Immunobiology 229 (2024) 152813

Contents lists available at ScienceDirect

Immunobiology

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

Altered serum concentrations of IL-8, IL-32 and IL-10 in patients with lung impairment 6 months after COVID-19☆

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ARTICLE INFO

Keywords:
Cytokines
Interleukin profiles
post-COVID-19

ABSTRACT

Post-COVID symptoms are reported in 10–35 % of patients not requiring hospitalization, and in up to 80 % of hospitalized patients and patients with severe disease. The pathogenesis of post-COVID syndrome remains largely unknown. Some evidence suggests that prolonged inflammation has a key role in the pathogenesis of most post-COVID manifestations. We evaluated a panel of inflammatory and immune-mediated cytokines in individuals with altered HRCT features and in patients without any long-term COVID symptoms. Blood samples of 89 adult patients previously hospitalized with COVID-19 were collected and stratified as patients with and without HRCT evidence of fibrotic lung alterations. Serum analyte concentrations of IL-4, IL-2, CXCL10 (IP-10), IL-1β, TNF-α, CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN-γ, IL-12p70 and TGF-β1 (free active form) were quantified by bead-based multiplex assay. Clinical and functional data were recorded in a database.

With the use of machine learning approach, IL-32, IL-8, and IL-10 proved to be associated with the development of HRCT evidence of lung sequelae at follow-up. Direct comparison of cytokine levels in the two groups showed increased levels of IL-32 and decreased levels of IL-8 in patients with lung impairment. After further stratification of patients by severity (severe versus mild/moderate) during hospitalization, IL-10 emerged as the only cytokine showing decreased levels in severe patients. These findings contribute to a better understanding of the immune response and potential prognostic markers in patients with lung sequelae after COVID-19.

1. Introduction

Coronavirus disease 2019 (COVID-19) is an infectious respiratory disorder caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), that broke out in 2019 and quickly became a pandemic (Bergantini et al., 2022). Although most of the literature focuses on acute illness, it is now evident that long-term consequences occur in 5–10 % of individuals (Subramanian et al., 2022; Havervall et al., 2021).

The World Health Organization defines "long COVID" as symptoms lasting for at least 2 months, occurring at least 3 months after the onset of COVID-19 or a positive SARS-CoV-2 test, without evidence suggesting any alternative diagnosis (WHO, 2021). Symptoms may involve several organs and may last for several months, affecting the ability to work and quality of life (Maltezou et al., 2021; Daga et al., 2021). Post-COVID symptoms are reported in 10–35 % of patients not requiring hospitalization, and in up to 80 % of hospitalized patients and patients with severe disease (Nalbandian et al., 2021; Becker, 2021).

The pathogenesis of post-COVID syndrome remains largely unknown. Some evidence suggests that prolonged inflammatory status has a principal feature in the pathogenesis of most post-COVID manifestations. Post-COVID fatigue may be attributed to lung dysfunction since lymphopenia has been significantly associated with chest tightness after discharge (Liang et al., 2020).

Regarding pulmonary sequelae, most individuals who had mild to moderate COVID-19 do not show any persisting abnormalities, whereas evidence of lung impairment may persist, with altered pulmonary function tests and radiological picture, in patients who had more severe acute COVID-19 (Whiteson, 2023; Orzes et al., 2021). There is growing awareness of the increased risk of thromboembolic disease and lung fibrosis in survivors of COVID-19; both could contribute to persistent

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

☆ The present study was conducted at Siena University without funding sponsors.

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https://doi.org/10.1016/j.imbio.2024.152813
Received 13 December 2023; Received in revised form 3 May 2024; Accepted 23 May 2024
Available online 24 May 2024

breathlessness (Stewart et al., 2023).

Recent studies have demonstrated that inflammation persist after resolution of COVID-19. This phenomenon has been evaluated in terms of increased levels of several cytokines, including IL-1β, IL-6, and TNF-α (Schultheiß et al., 2021). This persistent immune activation has been suggested to be associated with ongoing symptoms after COVID-19 infection (Peluso et al., 2021; Klein et al., 2022).

However, while a panel of biomarkers for the diagnosis of long-COVID has been proposed (Allan-Blitz et al., 2023), no specific biomarkers that distinguish patients with and without specific high resolution computer tomography (HRCT) evidence of lung fibrosis have been identified. To explore this aspect, we evaluated a panel of inflammatory and immune-mediated cytokines in individuals with altered HRCT features and in patients without any long-term COVID symptoms.

2. Methods

2.1. Study population

Blood samples of 89 adult patients previously hospitalized with COVID-19 were collected in the period August 2021 to February 2023. The clinical data and medical history were available for 80/89 patients.

To be included in the study, patients had to participate in the follow-up program organized by Siena University Hospital for those hospitalized in the Siena COVID Unit. The follow-up protocol included medical examination, high-resolution computed tomography (HRCT) of the chest, blood tests and lung function tests, to be performed in the interval between weeks 12 and 24 after discharge from hospital. All data, including clinical, sociodemographic, comorbidities and the main HRCT findings, including air-trapping and ground glass opacity, were entered in an electronic database.

The study complied with the principles of the Declaration of Helsinki. The University Ethics Committee approved the study (CEAVSE PAN_HUB_2021, code number 17431_0_1). All patients gave their written informed consent to participate in the study and to use their data.

2.2. Patient stratification and analysis

To evaluate the main cytokines associated with pulmonary sequelae after COVID, we initially analysed two groups: patients with and without HRCT evidence of fibrotic lung alterations. In this case, we used a double supervised and unsupervised statistical approach. To evaluate the association of proteins in post-COVID patients with the severity of COVID19 during hospitalization, patients were classified as mild, moderate and severe according to WHO criteria (World Health Organization. Clinical Management of COVID-19. World Health Organization, 2020, pp. 1–62).

2.3. Lung function tests

The following lung function parameters were recorded according to standard ATS/ERS criteria using with correction for temperature and barometric pressure: forced expiratory volume in the first second (FEV1), forced vital capacity (FVC), carbon monoxide diffusing capacity (DLCO), total lung capacity (TLC) (Miller et al., 2005). All parameters were expressed as percentages of predicted values.

2.4. Analyte detection

Serum analyte concentrations of IL-4, IL-2, CXCL10 (IP-10), IL-1β, TNF-α, CCL2 (MCP-1), IL-17A, IL-6, IL-10, IFN-γ, IL-12p70 and TGF-β1 (free active form) were detected by bead-based multiplex LEGENDplex™ analysis (LEGENDplex™ Custom Human Assay, Biolegend, San Diego, CA, USA) according to the manufacturer's instructions. Reactions were run in duplicate with a BD FACSLyric flow cytometer (BD-Biosciences San Jose, CA, USA). The data was analysed using LEGENDplex™ Data Analysis Software Suite (QOGNIT).

2.5. ELISA kit

Serum concentrations of IL-8 and IL-32 were determined using enzyme-linked immuno-sorbent assay (ELISA) kits from Invitrogen (Waltham, MA, USA) and MyBioSource (San Diego, CA, USA), following the manufacturer's instructions. Concentrations were read at 450 nm with a Victor X4 fluorimeter (Perkin Elmer, Waltham, MA, USA) and expressed in pg/mL.

2.6. Statistical analysis

To compare cytokine levels in the two groups, non-parametric Mann–Whitney tests were used for continuous numerical variables. To compare the relative frequencies of different levels of nominal/categorical variables, the Fisher's exact and Chi squared tests were used. Multivariate analysis with machine-learning approach, based on the "caret" package (Classification And REgression Training), was performed. The model was designed to select the variables best associated with survival by Cox regression analysis. Supervised principal component analysis (PCA) was used to reduce the dimensions of the data hyperspace and to cluster samples on the basis of cell subsets. Spearman correlation coefficient was determined to look for correlation among cytokine concentrations and clinical data. Probability values less than 0.05 were considered statistically significant. We also performed binomial logistic regression to identify the cytokines and comorbidities that most influenced the development of HRCT evidence of fibrosis. Statistical analysis was performed with GraphPad Prism 9.2 software and Jamovi free software version 2.3.26.

3. Results

3.1. Clinical characteristics of patients after stratification by HRCT features

Table 1 shows the main clinical and functional features of our cohort of samples. Patients with HRCT evidence of fibrotic abnormalities proved to be older and showed a trend oflower DLCO percentages (although without reaching significance), while the distribution of comorbidities did not differ.

3.2. Patients with lung sequelae were distinguished by different cytokine levels

After stratification of our population by presence or absence of lung fibrotic sequelae, machine-learning analysis with variable-importance plot was used to select the best variables for identifying the most accurate predictors. The variables entered were all cytokines and age were excluded from the model. The model selected the first eight variables as the best predictors of lung sequelae with a model accuracy of 0.848 (Fig. 1a). IL-32, IL-8 and IL-10 proved to be associated with the development of HRCT evidence of lung sequelae at follow-up.

Focusing on the main cytokine levels (including IL-32, IL8, IL-10 and IP-10) involved in the development of lung sequelae, we performed supervised principal component analysis (PCA) to determine whether these cytokines divided the cohort into two groups. The PCA plot showed that samples from both groups clustered well, corroborating the evidence that the two groups featured differential cytokine profiles. Patients without lung impairment after COVID-19 showed similar values, forming a very well defined and homogeneous group (blue circle) while the other group was more heterogeneous (yellow circle) (Fig. 1b).

Direct comparison of cytokine levels in the two groups showed increased levels of IL-32 and decreased levels of IL-8 (Suppl. Table1) (Fig. 1c) in patients with lung impairment, whereas IL-10 concentrations

Table 1
Clinical and functional data of the two groups of the study participatants.

	Fibrosis at HRCT		p-value
	no (N = 27)	yes (N = 53)	
GENDER			0.225
M	20.0 (74.1 %)	32.0 (60.4 %)	
F	7.0 (25.9 %)	21.0 (39.6 %)	
AGE			**0.005**
Mean (SD)	66.0 (12.0)	73.0 (8.6)	
Range	34.0–86.0	46.0–87.0	
SMOKING			0.162
Former	11.0 (57.9 %)	10.0 (32.3 %)	
Never	7.0 (36.8 %)	20.0 (64.5 %)	
Current	1.0 (5.3 %)	1.0 (3.2 %)	
Comorbidities			
RESPIRATORY DISEASES			0.134
No	27.0 (100.0 %)	47.0 (88.6 %)	
Yes	0.0 (0.0 %)	6.0 (11.3 %)	
NEOPLASIA			0.123
No	23.0 (85.1 %)	50.0 (94.3 %)	
Yes	4.0 (14.9 %)	3.0 (5.6 %)	
DIABETES			0.788
Yes	6.0 (22.3 %)	12.0 (22.6 %)	
No	21.0 (77.7 %)	41.0 (77.3 %)	
CARDIOMIOPATHY			0.892
No	21.0 (77.7 %)	39.0 (73.5 %)	
Yes	6.0 (22.3 %)	14.0 (26.4 %)	
RENAL FAILURE			0.508
No	25.0 (92.3 %)	49.0 (92.4 %)	
Yes	2.0 (7.7 %)	4.0 (7.5 %)	
Pulmonary function tests			
FEV1 (%)			0.820
Mean (SD)	101.2 (14.2)	100.1 (17.6)	
Range	71.0–123.0	44.0–144.0	
FVC (%)			0.845
Mean (SD)	95.6 (15.3)	94.7 (17.0)	
Range	61.0–122.0	34.0–135.0	
FEV1/FVC ratio			**0.002**
Mean (SD)	91.7 (15.1)	103.3 (11.6)	
Range	63.0–118.0	76.0–128.0	
TLC (%)			0.289
Mean (SD)	94.7 (3.5)	82.2 (18.4)	
Range	91.0–98.0	44.0–102.0	
DLCO (%)			0.065
Mean (SD)	81.5 (21.4)	72.4 (14.7)	
Range	48.0–122.0	41.0–106.0	

were similar in the two groups.

3.3. Variables impacting the development of lung sequelae: IL-8 and IL-32 proved to be the best predictors

The logistic regression model was designed to explore how previous comorbidities can affect the development of post-acute COVID-19 lung sequalae. We performed binomial logistic regression using presence/absence of HRCT evidence of fibrosis as dependent variable and none of the comorbidities influenced stratification of the groups (Table 2).

A second model was built using the two groups as coefficient and cytokine levels as variables. In this case, serum concentrations of IL-8 (z score: 0.381p = 0.017) appeared to significantly influence the development of lung sequelae after COVID-19 (Table 3).

IL-32 (z score: −1.674, p = 0.094) resulted at the limit of statistical significance. ROC analysis from this model showed an AUC of 0.93, specificity 0.976 and sensitivity 0.824 (Fig. 2a). A third model was built from these models using only the significant results, therefore including values of both IL-8 and IL-32. ROC analysis of the model showed an AUC of 0.891, specificity 0.898 and sensitivity 0.571 (Fig. 2a).

3.4. Post COVID-19 lung sequalae and relationship with severity of acute disease

When we investigated the existence of a relationship between severity of COVID-19 and post-COVID-19 symptoms, no relationship emerged, although it was clear from the Fig. 2b that many moderate and severe patients developed lung sequelae detectable by HRCT. After further stratification of patients by severity (severe versus mild/moderate), IL-10 emerged as the only cytokine showing decreased levels in severe patients (Fig. 2c).Of these severe group of patients, 69,8 % resulted belong to severe group during hospitalization (Fig. 2d).

3.5. Correlation analysis

Fig. 3a reported the correlation matrix among interleukins and pulmonary function tests. In line with the increase concentration of IL-32 in fibrotic group, IL-32 also showing indirect correlations with the percentage of FEV1 (r = −0.67 p = 0,03) and FVC (r = −0.74 p = 0,003). Also, IL-6 inversely correlate with DLCO (r = −0.74 p = 0.045). Moreover, as reported in the correlation matrix, the fourth cluster evidenced that the majority of cytokines directly corelated among them, with particular regards on IFN-γ, TNF-α and IL-4.

Fig. 3b also reported the dendrogram with cluster analysis based in correlation analysis.

4. Discussion

Post-COVID syndrome affect a significant healthcare and social burden, since symptoms may persist for months or even years (Low et al., 2023). The pathogenesis of so-called “long COVID syndrome” is still unclear, but the main hypothesis is based on evidence that the immune system of some patients infected with SARS-CoV-2 may be unable to rapidly clear the virus, which therefore persists in certain organs or tissue reservoirs after acute infection, affecting immunity and causing chronic symptoms (Miyata et al., 2023).

Most COVID-19 patients recover from their acute infection within two weeks. Some patients harbor these viral RNA for weeks to months after their primary COVID-19 symptoms resolve (D’Antonio et al., 2022). The persistence of the virus may also play a role in long COVID, the debilitating suite of symptoms that can last for months (d’Alessandro et al., 2021). When the virus is seeded in deep tissues, it potentially causes the immune system to shift into a dysregulated inflammatory state (Batra et al., 2022). The findings help explain some unusual aspects of COVID-19 and suggest a previously unappreciated way that viruses can make people sick (National Institutes of Health (NIH), 2024).

Although several cytokines have been evaluated in hospitalized COVID-19 patients, the first data on a relationship between pro/anti-inflammatory mediators and post-Covid syndrome was only reported recently (Bergantini et al., 2023). Here we investigated levels of several mediators in patients with lung sequelae 6 months after hospitalization with COVID-19, focusing on the possibility of an association with lung fibrotic alterations.

Our study revealed significant differences in cytokine profiles between long COVID-19 patients with and without lung impairment, 6 months after hospitalisation. IL-8, IL-32 and IL-10 seemed to be the main proteins associated with lung fibrotic alterations after COVID-19 infection.

An association between SARS-CoV-2 infection and lung fibrosis is still far from certain, even though similar molecular, genetic and immunological patterns with lung fibrosis of unknown origin (such as idiopathic pulmonary fibrosis) have been reported (Bergantini et al., 2022; Bergantini et al., 2023).

Additionally, the hyperstimulation of the immune system associated with systemic inflammation secondary to COVID-19 can trigger autoimmune responses, producing cytokines and autoantibodies that may contribute to the development and progression of lung parenchymal

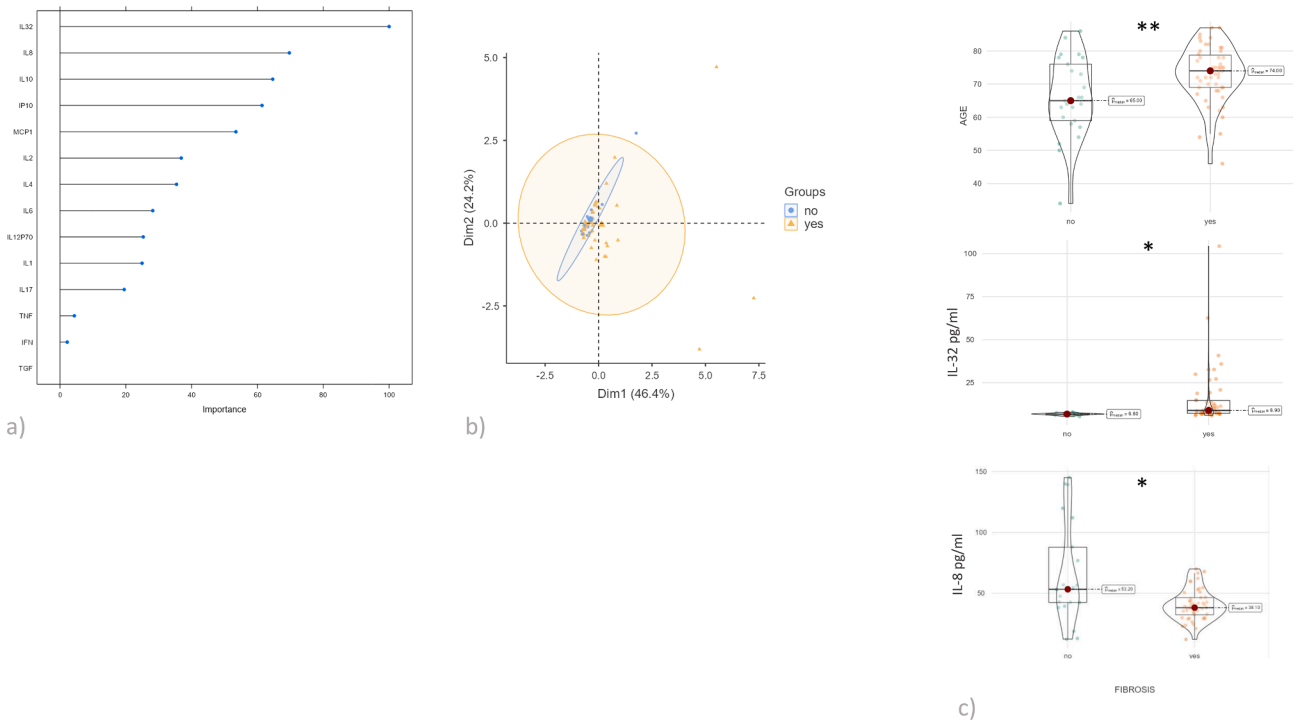


Fig. 1. (a) Random Forest variable importance plot for the machine learning model of long COVID lung sequelae. These machine-learning approach, based on the "caret" package (Classification And REgression Training), was performed. The model was designed to select the variables best associated with survival by Cox regression analysis. Patients are stratified as live/dead. Graph reported features importance for survival, extracted from the Random Forest with the best accuracy. The blue dot are the feature importance of the forest, along with their inter-trees variability. (b) Supervised PCA focusing on the main cytokine levels (including IL-32, IL8, IL-10 and IP-10) involved in the development of lung sequelae to determine whether these cytokines divided the cohort into two groups. patients without lung impairment after COVID-19 (blue circle), patients with lung impairment after COVID-19(yellow circle) (c) Comparison analysis based on non-parametric Mann–Whitney tests of serum IL-32, and IL-8 concentrations and age in the study population according to the presence or not of lung sequelae post Covid. Data are reported in the violin and box plot to observe the distribution, median and range of the reported variables. Red dots indicate the median of the variables. *$p < 0.05$ **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The logistic regression model was designed to explore whether previous comorbidities can affect the development of post-acute COVID-19 lung sequelae. Binomial logistic regression using presence/absence of HRCT evidence of fibrosis as the dependent variable and comorbidities as predictors.

Predictors	Estimates	SE	Z	p
Intercept	−14.272	1898.921	−0.00752	0.994
RESPIRATORY DISEASES:				
no – yes	17.400	1898.921	0.00916	0.993
NEOPLASIA:				
no – yes	−2.192	1.160	−1.89029	0.059
DIABETES:				
no – yes	−0.267	0.645	−0.41463	0.678
CARDIOPATHY:				
no – yes	−0.176	0.645	−0.27317	0.785
RENAL FAILURE:				
no – yes	−1.348	1.288	−1.04686	0.295

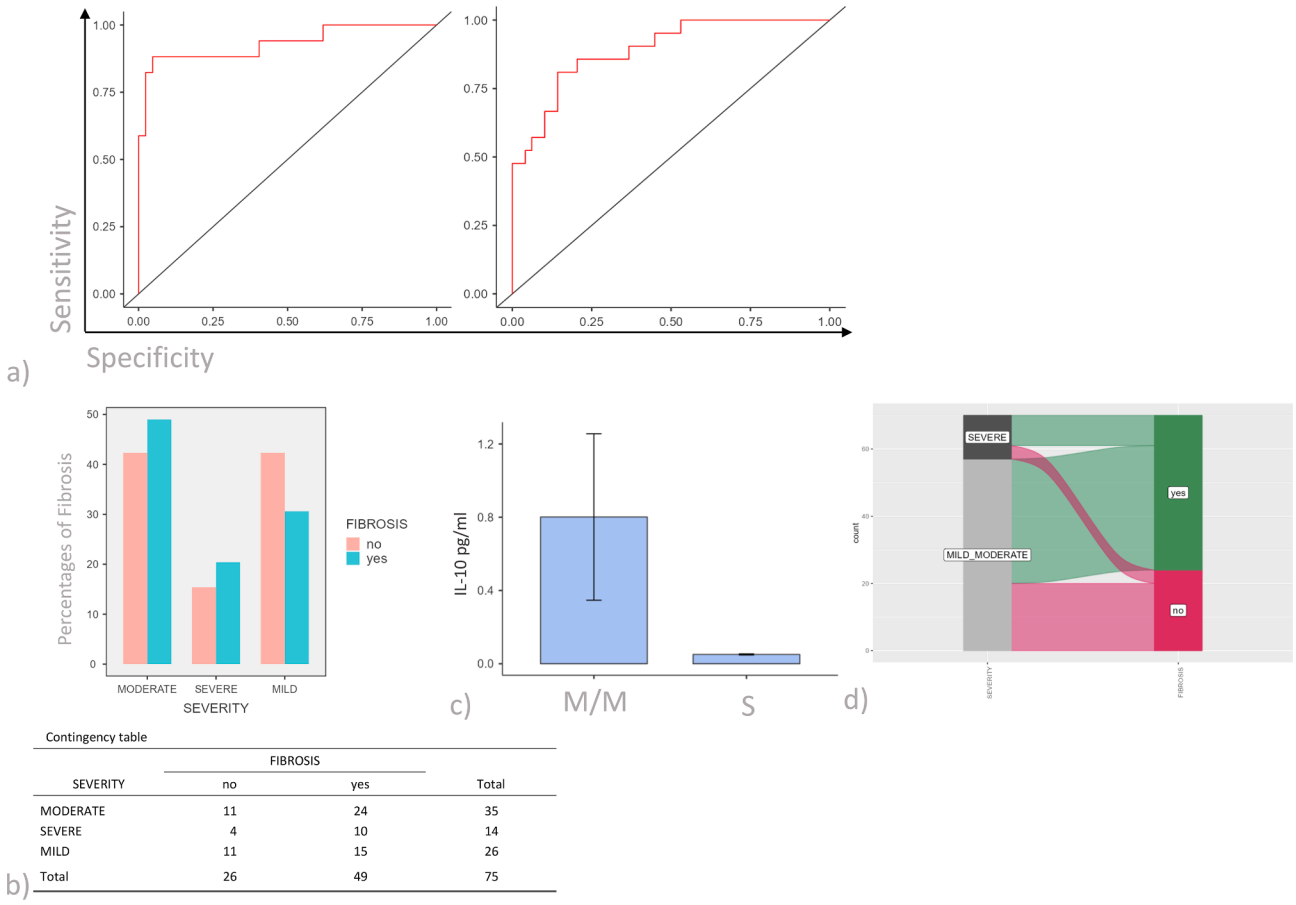
Table 3
Binomial logistic regression using presence/absence of HRCT evidence of fibrosis as the dependent variable and serum cytokine concentrations as predictors.

Predictors	Estimates	SE	Z	p
Intercept	1.60154	4.6407	0.345	0.730
IL-8	0.06743	0.0283	2.381	**0.017**
IL-32	−0.99991	0.5972	−1.674	0.094
IL-4	0.22435	1.7323	0.130	0.897
IL-2	9.65058	9.6776	0.997	0.319
IP-10	−0.01730	0.0169	−1.023	0.306
IL1β	1.93836	3.6002	0.538	0.590
TNF-α	−15.76195	42.4147	−0.372	0.710
MCP-1	0.00194	0.0154	0.125	0.900
IL-17A	18.98555	13.0768	1.452	0.147
IL-6	0.79834	1.5918	0.502	0.616
IL-10	21.53196	35.7203	0.603	0.547
IFN-γ	−2.85723	4.0224	−0.710	0.478
IL12P70	−165.34689	147.3375	−1.122	0.262
TGF-β	−3.47228	4.8488	−0.716	0.474

lesions (Sher et al., 2023).

Recent research has evidenced differences in innate immune cells and elevated expression of peripheral pro-inflammatory cytokines among individuals with long COVID (Phetsouphanh et al., 2022). In contrast, the finding of reduced markers of T-cell-mediated immunity has led some scholars to postulate "immune exhaustion," involving failure to heal of tissues injured during acute infection (Klein et al., 2022; Williams et al., 2022; Davis et al., 2023; Bergersen et al., 2023; Bergantini et al., 2021). Williams *et al.* (Williams et al., 2022) found that patients with long-COVID showed 100 % reductions in circulating levels of IFNγ and IL-8. This supports the hypothesis that immune exhaustion drives long-COVID and prevents the lungs and other organs from healing after acute infection. In line with this hypothesis, our results showed that patients with post-COVID-19 fibrotic lung sequelae had depressed serum concentrations of IL-8.

Post-COVID-19 patients also showed a lower percentage of Treg cells (Silva et al., 2023). IL-10 is the main cytokine released by Treg cells. In our study, we did not observe any significant difference in IL-10 concentrations between patients with and without lung fibrotic alterations, but interestingly, we found higher levels of IL-10 in patients with mild to moderate than with severe forms of infection. These results suggest that Treg cells contribute to failed recovery of the immune system in severe



Contingency table

SEVERITY	FIBROSIS no	FIBROSIS yes	Total
MODERATE	11	24	35
SEVERE	4	10	14
MILD	11	15	26
Total	26	49	75

b)

Fig. 2. (a) Logistic regression model (with ROC curve) of the two groups as coefficient and cytokine levels as variables. A third model was built from these models using only the significant results, therefore including values of both IL-8 and IL-32. ROC analysis of the model showed an AUC of 0.891, specificity 0.898 and sensitivity 0.571. (b) χ squared test and contigency table test after the stratification of our cohort based in severity progression of COVID-19 during hospitalization. (C) Comparison test of IL-10 values between mild to moderate (M/M) and severe patients (S) during hospitalization. (d) Alluvial plot stratified the relationship between mild to moderate and severe patients during hospitalization with the following development of fibrosis at HRCT.

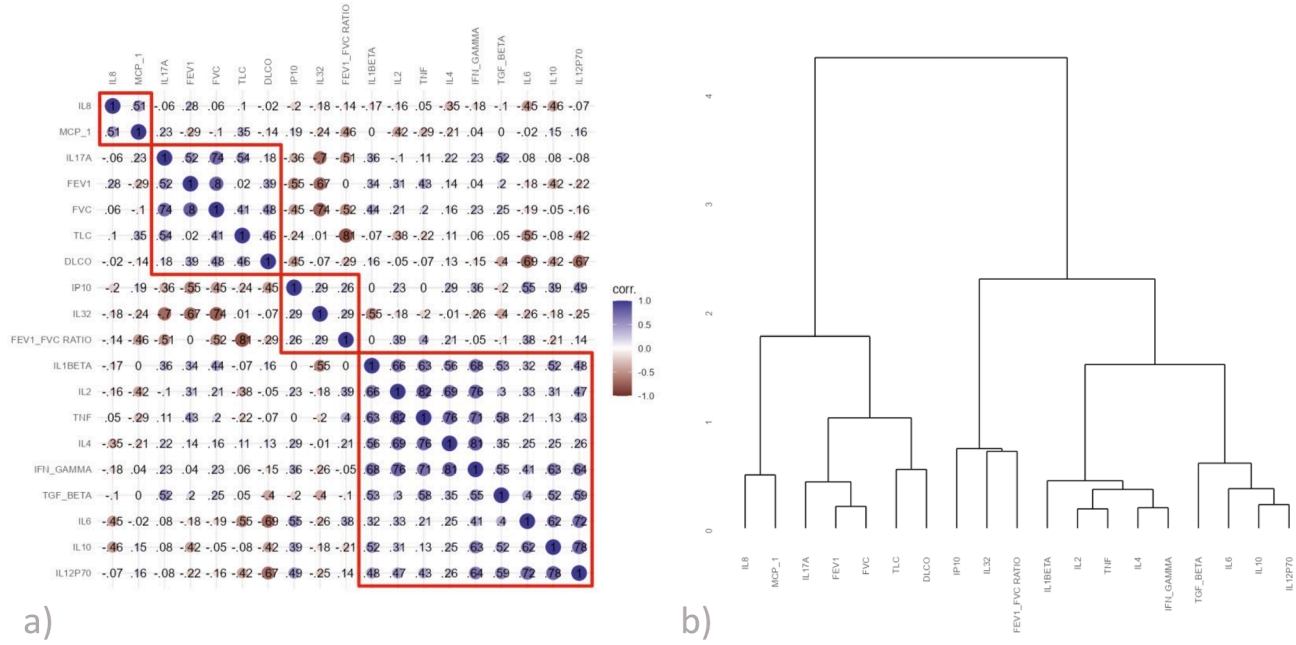


Fig. 3. (a) Correlation matrix based on spearman correlations among cytokines analysed and pulmonary function tests. The red line represents cluster analysis. (b) Dendrogram reported the four clusters which emerged from our data, based on correlation analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cases during hospitalization, as they apparently do not influence healing and fibrogenic processes. These results also confirm that Tregs may play nefarious roles in COVID-19 by suppressing antiviral T cell responses in the acute phase of the disease (Galván-Peña et al., 2021). Although further experiments on Treg cells need to be performed, to establish that the release of IL-10 came from these kinds of cells.

IL-32 is associated with inflammation, virus infections and cancer through its role in several processes such as regulation of apoptosis, accentuation of inflammation, and angiogenesis (Gong et al., 2020). Decreased levels of this cytokine have been associated with poor prognosis and limited survival (Bergantini et al., 2023; Bergantini et al., 2021). Here we observed that IL-32 was significantly higher in patients with post-COVID-19 fibrotic sequelae, although IL-32 pathway mechanisms in such patients have not been established (Gong et al., 2020). These results may be explained by the evidence that IL-32 promotes the epithelial to mesenchymal transition in lung alveolar epithelial cells by triggering oxidative stress.

As reported in other studies, the influence of age on the clinical course of COVID-19 is a crucial point. The decline in immune function may affect cytokine responses and disease severity during viral infections but also progression and complications of COVID-19 (Reeves et al., 2022; Romero Starke et al., 2021; d'Alessandro et al., 2021). Although very little data is available on age in connection with fibrosis after SARS-CoV-2 infection (Duong-Quy et al., 2023), in our study the influence of age was closely linked to the development of respiratory symptoms, especially pulmonary fibrosis, after COVID-19.

Cytokines played a crucial role in determining the severity of COVID-19 both during acute infection and also in terms of lung complications after resolution of the infection. These findings contribute to a better understanding of the immune response and potential prognostic markers in patients with lung sequelae after COVID-19.

CRediT authorship contribution statement

Laura Bergantini: Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Conceptualization. **Sara Gangi:** Methodology, Formal analysis. **Miriana d'Alessandro:** Writing – original draft, Data curation, Conceptualization. **Paolo Cameli:** Resources, Methodology, Investigation. **Beatrice Perea:** Resources, Methodology. **Martina Meocci:** Resources, Investigation. **Gaia Fabbri:** Resources, Investigation. **Francesco Bianchi:** Resources, Investigation. **Elena Bargagli:** Writing – review & editing, Supervision, Project administration, 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.

Data availability

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

Acknowledgement

None.

Author Contributions

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imbio.2024.152813.

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