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# Title page

**Type of manuscript:** Brief report

**Title:** High SARS-CoV-2 viral load and low CCL5 expression levels in the upper respiratory tract are associated with COVID-19 severity

**Running heading:** Biomarkers related to severe COVID-19

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**Summary:** SARS-CoV-2 replication in the nasopharyngeal mucosa induces the expression of several innate immune genes. High SARS-CoV-2 viral load and low CCL5 expression levels were associated with ICU admission or death, although CCL5 was the best predictor of COVID-19 severity.

## Footnote Page

### Competing interests

The authors declare that they have no competing interests.

The funding sources played no role in the study's design, collection, analysis, interpretation of the data, or manuscript writing.

### Funding

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## **Abstract**

Mucosal immune response in the upper respiratory tract is crucial for the initial control of viral replication, the clearance of SARS-CoV-2, and the progression of the coronavirus disease 2019 (COVID-19). We analyzed the SARS-CoV-2 RNA load and the expression of selected immune genes in the upper respiratory tract (nasopharynx) of 255 SARS-CoV-2 infected patients and evaluated their association with severe COVID-19. SARS-CoV-2 replication in the nasopharyngeal mucosa induces the expression of several innate immune genes. High SARS-CoV-2 viral load and low CCL5 expression levels were associated with ICU admission or death, although CCL5 was the best predictor of COVID-19 severity.

## **Key Words**

SARS-CoV-2; COVID-19; gene expression; CCL5; innate immunity; nasopharynx; death; ICU; viral load.

## Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection causes the coronavirus disease 2019 (COVID-19), a disease with high mortality and morbidity rates worldwide. Clinical manifestations range from mild symptoms to pneumonia and severe acute respiratory syndrome. The proinflammatory response and viral replication are closely related to the severity of the disease and risk of death (1).

SARS-CoV-2 infection initiates in the upper respiratory tract, mainly in nasal epithelial cells, where the binding of the virus to the angiotensin-converting enzyme 2 (ACE2) cell receptor occurs (1). After cellular and viral membrane fusion, the viral genome replicates inside the cell. Viral RNAs activate the innate immune system, triggering signaling pathways leading to the expression of type I and III interferons (IFN- $\alpha/\beta$  and IFN- $\lambda$ ), proinflammatory cytokines, and chemokines, and the recruitment of inflammatory myeloid cells. Interferon-mediated signaling, in turn, upregulates the expression of multiple antiviral interferon-stimulated genes (ISGs) (1).

Mucosal immune response in the upper respiratory tract is crucial for the initial control of viral replication, the clearance of SARS-CoV-2, and the progression of the COVID-19 (1, 2). A characteristic of SARS-CoV-2 infection is the high viral loads in the upper airways before symptom onset, which may be related to the reduced activation of some innate immunity pathways (3, 4). Also, dysregulated antiviral immunity in the nasal epithelium seems to predict progression to severe COVID-19 (5, 6). Therefore, differentiating protective immune response in the nasopharynx from that leading to fatal outcomes is essential in developing preventative and therapeutic strategies against SARS-CoV-2 infection. This study aimed to analyze the viral RNA load and the expression of selected immune genes in the upper respiratory tract (nasopharynx) of SARS-CoV-2 infected patients and evaluate their association with severe COVID-19.

## Methods

### Study design and patients

We conducted a retrospective study of 255 SARS-CoV-2 infected patients from Hospital Universitario Príncipe de Asturias between November 9th, 2020, and March 8th, 2021, who had a positive Real-Time Polymerase Chain Reaction (RT-PCR) test at the emergency admission. Patients were initially collected to complete three groups with different severity stages: 85 outpatients that were examined at the emergency room and discharged within the first 24 hours (mild cases), 87 hospitalized in medicine wards that did not require critical care (moderate cases), and 83 critical patients that were admitted to the intensive care unit (ICU) or died within 28 days after hospital admission (severe cases). In parallel to the COVID-19 patients, we collected 30 healthy individuals with a negative PCR test for SARS-CoV-2 and no suspicion of any other respiratory infection. Demographic and clinical data were extracted from medical records.

This study was approved by the “Hospital Universitario Príncipe de Asturias” Ethics Committee (Ref.: EXPRES-INMUNE-COVID) and the Hospital's Institutional Review Board. Informed consent waiver was authorized by the Ethics Committee.

### Laboratory assays

#### Nasopharyngeal swabs samples

Biological samples (nasopharyngeal swabs) were obtained during the first 24 hours after the emergency admission. Samples were placed in a transport medium with guanidinium isothiocyanate (NEST Disposable Nasopharyngeal VTM Sampler kit, Wuxi NEST Biotechnology, Wuxi, China) for COVID-19 testing, and stored at -80°C. This medium served as an inactivator and preservative for SARS-CoV-2 RNA and mucosal biomarkers mRNA.

#### RT-PCR assay for COVID-19 diagnosis

Viral RNA was obtained at the hospital from clinical samples using two automatic extractors: MagCore HF16 (RBC bioscience, Taipei, Taiwan) and ELITE Ingenius (ELITechGroup, Puteaux,

France). RNA amplification was performed using two Real-Time PCR platforms randomly according to the usual laboratory workflow: Viasure SARS-CoV-2 RealTime PCR Detection Kit (Certest Biotech S.L.; detected genes: ORF1ab and N) and GeneFinder COVID-19 Plus RealAmp Kit (Osang Healthcare Co.; detected genes: E, N, and RdRP). The concordance of these two platforms showed 100% agreement and similar Ct values (see **Supplementary Table 1**). Samples were considered positive when all SARS-CoV-2 genes included in each RT-PCR assay were amplified.

### **RT-PCR for quantification of mucosal biomarkers**

RNA previously extracted as above was reverse transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions.

The expression of the selected genes (mucosal biomarkers) was quantified in the generated cDNA by real-time PCR using TaqMan Gene Expression Assays specific for each gene (Applied Biosystems, Foster City, CA, USA). The PCRs were done in triplicate in a Step One instrument (Applied Biosystems) following the manufacturer's instructions. TaqMan Gene Expression Assays containing TaqMan MGB probes (FAM dye-labeled, Applied Biosystems) were used for the following cellular genes: Actin- $\beta$  (ACTB) (Hs99999903\_m1), DExD/H-Box Helicase 58 (DDX58) (RIG-I, Hs00204833\_m1), tumor necrosis factor (TNF) (Hs00174128\_m1), interleukin 6 (IL6) (Hs00985639\_m1), interleukin 8 (IL8) (CXCL8, Hs00174103\_m1), interferon- $\beta$ 1 (IFNB1) (Hs01077958\_s1), interferon-stimulated gene 15 (ISG15) (Hs00192713\_m1), interferon-induced protein with tetratricopeptide (IFIT1) (Hs00174103\_m1), chemokine C-X-C motif ligand 10 (CXCL10) (Hs00171042\_m1) and chemokine C-C motif ligand 5 (CCL5) (Hs00982282\_m1). We used the ACTB (actin- $\beta$  mRNA) as an endogenous control to normalize the quantitation of an mRNA target for differences in the amount of total RNA added to each reaction. We also performed a relative quantification by the comparative Ct ( $\Delta\Delta$ Ct) method (Applied Biosystems User Bulletin no. 2) using as a reference sample (calibrator) a random mixture of samples from 100 SARS-CoV-2 positive individuals. Target mRNA expression was quantified relative to the calibrator for all experimental samples and expressed as n-fold.

Expression of SARS-CoV-2 nucleocapsid gene was performed, according to the manufacturer's instructions, using SYBR-Green reaction mix (Power-Up SYBR-Green Master MIX, Applied Biosystems) and the following primers: ACTB (forward: 5'-CACCAACTGGGACGACAT-3', reverse: 5'-ACAGCCTGGATAGCAACG-3') and N (forward: 5'-GGGAGCCTTGAATACACCAAAA-3', reverse: 5'-TGTAGCACGATTGCAGCATTG-3'). The expression of ACTB was used as endogenous control, and relative quantification was made by the comparative Ct ( $\Delta\Delta$ Ct) method using the same calibrator as above.

### **Severe COVID-19 outcomes**

The primary endpoint during hospital admission was ICU admission or death within 28 days (ICU admission/death). A single episode was considered for each patient. When a patient was discharged from the emergency department and later readmitted during the study period, only the first hospital admission episode was considered for the analysis, and the corresponding respiratory sample for mucosal biomarkers.

### **Statistical analysis**

Statistical analysis was performed by Stata IC 15.1 (StataCorp, Texas, USA). Figures were generated using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA, USA). All p-values were two-tailed, and the significance level was set at 0.05 (2-tailed).

The differences between independent groups were assessed using the Mann-Whitney U test, Chi-square test, or Fisher's exact test. Correlation analysis between mucosal biomarkers was performed using the Pearson coefficient (r). Logistic regression analysis was employed to determine the association of mucosal biomarkers with COVID-outcomes, providing the odds ratio (OR) and their 95% confidence intervals (CIs). In all cases, we performed univariate and multivariate regression analyses adjusted by significant covariables prior to SARS-COV-2 infection (age, gender, and comorbidities) and covariables at baseline (time from COVID-19 symptoms to sample collection,

international normalized ratio, C-reactive protein, ferritin, lactate dehydrogenase, alanine aminotransferase, albumin, eGFR, creatinine, glucose, thrombocytes, neutrophils, lymphocytes, and hematocrit). Covariables were selected by a stepwise forward selection method ( $p_{in} < 0.05$  and  $p_{out} < 0.10$ ) to avoid model overfitting. For association analysis, mucosal biomarker values were log<sub>2</sub>-transformed (base-2 logarithms). The predictive performance of severe COVID-19 was assessed by area under the receiver-operating characteristic curve (AUC), which was considered excellent (0.90–1), good (0.80–0.90), reasonable (0.70–0.80), and poor (0.60–0.70). Also, we divided the study population in half into training and validation cohorts to evaluate the predictive value of the tested genes.

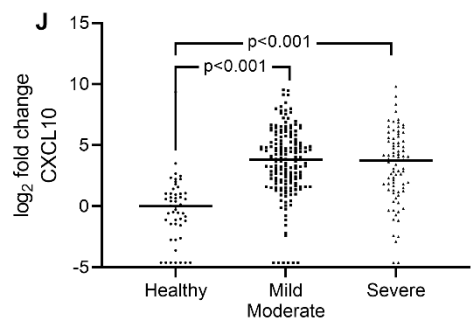
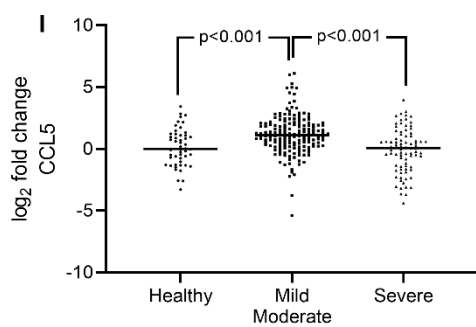
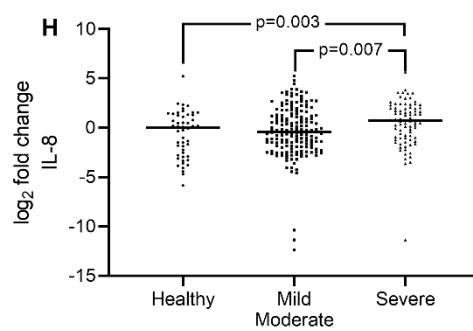
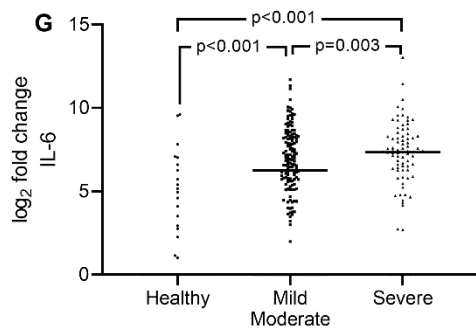
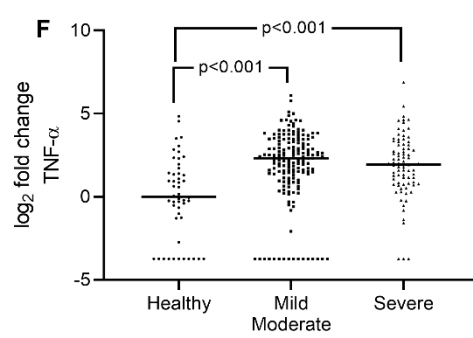
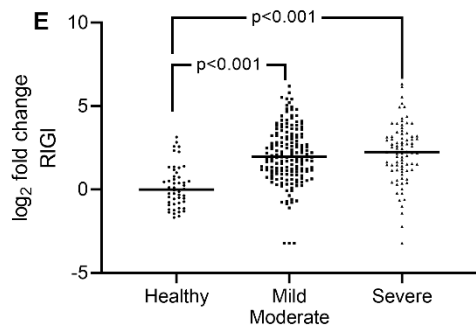
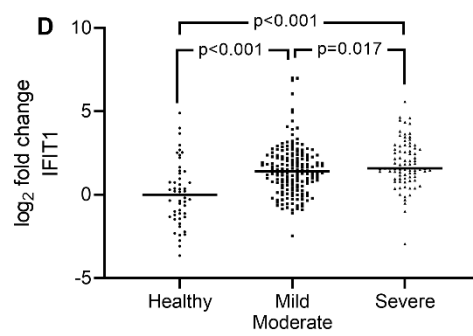
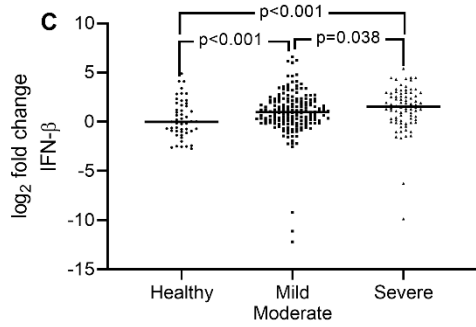
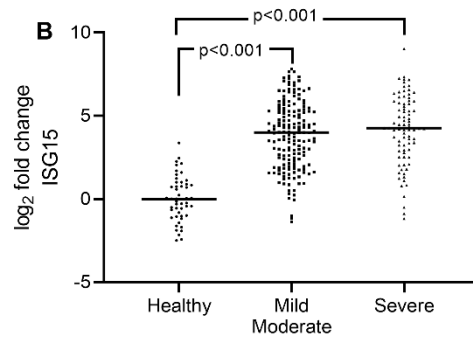
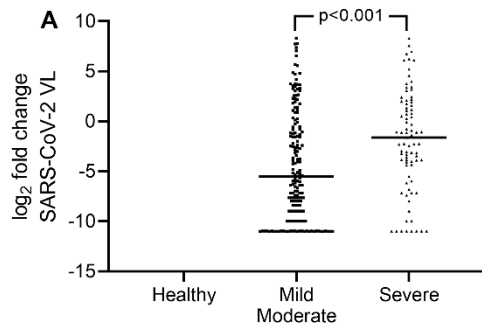
## Results

### Characteristics of COVID-19 patients

Baseline characteristics of COVID-19 patients are shown in Supplementary Table 2. Patients were 60% males, the median age was 63.8 years, and the main comorbidities were chronic heart disease, hypertension, chronic obstructive pulmonary disease, obesity, diabetes, and dyslipidemia. After hospital admission, 19.6% entered the ICU, 16.1% had invasive mechanical ventilation, and 14.1% died within 28 days. Moreover, healthy controls were 47.7% males, and the median age was 60 years.

### Innate immunity genes are upregulated in COVID-19 patients

COVID-19 patients had higher levels of ISG15, IFN- $\beta$ , IFIT1, RIGI, TNF- $\alpha$ , IL-6, CCL5, and CXCL10 than healthy controls ( $p$ -value $<0.05$ ; **Supplementary Figure 1**). Patients with severe COVID-19 had significantly higher values for SARS-CoV-2 viral load, IFN- $\beta$ , IFIT1, IL-6, and IL-8 than patients with mild or moderate disease, while CCL5 values were substantially lower in patients with severe COVID-19 ( $p$ -value $<0.05$ ; **Figure 1**). Moreover, we found a strong direct correlation between SARS-CoV-2 viral load and ISG15, RIGI, TNF- $\alpha$ , IL-6, and CXCL10 ( $p$ -value $<0.001$ ; **Supplementary Figure 2**).





**Figure 1.** Relative expression of innate immunity genes and SARS-CoV-2 viral load in the upper respiratory tract of COVID-19 patients. Values are expressed as  $\log_2$  (fold-changes) to the median of healthy controls to facilitate comparisons between groups.

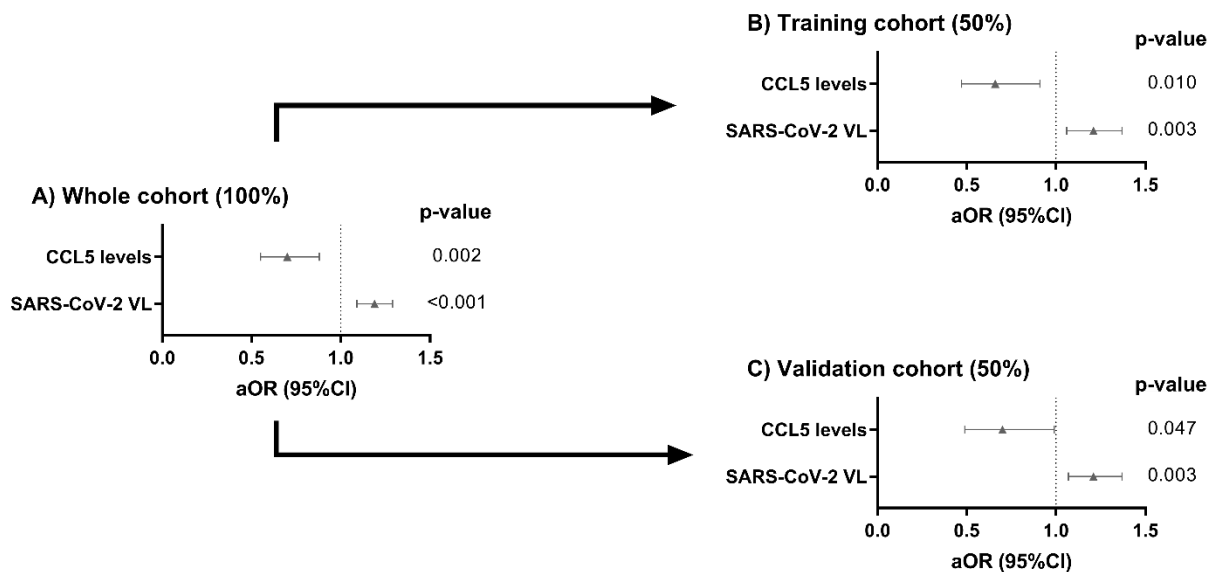
**Statistics:** The differences between groups were assessed using the Mann-Whitney U test.

**Abbreviations:** COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ISG15, interferon-stimulated gene 15; IFN- $\beta$ , interferon-beta; IFIT1, Interferon-induced protein with tetratricopeptide repeats 1; TNF- $\alpha$ , tumor necrosis factor-alpha; IL-6, interleukin 6; IL-8, interleukin 8; CCL5, chemokine (C-C motif) ligand 5; CXCL10, C-X-C motif chemokine ligand 10; RIGI, retinoic acid-inducible gene I; p-value, level of significance.

### Mucosal biomarkers predict COVID-19 outcomes

In adjusted regression models (Figure 2; the full description in Supplementary Table 3), SARS-CoV-2 viral load was a risk factor (aOR=1.19,  $p$ -value<0.001), and CCL5 was a protective factor for ICU admission or death during hospital admission (aOR=0.70,  $p$ -value=0.002). When the whole cohort was divided in half (training and validation cohorts), we also found significant associations for SARS-CoV-2 and CCL5 viral load in both cohorts ( $p$ -value<0.05), supporting that the association between high levels of SARS-CoV-2 viral load and low levels of CCL5 expression with COVID-19 severity is robust.

Moreover, CCL5 was the only one with an AUC > 0.70 (Supplementary Figure 3A), and the combination with other mucosal biomarkers did not significantly improve the AUC value (data not shown). CCL5 was also directly correlated with higher peripheral oxygen saturation (SpO<sub>2</sub>) values at emergency admission ( $r=0.295$ ;  $p<0.001$ ) (Supplementary Figure 3B).



**Figure 2.** Association between mucosal biomarkers ( $\log_2$  values) in the upper respiratory tract and ICU admission or death during hospital admission.

**Statistics:** The association analysis was performed by logistic regression adjusted by the most significant covariables.

**Abbreviations:** SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ICU, intensive care unit; OR, odds ratio; aOR, adjusted odds ratio; 95% CI, 95% confidence interval; p-value, level of significance; CCL5, Chemokine (C-C motif) ligand 5; VL, viral load.

## Discussion

This study found that decreased expression of CCL5 and elevated SARS-CoV-2 viral load in nasopharyngeal samples were associated with poor COVID-19 outcomes.

In our study, COVID-19 patients had upregulated innate immunity genes, which were directly associated with SARS-CoV-2 viral load. Characterizing the immune response triggered in the upper respiratory tract by SARS-CoV-2 is essential since the virus's infection, replication, and dissemination begins in this place. An adequate immune response in the nasopharynx contributes to controlling virus replication, preventing its spread towards the lower respiratory tract, and avoiding disease complications. Conversely, an unbalanced innate immune response is closely related to the COVID-19 severity and the risk of death (1).

We found that COVID-19 patients with high values of SARS-CoV-2 viral load had higher odds of ICU admission or death. In agreement with our data, some studies have found that SARS-CoV-2 viral loads on the upper respiratory tract are positively associated with disease severity (7-9).

However, others have found no correlation (4, 10, 11). This difference may be related to different times between sample collection and SARS-CoV-2 viral load quantification.

Moreover, higher levels of CCL5 were associated with better COVID-19 outcomes, and it was also the only biomarker with acceptable predictive performance. CCL5 (also known as RANTES) is a potent chemoattractant for several immune cells such as monocytes and NK cells and promotes interaction between T cells and dendritic cells, essential for virus control (12). Thus, the CCL5 expression may help eliminate SARS-CoV-2 infection and prevent patients from developing severe COVID-19 (13). In agreement with this, it has been described that in patients with critical COVID-19, CD8<sup>+</sup> cytotoxic lymphocytes expressed lower levels of CCL5 (14). Besides, COVID-19 patients with mild disease had higher nasopharyngeal CCL5 expression than those with severe pneumonia at the emergency room (15). Thus, profiling the expression of the CCL5 gene in the nasopharyngeal mucosa with the same sample used for diagnosis could help improve the prognosis of patients at the initial phase of infection and guide potential treatments.

However, our study can be considered preliminary and has several limitations, such as it was retrospective, had a limited sample size, and only a few biomarkers were evaluated. Further studies should confirm our findings and corroborate the potential use of nasopharyngeal biomarkers to predict the clinical course of COVID-19.

### Conclusion

SARS-CoV-2 replication in the nasopharyngeal mucosa induces the expression of several innate immune genes. High SARS-CoV-2 viral load and low CCL5 expression levels were associated with ICU admission or death, although CCL5 was the best predictor of COVID-19 severity.

## **Declarations**

### **Ethics approval and consent to participate**

This study was approved by the “Hospital Universitario Príncipe de Asturias” Ethics Committee (Ref.: EXPRES-INMUNE-COVID). The Institutional Review Board of the Hospital also approved this study. The Ethics Committee authorized the informed consent waiver.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and analyzed during the current study are available from the corresponding authors upon reasoned request.

### **Author contributions**

Funding acquisition: JFBM, SR, and IM.

Study concept and design: SR and IM.

Patients' selection and clinical data acquisition: FPG, LCG, IHF, VGV, and FCG.

Sample preparation, RNA isolation, and RT-PCRs: MMV, RLRG, MJMG, and EJVA.

Statistical analysis and interpretation of data: FPG, SR, and IM.

Writing – original draft preparation: FPG, MMV, SR, and IM.

Writing – Review & Editing: JFBM.

Supervision and visualization: SR and IM.

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### **Authors' information**

Not applicable.

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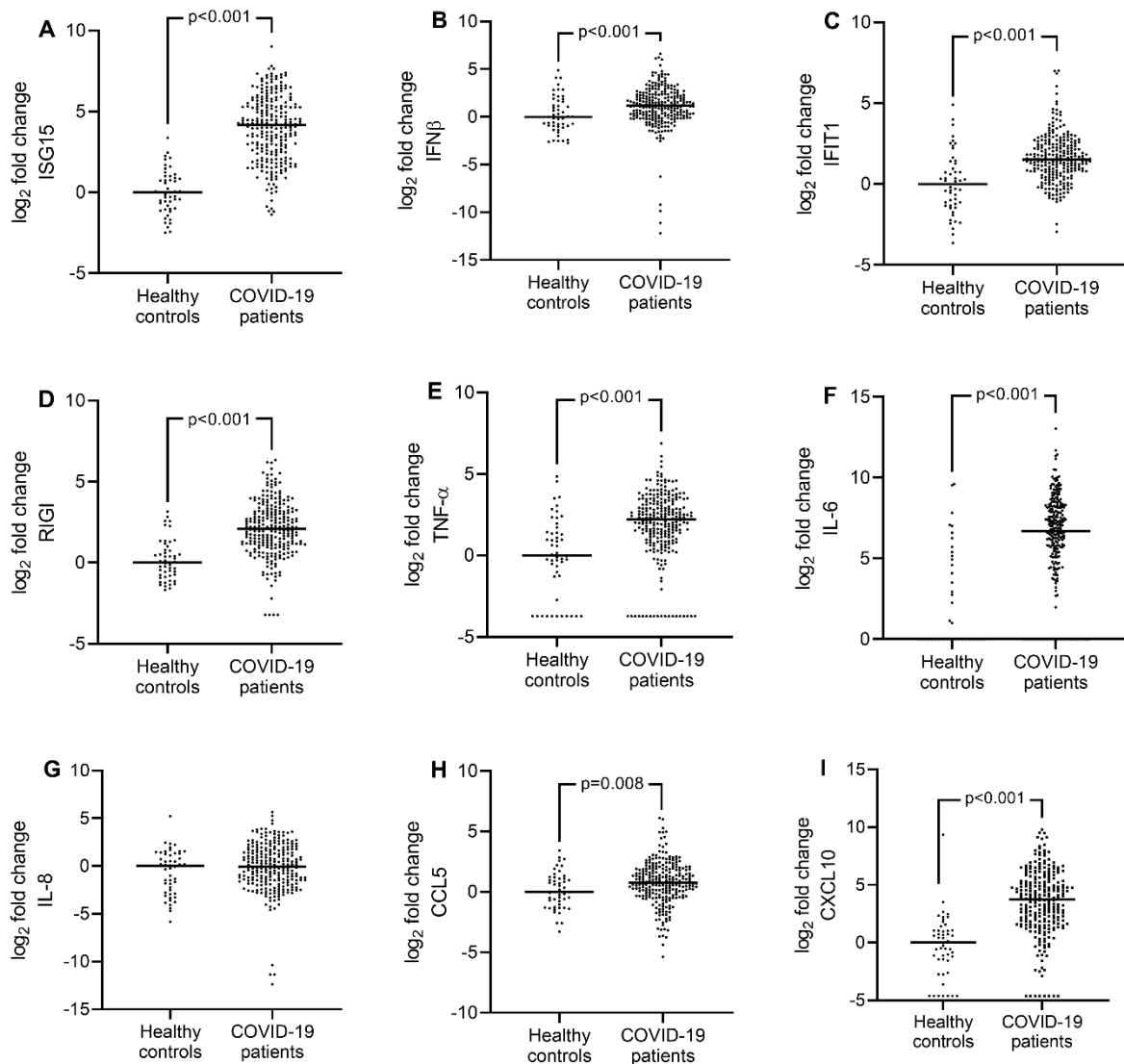
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## Supplementary file

**Supplementary Figure 1.** Relative expression of innate immunity genes ( $\log_2$  values) in the upper respiratory tract of COVID-19 patients and healthy controls. Values are expressed as  $\log_2$  (fold-changes) to the median of healthy controls to facilitate comparisons between groups.

**Statistics:** The differences between groups were assessed using the Mann-Whitney U test.

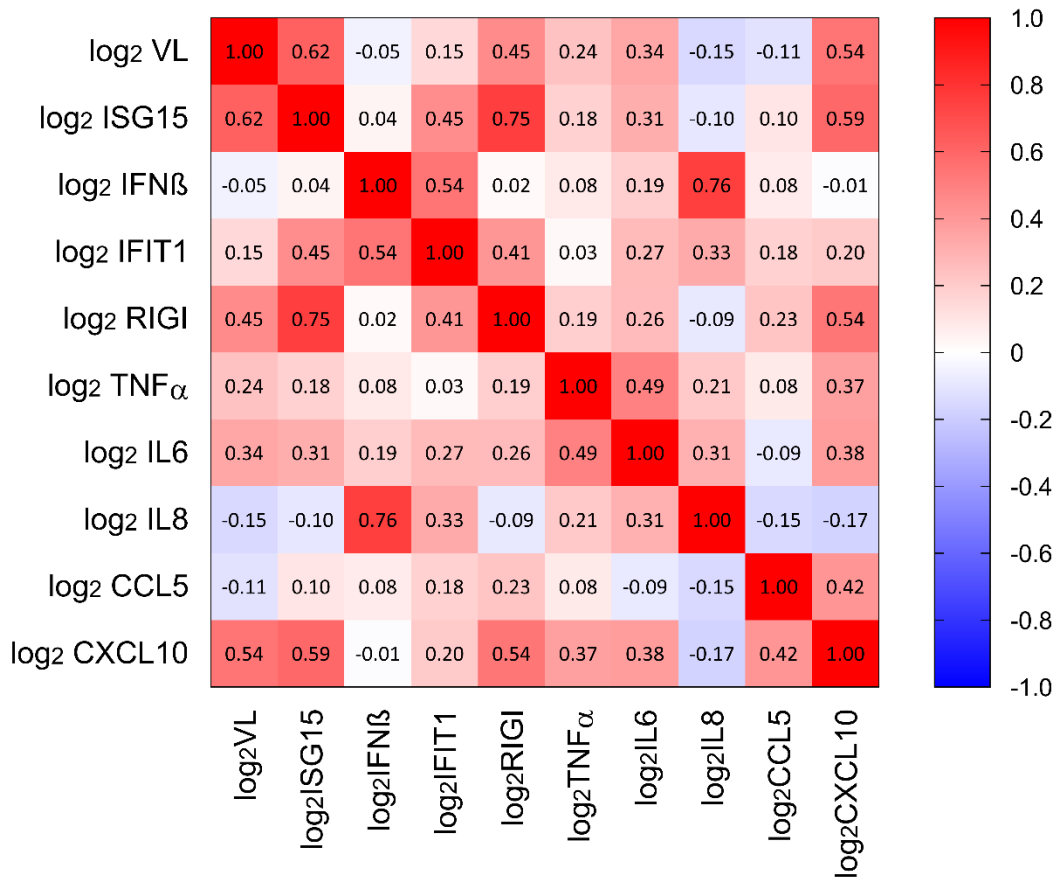
**Abbreviations:** COVID-19, coronavirus disease 2019; ISG15, interferon-stimulated gene 15; IFN- $\beta$ , interferon-beta; IFIT1, Interferon-induced protein with tetratricopeptide repeats 1; TNF- $\alpha$ , tumor necrosis factor-alpha; IL-6, interleukin 6; IL-8, interleukin 8; CCL5, Chemokine (C-C motif) ligand 5; CXCL10, C-X-C motif chemokine ligand 10; RIGI, retinoic acid-inducible gene I; p-value, level of significance.



**Supplementary Figure 2.** Correlation between mucosal biomarkers ( $\log_2$  values) in the upper respiratory tract of COVID-19 patients.

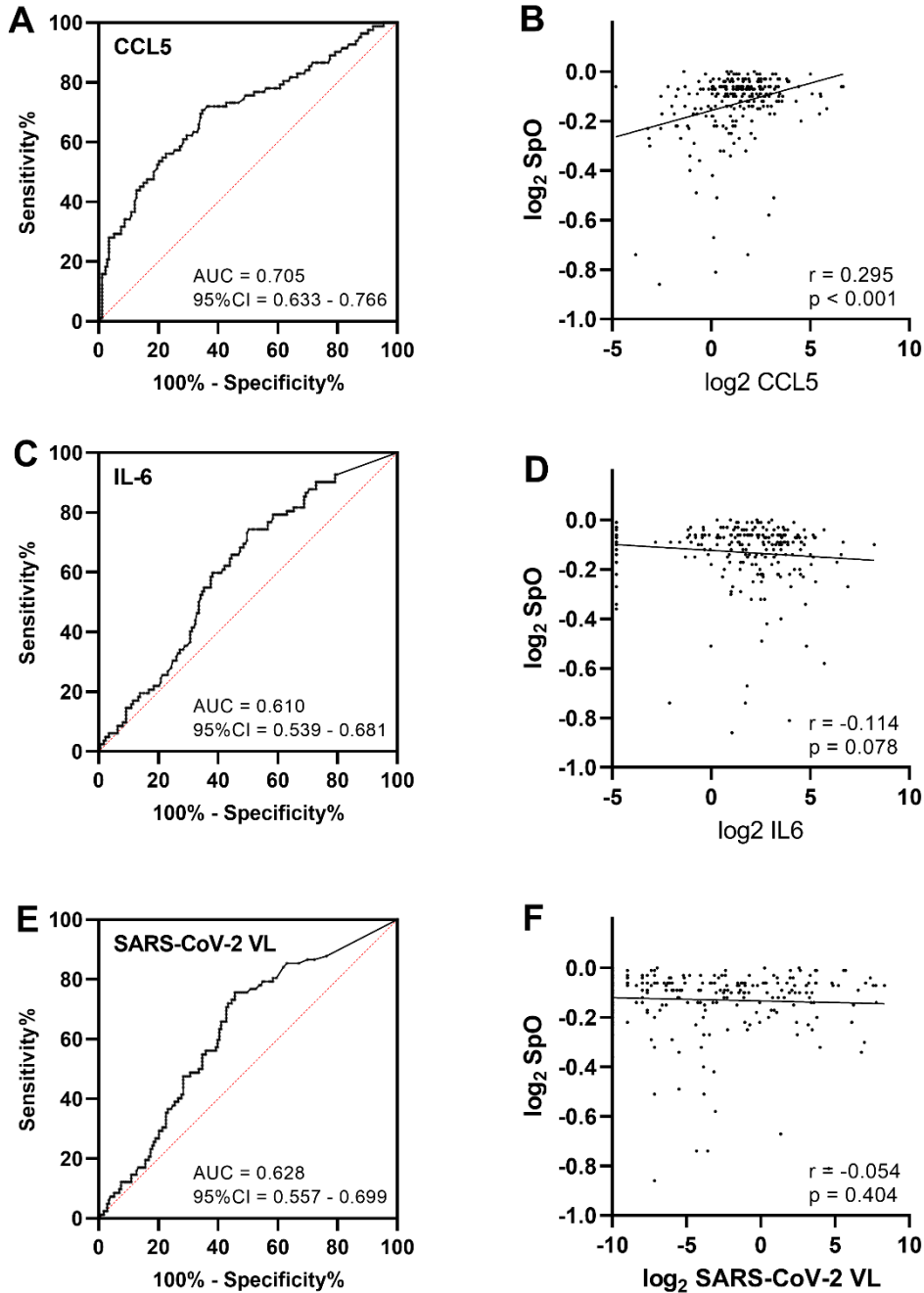
**Statistics:** The correlation analysis was performed by Pearson correlation.

**Abbreviations:** SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ISG15, interferon-stimulated gene 15; IFN- $\beta$ , interferon-beta; IFIT1, Interferon-induced protein with tetratricopeptide repeats 1; TNF- $\alpha$ , tumor necrosis factor-alpha; IL-6, interleukin 6; IL-8, interleukin 8; CCL5, Chemokine (C-C motif) ligand 5; CXCL10, C-X-C motif chemokine ligand 10; RIGI, retinoic acid-inducible gene I.



**Supplementary Figure 3.** AUC values of CCL5, IL-6, and SARS-CoV-2 viral load in the upper respiratory tract to predict severe COVID-19 (A, C, and E), and Person correlations of CCL5, IL-6, and SARS-CoV-2 viral load with SpO at the emergency room (B, D, and F).

**Abbreviations:** COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; AUC, area under the receiver-operating characteristic curve; SpO, peripheral oxygen saturation; IL-6, interleukin 6; CCL5, Chemokine (C-C motif) ligand 5; p-value, level of significance.





**Supplementary Table 1.** Agreement between RT-PCR assays.

Sample	<i>Viasure SARS-CoV-2 RealTime PCR Detection Kit</i>					<i>GeneFinder COVID-19 Plus RealAmp Kit</i>						
	<i>ORF1b gene</i>		<i>N gene</i>		Result	<i>E gene</i>		<i>RdRP gene</i>		<i>N gene</i>		Result
	Interpretation	Ct value	Interpretation	Ct value		Interpretation	Ct value	Interpretation	Ct value	Interpretation	Ct value	
71470454	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
71521754	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
71558577	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
71445944	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
71553033	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
71558573	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
71446047	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
60091239	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
32674737	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
30138045	Negative	N/A	Negative	N/A	Negative	Negative	N/A	Negative	N/A	Negative	N/A	Negative
71470455	Positive	31,1	Positive	32,0	Positive	Positive	33,9	Positive	35,7	Positive	33,3	Positive
11157975	Positive	33,2	Positive	32,7	Positive	Positive	34,3	Positive	32,3	Positive	32,7	Positive
71501223	Positive	19,6	Positive	20,5	Positive	Positive	21,6	Positive	22,4	Positive	21,5	Positive
71521947	Positive	19,7	Positive	20,5	Positive	Positive	21,4	Positive	21,7	Positive	21,0	Positive
71543004	Positive	30,5	Positive	30,5	Positive	Positive	30,8	Positive	33,2	Positive	30,9	Positive
71465878	Positive	34,5	Positive	35,2	Positive	Positive	35,8	Positive	35,9	Positive	34,8	Positive
71494627	Positive	24,8	Positive	27,0	Positive	Positive	26,7	Positive	26,0	Positive	27,4	Positive
71547774	Positive	21,6	Positive	20,5	Positive	Positive	22,5	Positive	23,5	Positive	20,6	Positive

<b>71558588</b>	Positive	18,6	Positive	19,5	Positive	Positive	17,9	Positive	18,3	Positive	18,2	Positive
<b>71446052</b>	Positive	28,1	Positive	27,0	Positive	Positive	27,5	Positive	27,2	Positive	26,1	Positive
<b>71558587</b>	Positive	33,4	Positive	32,2	Positive	Positive	31,4	Positive	32,4	Positive	31,4	Positive
<b>71465905</b>	Positive	36,1	Positive	35,2	Positive	Positive	35,7	Positive	36,7	Positive	35,4	Positive
<b>71553034</b>	Positive	23,1	Positive	22,6	Positive	Positive	22,3	Positive	23,2	Positive	23,0	Positive
<b>71446051</b>	Positive	30,4	Positive	29,0	Positive	Positive	29,1	Positive	30,4	Positive	29,5	Positive
<b>71558589</b>	Positive	29,1	Positive	27,5	Positive	Positive	27,7	Positive	28,8	Positive	28,4	Positive
<b>71445945</b>	Positive	27,5	Positive	26,2	Positive	Positive	25,8	Positive	25,4	Positive	25,1	Positive
<b>32672291</b>	Positive	23,4	Positive	22,1	Positive	Positive	20,7	Positive	21,7	Positive	20,5	Positive
<b>42672474</b>	Positive	30,2	Positive	31,0	Positive	Positive	32,5	Positive	33,1	Positive	31,8	Positive
<b>42671498</b>	Positive	19,3	Positive	17,8	Positive	Positive	18,3	Positive	18,9	Positive	18,2	Positive
<b>11157674</b>	Positive	20,5	Positive	22,9	Positive	Positive	22,7	Positive	22,7	Positive	22,1	Positive

**Abbreviations:** COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; Ct, cycle threshold; N/A, not applicable; RT-PCR: real-time polymerase chain reaction.

**Supplementary Table 2.** Characteristics of COVID-19 patients.

<b>Characteristic</b>	<b>All</b>	<b>Mild</b>	<b>Moderate</b>	<b>Severe</b>
<b>No. patients</b>	255	85	87	83
<b>Age (years)</b>	63.8 (49.0 – 74.9)	47.3 (31.5 – 61.4)	66.1 (54.8 – 75.1)	71.0 (62.5 – 81.9)
<b>Gender (male)</b>	153 (60.0%)	41 (48.2%)	49 (56.3%)	63 (75.9%)
<b>Comorbidities</b>				
Chronic heart disease	44 (17.3%)	3 (3.5%)	18 (20.7%)	23 (27.7%)
Hypertension	113 (44.3%)	17 (20.0%)	50 (57.5%)	46 (55.4%)
COPD	31 (12.2%)	4 (4.7%)	13 (14.9%)	14 (16.9%)
Asthma	13 (5.1%)	4 (4.7%)	4 (4.6%)	5 (6.0%)
Chronic kidney disease	18 (7.1%)	1 (1.2%)	6 (6.9%)	11 (13.3%)
Liver cirrhosis	13 (5.1%)	3 (3.5%)	2 (2.3%)	8 (9.6%)
Cancer	4 (1.6%)	0 (0.0%)	2 (2.3%)	2 (2.4%)
Obesity	89 (34.9%)	5 (5.9%)	37 (42.5%)	47 (56.6%)
Diabetes	44 (17.3%)	4 (4.7%)	12 (13.8%)	28 (33.7%)
Dyslipidemia	80 (31.4%)	10 (11.8%)	31 (35.6%)	39 (47.0%)
Smoker	27 (10.6%)	12 (14.1%)	12 (13.8%)	3 (3.6%)
Charlson comorbidity index	2 (0 – 4)	0 (0 – 2)	3 (1 – 5)	4 (2 – 5)
<b>Time from COVID-19 symptoms to samples collection (days)</b>	6.6 (3.5 – 8.8)	5.6 (2.9 – 7.8)	7.5 (3.9 – 9.5)	6.9 (3.3 – 8.8)
<b>Oxygen saturation in room air (%)</b>	94 (90 – 96)	97 (95 – 98)	94 (91 – 95)	88 (83 – 93)
<b>Baseline laboratory findings</b>				
Hematocrit (%)	42.7 (39.7 – 45.4)	43.7 (41.1 – 46.8)	42.1 (39.4 – 44.4)	42.7 (39.5 – 46.8)
White blood cells (x 10 <sup>3</sup> cells/μl)	6.0 (4.7 – 8.3)	5.8 (4.8 – 7.6)	6.2 (4.8 – 8.4)	6.0 (4.4 – 9.1)
Lymphocytes (x 10 <sup>3</sup> cells/μl)	1.0 (0.7 – 1.4)	1.3 (1.1 – 1.7)	1.0 (0.9 – 1.4)	0.8 (0.5 – 1.1)
Neutrophils (x 10 <sup>3</sup> cells/μl)	4.4 (3.3 – 6.4)	4.0 (3.0 – 5.4)	4.4 (3.4 – 6.0)	4.7 (3.4 – 7.5)
Thrombocytes (x 10 <sup>9</sup> cells/L)	186 (143 – 239)	188 (156 – 244)	202 (170 – 263)	155 (122 – 208)
International normalized ratio	1.00 (0.95 – 1.10)	0.98 (0.94 – 1.06)	0.98 (0.94 – 1.07)	0.96 (1.05 – 1.13)
Glucose (mg/dL)	117 (101 – 142)	104 (94 – 111)	116 (101 – 145)	124 (113 – 164)
Creatinine (mg/dL)	0.92 (0.78 – 1.21)	0.81 (0.69 – 0.94)	0.92 (0.75 – 1.18)	1.05 (0.84 – 1.43)
eGFR (mL/min)	78.4 (56.3 – 91.7)	90.2 (76.1 – 103.5)	80.8 (55.8 – 94.3)	69.3 (45.3 – 85.4)
Albumin (g/dL)	4.0 (3.7 – 4.3)	4.4 (4.1 – 4.5)	4.1 (3.8 – 4.3)	3.8 (3.6 – 4.0)
Alanine aminotransferase (IU/L)	29 (19 – 44)	24 (19 – 35)	27 (17 – 40)	31 (19 – 67)
Lactate dehydrogenase (IU/L)	299 (238 – 414)	214 (194 – 271)	284 (243 – 363)	407 (302 – 506)
Ferritin (ug/L)	444 (195 – 872)	197 (88 – 429)	406 (210 – 835)	657 (265 – 1200)
C-reactive Protein (mg/L)	70.3 (23.3 – 123.3)	8.6 (3.2 – 32.5)	73.8 (28.5 – 122.2)	83.2 (57.3 – 163.1)

**Clinical outcomes**

Invasive mechanical ventilation	41 (16.1%)	0 (0.0%)	0 (0.0%)	41 (49.4%)
ICU admission	50 (19.6%)	0 (0.0%)	0 (0.0%)	50 (60.2%)
Mortality within 28 days	36 (14.1%)	0 (0.0%)	0 (0.0%)	36 (43.4%)
ICU/death	83 (32.6%)	0 (0.0%)	0 (0.0%)	83 (100.0%)

**Statistics:** Values are expressed as the median and interquartile range (IQR) for continuous variables and absolute count (percentage) for categorical variables.

**Abbreviations:** IQR, interquartile range; COPD, chronic obstructive pulmonary disease; eGFR, estimated glomerular filtration rate; COVID-19, coronavirus disease 2019; ICU, intensive care unit; IU, international units;  $\mu$ l: microliter; L, liter; mg, milligrams.

**Supplementary Table 3.** Association between mucosal biomarkers (log<sub>2</sub> values) in the upper respiratory tract and ICU/death during hospital admission.

<b>A) Whole cohort (100%)</b>				
<b>Biomarkers</b>	<b>Unadjusted regression</b>		<b>Adjusted regression</b>	
	<b>OR (95%CI)</b>	<b>p-value</b>	<b>aOR (95%CI)</b>	<b>p-value</b>
<b>SARS-CoV-2</b>				
Viral load	1.09 (1.04 – 1.15)	<b>0.001</b>	1.19 (1.09 – 1.29)	<b>&lt;0.001</b>
<b>Antiviral genes</b>				
ISG15	1.08 (0.95 – 1.24)	0.227	1.21 (0.97 – 1.51)	0.090
IFN-β	1.09 (0.96 – 1.24)	0.183	0.98 (0.83 – 1.16)	0.817
IFIT1	1.19 (1.00 – 1.43)	0.054	1.08 (0.84 – 1.38)	0.570
RIGI	1.05 (0.90 – 1.22)	0.569	1.05 (0.83 – 1.33)	0.673
<b>Cytokine genes</b>				
TNF-α	1.01 (0.89 – 1.14)	0.924	1.11 (0.92 – 1.35)	0.261
IL-6	1.16 (1.05 – 1.28)	<b>0.002</b>	1.08 (0.96 – 1.22)	0.225
IL-8	1.15 (1.02 – 1.29)	<b>0.022</b>	1.02 (0.88 – 1.17)	0.840
<b>Chemokine genes</b>				
CCL5	0.62 (0.52 – 0.75)	<b>&lt;0.001</b>	0.70 (0.55 – 0.88)	<b>0.002</b>
CXCL10	0.96 (0.88 – 1.05)	0.364	1.05 (0.91 – 1.21)	0.503
<b>B) Training cohort (50%)</b>				
<b>Biomarkers</b>	<b>Unadjusted regression</b>		<b>Adjusted regression</b>	
	<b>OR (95%CI)</b>	<b>p-value</b>	<b>aOR (95%CI)</b>	<b>p-value</b>
<b>SARS-CoV-2</b>				
Viral load	1.10 (1.02 – 1.18)	<b>0.010</b>	1.21 (1.06 – 1.37)	<b>0.003</b>
<b>Antiviral genes</b>				
ISG15	1.05 (0.89 – 1.25)	0.553	1.32 (0.93 – 1.87)	0.115
IFN-β	1.12 (0.93 – 1.36)	0.243	1.15 (0.91 – 1.45)	0.240
IFIT1	1.09 (0.84 – 1.40)	0.515	0.89 (0.61 – 1.32)	0.566
RIGI	0.98 (0.79 – 1.20)	0.816	0.92 (0.68 – 1.23)	0.570
<b>Cytokine genes</b>				
TNF-α	1.04 (0.89 – 1.23)	0.608	1.04 (0.80 – 1.34)	0.777
IL-6	1.26 (1.09 – 1.46)	<b>0.002</b>	1.10 (0.92 – 1.32)	0.277
IL-8	1.36 (1.10 – 1.68)	<b>0.004</b>	1.23 (0.97 – 1.57)	0.088
<b>Chemokine genes</b>				
CCL5	0.62 (0.48 – 0.79)	<b>&lt;0.001</b>	0.66 (0.47 – 0.91)	<b>0.010</b>
CXCL10	0.96 (0.85 – 1.09)	0.542	1.03 (0.84 – 1.27)	0.769
<b>C) Validation cohort (50%)</b>				
<b>Biomarkers</b>	<b>Unadjusted regression</b>		<b>Adjusted regression</b>	
	<b>OR (95%CI)</b>	<b>p-value</b>	<b>aOR (95%CI)</b>	<b>p-value</b>
<b>SARS-CoV-2</b>				
Viral load	1.09 (1.01 – 1.17)	<b>0.024</b>	1.21 (1.07 – 1.37)	<b>0.003</b>
<b>Antiviral genes</b>				
ISG15	1.13 (0.92 – 1.38)	0.246	1.12 (0.81 – 1.55)	0.481
IFN-β	1.06 (0.90 – 1.26)	0.480	0.88 (0.67 – 1.16)	0.359
IFIT1	1.30 (1.01 – 1.69)	<b>0.041</b>	1.11 (0.78 – 1.57)	0.572
RIGI	1.15 (0.90 – 1.46)	0.253	1.15 (0.80 – 1.65)	0.459
<b>Cytokine genes</b>				
TNF-α	0.95 (0.78 – 1.15)	0.615	1.04 (0.78 – 1.39)	0.781
IL-6	1.08 (0.95 – 1.22)	0.267	1.03 (0.87 – 1.21)	0.739
IL-8	1.04 (0.90 – 1.20)	0.565	0.89 (0.71 – 1.10)	0.278
<b>Chemokine genes</b>				
CCL5	0.61 (0.46 – 0.81)	<b>0.001</b>	0.70 (0.49 – 0.99)	<b>0.047</b>
CXCL10	0.95 (0.83 – 1.09)	0.497	1.06 (0.86 – 1.30)	0.605

**Statistics:** The association analysis was performed by logistic regression adjusted by the most significant covariables. The statistically significant differences are shown in bold.

**Abbreviations:** COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ICU, intensive care unit; OR, odds ratio; aOR, adjusted odds ratio; 95%CI, 95% confidence interval; p-value, level of significance; ISG15, interferon-stimulated gene 15; IFN- $\beta$ , interferon-beta; IFIT1, Interferon-induced protein with tetratricopeptide repeats 1; TNF- $\alpha$ , tumor necrosis factor-alpha; IL-6, interleukin 6; IL-8, interleukin 8; CCL5, Chemokine (C-C motif) ligand 5; CXCL10, C-X-C motif chemokine ligand 10; RIGI, retinoic acid-inducible gene I.