conclusion, our results provide new insights into the cytokine storm in COVID-19 patients, highlighting specific cytokine signatures among patients with different severity of infection. An emerging role of IL-32 is outlined, suggesting complex roles of these cytokines in viral infection and possible cross-talk with IL-8.

## 6. Consent for publication

All subjects gave their written informed consent to the study.

## 7. Availability of data and materials

The data will be available if request to the corresponding authors

## 8. Data availability statement

All data generated or analysed during this study are included in this published article (and its supplementary information files).

## Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by FB, AO and SL. The first draft of the manuscript was written by LB and Md, all authors commented on previous versions of the manuscript. EB and PC supervised the manuscript. All authors read and approved the final manuscript.

## Ethical approval

The study was approved by Local Ethics Committee CEAVSE (code number 180712 and Markerlung 17431).

## CRedit authorship contribution statement

**Laura Bergantini:** Conceptualization. **Miriana d'Alessandro:** Methodology. **Paolo Cameli:** Funding acquisition, Writing – original draft. **Ambra Otranto:** Formal analysis, Investigation. **Simona Luzzi:** Formal analysis, Investigation. **Francesco Bianchi:** Formal analysis, Investigation. **Elena Bargagli:** Funding acquisition.

## 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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