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Circulating epithelial cell as viral infection and tissue origin marker in patients with severe COVID-19

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ABSTRACT

Liquid biopsy (LB) is a minimally invasive procedure that detects biomarkers in body fluids for real-time monitoring of patients. This study developed a new LB approach to analyze Circulating Epithelial Cells (CECs) in Intensive Care Unit (ICU) patients with severe COVID-19 and High-Exposure Negative Population to COVID-19 (HENPC) as the control group. The CECs were characterized by multispectral imaging flow cytometry, and an anti-SARS-CoV-2 Spike S1 protein (ProtS) antibody was used to detect infection. The results showed that CECs were present in most ICU patients ($p = 0.0412$), and their median number was significantly higher ($p = 0.0004$) than in controls. CEC clusters were only identified in patients, and high positive ProtS expression was observed in CECs from ICU patients compared to negative controls. In conclusion, LB could be a minimally invasive tool for detecting tissue damage caused by infectious agents and could provide real-time biological information about disease status and evolution. However, further validation in a larger population of patients is needed.

1. Introduction

Since 2019, SARS-CoV-2 infection and its disease (COVID-19) have threatened healthcare systems. The viral infection has a wide range of clinical presentations, from asymptomatic infection to severe illness, usually involving the respiratory system [1]. At the beginning of the pandemic, about 20% of the infected population was admitted to the hospital due to bilateral pneumonia, and 5% needed admission to the Intensive Care Unit (ICU) due to severe respiratory failure [2–5]. The positive evolution of these patients largely depends on the correct treatment, which will also depend on a proper diagnosis. Blood biomarkers such as C Reactive Protein (CRP) can guide treatment. However, there is no specific biomarker able to inform about the course of the infection in the lung or the potential presence of the virus in other organs.

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In the last decade, liquid biopsy (LB) has arisen great interest in different areas of medicine, especially in oncology; however, LB methodologies can also be applied to infectious diseases [6]. LB involves detecting and analyzing biomarkers in a body fluid through non- or minimally invasive methods. There are different types of biomarkers, such as Circulating Epithelial Cells (CECs), Circulating Tumor Cells (CTCs), circulating tumor DNA (ctDNA), extracellular vesicles, or tumor-educated platelets, which can be studied using LB [7]. Our group has recently demonstrated that isolating CECs with type II pneumocyte characteristics in patients with Chronic Obstructive Pulmonary Disease (COPD) is possible, suggesting their role as a marker of pulmonary injury [8]. SARS-CoV-2 infection involves an inflammatory process with diffuse alveolar damage that destroys the epithelial lung tissue during viral replication and dissemination. The damaged tissue can drain entire cells into the bloodstream; therefore, identifying tissue-specific markers in these cells allow us to determine the tissue origin of these drained cells, inferring the source of the infection. Thus, we hypothesized that CECs could be isolated from blood in patients with severe COVID-19 and used as a potential biomarker for disease and tissue origin.

This study aimed to identify CECs expressing SARS-CoV-2 Spike S1 protein (ProtS) in blood samples from patients with severe COVID-19 admitted to the ICU and compare them with a High-Exposure Negative Population to COVID-19 (HENPC) that tested negative for COVID-19 by Polymerase Chain Reaction (PCR).

2. Materials and methods

This cross-sectional and proof-of-concept study included patients with severe COVID-19 admitted to the ICU and a negative control group of healthcare workers with high exposure but PCR negative for SARS-CoV2 from two Spanish hospitals (University Hospital Virgen de las Nieves and University Hospital San Cecilio, Granada, Spain) during august and September 2020.

Inclusion criteria were defined as: adults over 18 years of age with severe COVID-19 (categorized as a positive diagnostic test of active infection for SARS-CoV-2, dyspnea, respiratory rate of 30 breaths per minute, blood oxygen saturation of 93%, Pao2:Fio2 ratio $<$300 mm Hg or infiltrates in more than 50% of the lung field), with ICU admission requirement due to the need of organ support, and exclusion of other conditions that could lead to Acute Respiratory Distress Syndrome (ARDS).

Exclusion criteria included: immunocompromised patients (due to solid/hematological malignancies, Human Immunodeficiency Virus (HIV), or chronic corticosteroid use) and other conditions that led to ICU admission apart from COVID-19. All participants signed informed consent before sample collection.

2.1. Cell cultures

A total of three different cell lines were used in this project. The hAELVi cell line (human alveolar epithelial lentivirus immortalized cell line) was purchased from InSCREENeX company (InSCREENeX GmbH, Germany) and maintained according to the manufacturer's recommendations. The hAELVi cells were cultured on T75 cell culture flasks in huAEC medium (InSCREENeX GmbH, Germany) supplemented with 1% penicillin/streptomycin and its basal supplements provided by the manufacturer. The HEP-G2 (hepatocellular carcinoma cell line) was obtained from the American Type Culture Collection (Manassas, VA, USA). It was grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Grand Island, NY, USA). The Jurkat cell line (an immortalized T lymphocyte cell line) was kindly donated by Dr. Francisco Martin (GENyO Center, Granada, Spain). Jurkat cells were cultured in RPMI 1640 medium with 2.5 mM glutamine and 10% fetal bovine serum. All cell lines were incubated in a humidified atmosphere containing 5% CO2 at 37 °C. Experiments were conducted when cells reached 80–90% of confluency.

2.2. Antibodies quality control assay using cell lines

Cultures containing approximately 50,000 cells were incubated with the different antibodies (Table 1).

The Jurkat cell line was used as a positive control for anti-CD8 antibody (Mouse anti-human CD8 monoclonal (OKT8), and the hAELVi cell line as a positive control of anti-Cytokeratin (CK) antibody (Mouse anti-human monoclonal Cytokeratin (CAM5.2). Mononuclear cells from healthy donors were positive controls for CD14 (Mouse anti-human CD14 monoclonal (M5E2). Nuclear staining (7-AAD) determines cell integrity, a requirement to define an epithelial cell. The stained cells were analyzed with the Amnis ImageStream MKII Imaging System (Luminex Corporation), a multispectral imaging flow cytometer designed to acquire up to 12 channels of fluorescence. By collecting large numbers of digital images per sample and providing a numerical representation of image-based features, the ImageStreamX MkII combines the per-cell information content provided by standard microscopy with the statistical significance afforded by large sample sizes common to traditional flow cytometry. With the ImageStreamX MkII System, fluorescence intensity measurements are acquired as with a conventional flow cytometer; however, the best applications for the ImageStreamX MkII take advantage of the system's systems imaging abilities to locate and quantify the distribution of signals on, in, or between cells.

Cultures containing approximately 50,000 cells were incubated with the different antibodies (Table 1). The hAELVi cell line was used as a positive control for RAGE protein (Rabbit polyclonal anti-RAGE-AF750, bs-0177R-A750, Bioss Inc), a specific lung tissue marker. The HEP-G2 cell line was a positive control for ASGR1 (primary antibody rabbit anti-Asialoglycoprotein-Goat and secondary antibody anti-rabbit Alexa Fluor 633), a specific liver tissue marker. Nuclear staining (7-AAD) determines cell integrity, a requirement to define an epithelial cell. The stained cells were analyzed with the Amnis ImageStream MKII Imaging System (Luminex Corporation).

2.3. Circulating epithelial cell detection and characterization in samples from ICU patients and HENPC

Ten ml of peripheral blood were drawn in vacutainer tubes with EDTA K2 from each ICU patient and HENPC control and processed within 4 h of collection. According to the manufacturer's protocol, blood samples were lysed using red blood cell lysis solution (Miltenyi Biotec. Germany). After lysis, mononuclear cells were fixed and permeabilized using the Fix & Perm cell permeabilization kit (ThermoFisher) for subsequent immunostaining. Samples containing approximately 50,000 cells were incubated with rabbit Anti-SARS-CoV-2 Spike S1 monoclonal (HL6) antibody for 30 min at room temperature. Cells were washed with 500 µl of PBS 1× and centrifuged at 350×*g* for 3 min. Then, an antibody cocktail was added in PBS 1× containing 7-AAD for nuclear staining, mouse anti-human monoclonal Cytokeratin (CAM5.2) for epithelial cells, mouse anti-human CD14 monoclonal (M5E2) for macrophages, and mouse anti-human CD8 monoclonal (OKT8) for lymphocytes labeling; and incubated for 30 min at room temperature (Table 1). Finally, another wash was performed with 500 µl of PBS 1× for subsequent imaging flow cytometry analysis (Fig. 1). The stained cells were analyzed with the Amnis ImageStream MKII Imaging System (Luminex Corporation).

2.4. Imaging flow cytometry analyses

Using the Inspire® Software, labeled samples were run onto an Amnis ImageStream MKII Imaging System (Luminex Corporation). Cells were selected based on nuclear staining with 7-AAD, and fluorescence was acquired with a power of 120 mW for the 405 nm laser, 200 mW for the

Table 1
Antibodies and fluorophores included in the study design, channels used for detection and cell lines used as controls.

Antibody	Fluorophore	Reference	Provider	Dilution	Channel used for detection	Cell line
Rabbit anti-SARS-CoV-2 (COVID-19) Spike S1 monoclonal (HL6)	AF488	GTX635654	Genetex	1:100	2	Only on samples
Mouse anti-human monoclonal Cytokeratin (CAM5.2)	PE-CF594	563615	BD Biosciences	1:100	4	hAELVi (human alveolar type I cell)
Nuclear staining	7-AAD	00-6993-50	ThermoFisher	1:100	5	Cell lines and samples
Mouse anti-human CD14 monoclonal (M5E2)	V450	561390	BD Biosciences	5:100	7	Mononuclear cells from healthy donors
Mouse anti-human CD8 monoclonal (OKT8)	APC	17-0086-42	ThermoFisher	1:100	11	Jurkat
Rabbit polyclonal anti-RAGE	AF750	bs-0177R-af750	Quimigen	1:50	12	hAELVi (human alveolar type I cell)
Rabbit anti-Asialoglycoprotein (ASGR1)	N/A	HPA012852	Merck	1:100	N/A	HEP-G2 (hepatocellular carcinoma)
Goat anti-rabbit Alexa Fluor 633	AF633	A-21070	Thermofisher	1:1000	11	

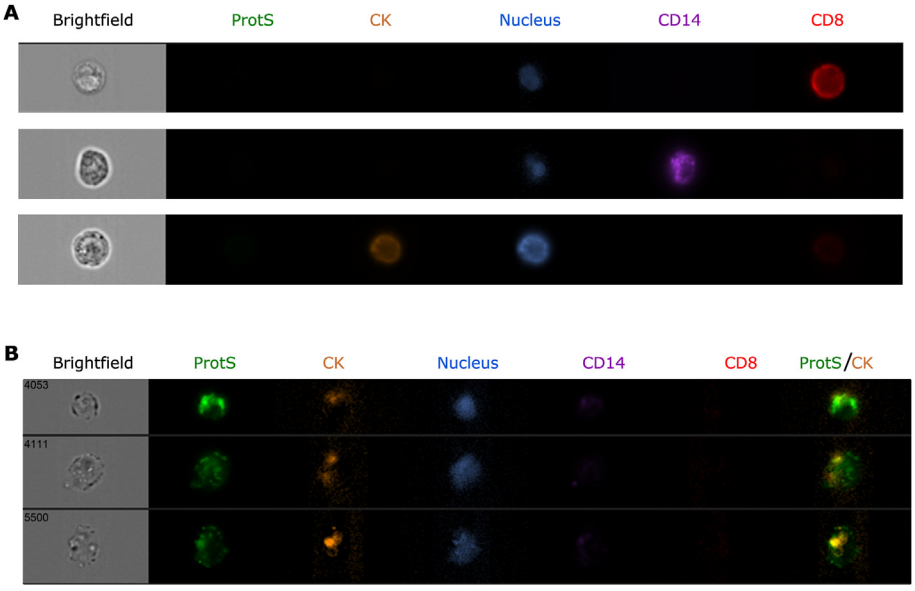

Fig. 1. Cell line characterization and patient phenotyping with the Amnis ImageStream MKII Imaging System. A-upper panel: CD8 positive expression (red) on the Jurkat cell line. A-middle panel: CD14 positive (purple) expression on healthy donors. A-lower panel: Cytokeratin (CK) expression (orange) in hAELVi cells. Nuclear staining (7-AAD) (blue) determines cell integrity, a requirement to define an epithelial cell. (B) Images showing in green the presence of SARS-CoV-2 Spike S1 protein (ProtS) in patient cells. The nucleus was identified using a 7AAD fluorescent intercalator able to bind to DNA (blue). Identification of the other cell types was performed with anti-cytokeratin (CK) in orange for epithelial cells, anti-CD14 in purple for macrophages, and anti-CD8 in red for lymphocytes. ProtS was detected in Intensive Care Unit (ICU) patients in cells expressing only CK. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

488 nm laser, and 150 mW for the 658 nm laser. Images were taken at 60× magnification with low flow and analyzed using the IDEAS® Software (Luminex Corporation) following the manufacturer's recommendation. First, the compensation matrix was done using single-stained controls. Then, cells were selected using the "Raw Max Pixel" properties below 1000 for every fluorophore to avoid saturation and "Gradient RMS" greater than 30 to choose cells in focus. Cell populations were generated using negative and positive controls.

2.5. Statistical analyses

Range, median (interquartile range), or mean ± standard deviation was analyzed. Categorical variables were compared using the chi-square ($\chi 2$) test, and continuous variables were compared using Student's t-test or the Mann-Whitney *U* test, as required. Statistical significance was defined as $p < 0.05$. All statistical analyses were performed using GraphPad Prism software (Dotmatics, San Diego, CA, USA).

3. Results

The epidemiological, clinical, and pathological characteristics of the patients recruited are shown in Table 2.

Seven out of 9 (78%) ICU patients were on invasive ventilation, and the mean time from the beginning of the symptoms to the hospital admission was 4.89 days. The mean time from hospital admission to ICU admission was 5.22 days, with 44% of mortality. These patients were in their middle 50 s, 56% were women, and 89% had at least one comorbidity.

3.1. Circulating epithelial cell SARS-CoV-2 positive characterization from peripheral blood from ICU patients

The antibody quality control assays using cell lines were optimal, as seen in Fig. 1A, which allowed us to analyze patients, as observed in Fig. 1B, where positive CECs expressed ProtS. Indeed, positive ProtS staining was identified in the CECs of ICU patients and the negative control group (Table 3).

The percentage of CECs positive for ProtS in the ICU patients and the control group was 18% vs. 8%, respectively ($p = 0.65$). We found expressions of ProtS in both CECs (CK+) and macrophages ($CD14^+$) (data not shown). Fig. 2A showed a median of 23.54 CECs (range 0.08–43.63) in ICU patients and 2.34 (range 0.08–18.31) in HENPC controls ($p = 0.0004$). Interestingly, we exclusively observed CEC clusters in ICU patients (Fig. 2B).

Table 2
Descriptive analysis of the clinicopathological features of the Intensive Care Unit (ICU) patients included in the study.

	n = 9
Age, mean ± SD	57.4 ± 11.9
Current smokers, n (%)	1 (11%)
Comorbidities	
Obesity, n (%)	5 (56%)
Type 2 diabetes, n (%)	5 (56%)
COPD, n (%)	1 (11%)
Cardiovascular disease, n (%)	4 (44%)
Respiratory failure	
Invasive ventilation, n (%)	7 (78%)
Prone ventilation, n (%)	6 (67%)
Pharmacological treatment	
Dexamethasone, n (%)	8 (89%)
Remdesivir, n (%)	2 (22%)
Clinical source	
Days to ICU admission, mean ± SD	5.22 ± 3.96
ICU days of stay, mean ± SD	28.3 ± 15.9
Mortality, n (%)	4 (44%)
Laboratory findings	
CRP, mean ± SD mg/dL	165.3 ± 46.2
Ferritin, mean ± SD mg/dL	702.8 ± 52.3

SD: Standard Deviation, M: Males, F: Females, COPD: Chronic Obstructive Pulmonary Disease, CRP: C Reactive Protein.

3.2. Circulating epithelial cell characterization according to tissue origin

The cell lines also showed positive expression of RAGE or ASGR1, specific lung, and liver tissue markers, respectively (Fig. 2C and D).

4. Discussion

This proof-of-concept study showed that isolating CECs infected with the SARS-CoV-2 virus (ProtS + CECs) from patients with severe COVID-19 could be used as a potential biomarker of pulmonary injury. These results are interesting because there is an urgent need to find SARS-CoV-2 biomarkers to determine which patients have an increased risk of severe respiratory failure and worse clinical outcomes. In addition, this biomarker could work as a follow-up tool for disease progression and help define treatment responses [9–12].

In this current work, we focused on detecting CECs based on CK expression, and we isolated CECs in both the ICU patients and the HENPC groups (67% vs. 26%, respectively) ($p = 0.0412$). The median number of CECs was significantly higher in ICU patients than in controls, 23.54 vs. 2.34, respectively ($p = 0.0004$). ProtS was expressed in 18% of the patients and 8% of the control population ($p = 0.65$). Surprisingly, CEC clusters were only observed in ICU patients (Fig. 2B). We have previously described that only clusters are disease markers, like in pancreatic cancer [13]. The finding of ProtS expression in CD14 + CECs could be explained as a mechanism of the innate immune response, supported by data from Han et al., who demonstrated that microvesicles released from macrophages could be engulfed by epithelial cells leading to decreased inflammatory responses [14].

Patients admitted to ICU due to a SARS-CoV-2 infection, and its disease (COVID-19) can have different organs affected; however, it is difficult to define whether the virus is housed in one or more organs. In addition to identifying the ProtS in CECs, cell markers such as RAGE or ASGR1 proteins could allow us to identify the specific infected tissue of origin [15]. The number and type of organs injured could have implications in administering the final treatment, showing this methodology's additional value.

Despite the novelty of our results, we acknowledge the limitations of this work, including the low number of analyzed cases. However, a larger cohort including additional antibodies associated with the cellular origin of these CECs to identify potential organs infected by the virus would be attractive.

To this day, the long-term complications of the organs affected by SARS-CoV-2 infection are still unknown. Thus, having minimally invasive tools to monitor these infected organs becomes essential.

5. Conclusions

We first described a methodology able to identify Circulating Epithelial Cells in the blood that allowed us to detect SARS-CoV-2 infection and potentially determine their organ of origin. These results are hypothesis generators and could be used as a non-invasive diagnostic tool for detecting tissue damage caused by an infectious agent if confirmed in a larger population. LB could be a minimally invasive tool for detecting tissue damage caused by infectious agents and could provide real-time biological information about disease status and evolution.

Author contributions

Conceptualization, M.J.S, B.A.N., and J.J.D.M.; methodology, M.P.M.V., M.C.G.N., and A.G.D.; validation and formal analysis, M.J.S., P.J.R.P., J.A.L.A., and J.E.H.; sources and data curation, J.M.P.V., M.C.R., and J.M.N.M.; writing—original draft preparation, M.J.S. and B.A.N.; writing—review and editing, V.D., D.M.P., and C.R. All authors have read and agreed to the published version of the manuscript.

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Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee (2709-N-20).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability statement

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

Table 3
Analysis results of Intensive Care Unit (ICU) patients' and High-Exposure Negative Population to COVID-19 (HENPC).

	n	PCR	Patient CEC-positive n (%)	Number CEC CK + patients mean (range)	Cluster	CEC ProtS-positive (%)
ICU patient	9	positive	6/9 (67)	23.54 (0.08–43.63)	Pos	18
HENPC controls	19	negative	5/19 (26)	2.34 (0.08–18.31)	Neg	8
p-value	–	–	0.0412	0.0004	–	0.65

n: number; PCR: polymerase chain reaction; CEC: Circulating Epithelial Cell; CK: Cytokeratin; ProtS: SARS-CoV-2 Spike S1 protein.

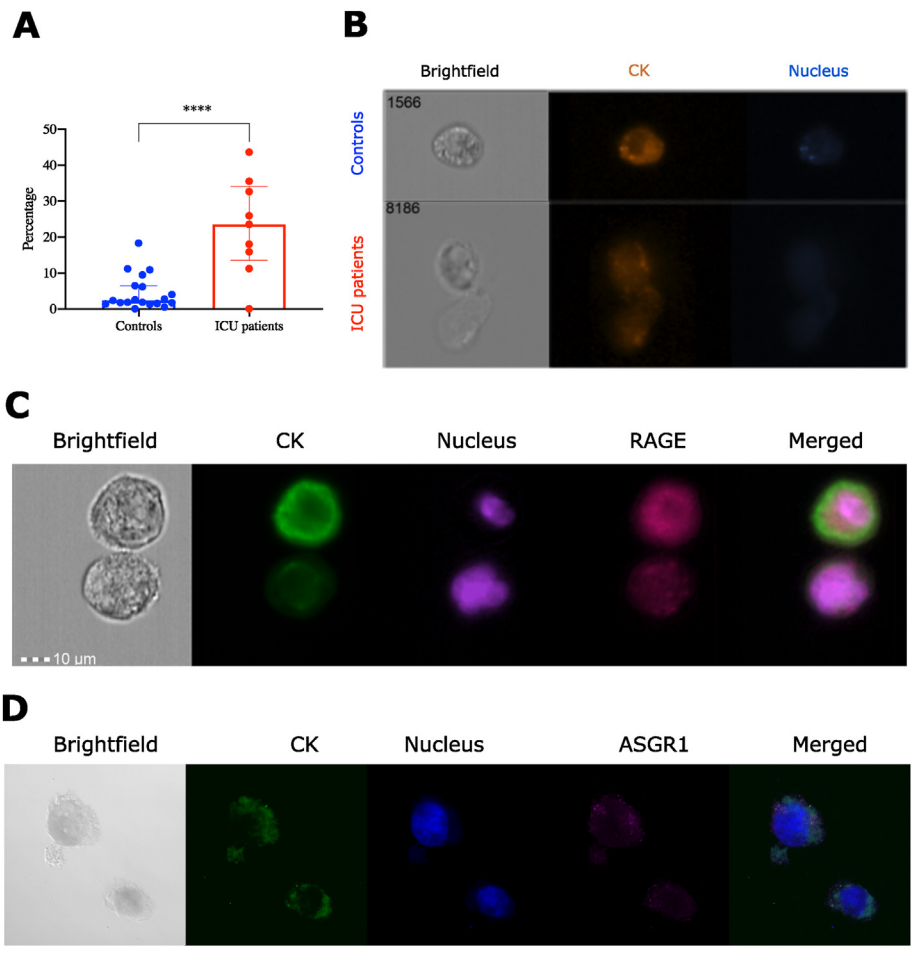

Fig. 2. Circulating epithelial cells (CECs) were identified based on cytokeratin (CK) expression. (A) Scatter plot of percentage of CK positiveness in High-Exposure Negative Population to COVID-19 (HENPC) controls and Intensive Care Unit (ICU) patients, with medians and interquartile ranges (p-value**** = 0.0004). (B) Amnis ImageStream MKII Imaging picture with cytokeratin (CK) in orange and nuclear staining showing a cluster. (C) CEC positive for RAGE expression, and D) CEC positive for ASGR1 expression. Both, specific markers of lung and liver tissue, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Patents

FIBAO-22009 - IPR-986 - P 202330087 patent entitled "Detection in circulating epithelial cells of viruses and/or bacteria causing inflammatory infections" on behalf of the Andalusian Health Service and the University of Granada.

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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Acronyms

ARDS: Acute Respiratory Distress Syndrome
CEC: Circulating Epithelial Cell
ctDNA: circulating tumor DNA
CK: Cytokeratin
CTC: Circulating Tumor Cell
COPD: Chronic Obstructive Pulmonary Disease
CRP: C Reactive Protein
HENPC: High-Exposure Negative Population to COVID-19
HIV: Human Immunodeficiency Vrus
ICU: Intensive Care Unit
LB: Liquid Biopsy
PCR: polymerase chain reaction
ProtS: SARS-CoV-2 Spike S1 protein