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THE PROGNOSTIC ROLE OF T-CELL IMMUNE RESPONSE AND LONG-TERM CLINICAL OUTCOMES IN CRITICALLY ILL COVID-19 PATIENTS TREATED WITH VV-ECMO

PhD thesis

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List of Abbreviations

ARDS: Acute Respiratory Distress Syndrome

BDI: Beck Depression Inventory

BMI: Body Mass Index

CMR: Cardiac Magnetic Resonance

COPD: Chronic Obstructive Pulmonary Disease

COVID-19: Coronavirus Disease 2019

CT: Computed Tomography

DLCO: Diffusing-Capacity of the Lungs for Carbon Monoxide

ECG: Electrocardiogram

ECMO: Extracorporeal Membrane Oxygenation

ELSO: Extracorporeal Life Support Organization

ESS: Epworth Sleepiness Scale

FEV1: Forced Expiratory Volume in the First Second

FIO2: Fraction of Inspired Oxygen

FSS: Fatigue Severity Scale

FVC: Forced Vital Capacity

GCS: Global Circumferential Strain

GGO: Ground-Glass Opacity

GLS: Global Longitudinal Strain

HR: Heart Rate

ICU: Intensive Care Unit

IFN-γ: Interferon-γ

IL-6: Interleukin-6

IMV: Invasive Mechanical Ventilation

IQR: Inter-Quartile Range

KLCO: Carbon Monoxide Transfer Coefficient

LGE: Late Gadolinium Enhancement

LV: Left Ventricle

LVEDD: Left Ventricular End-Diastolic Diameter

LVEDVi: Left Ventricular End-Diastolic Volume Index

LVEF: Left Ventricular Ejection Fraction

LVEFi: Left Ventricular Ejection Fraction Index

LVESVi: Left Ventricular End-Systolic Volume Index

LV GCS: Left Ventricular Global Contractile Strain

LV GLS: Left Ventricular Global Longitudinal Strain

LVMi: Left Ventricular Mass Index

LVSVi: Left Ventricular Stroke Volume Index

NAbs: Neutralizing Antibodies

NC: Nucleocapsid

NT-proBNP: N-Terminal Pro-B-Type Natriuretic Peptide

PANAS: Positive and Negative Affect Schedule

PaO2: Partial Pressure of Arterial Oxygen

PICS: Post-Intensive Care Syndrome

PDS: Post-traumatic Diagnostic Scale

PFT: Pulmonary Function Tests

PSQI: Pittsburgh Sleep Quality Index

QF: Quantiferon

RBD: Receptor Binding Domain

RV: Right Ventricle

RVEDD: Left Ventricular End-Diastolic Diameter

RVFAC: Left Ventricular Fractional Area Change

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

6MWT: 6-Minute Walk Test

SF-36: 36-Item Short Form Health Survey

SL: Satisfaction with Life

S1: Spike Protein 1

SpO2: Peripheral Oxygen Saturation

S-specific: Spike Specific

STAI: Stait-Trait Anxiety Inventory

VAS: Visual Analogue Scale

VV: Venovenous

VV-ECMO: Venovenous Extracorporeal Membrane Oxygenation

WBC: White Blood Cell

WHO: World Health Organization

1. Introduction

1.1. SARS-CoV-2 pandemic

The emergence of COVID-19 in late 2019 marked one of the most significant global health challenges of the 21st century. Quickly developing into a pandemic, it has significantly altered the functioning of healthcare systems worldwide. The first cases appeared in December 2019 in the Chinese city of Wuhan, where a cluster of pneumonia cases of unknown origin was detected. Following the genetic sequencing of the virus, it quickly became clear that a new strain of coronavirus with significant human-to-human transmissibility has emerged. The pathogen causing COVID-19, SARS-CoV-2, quickly triggered a global health emergency. Due to the rapid international spread of the infection, the World Health Organization officially declared a pandemic on March 11, 2020.

In the early stages, doctors and researchers found themselves facing a pathogen that was then still unknown; they had to make serious decisions in a situation where the available scientific evidence was extremely limited. The medical community was suddenly confronted with the diagnostic, therapeutic, and epidemiological challenges of a new infectious disease, while the exponential spread of the infection placed an increasing burden on healthcare systems.

The clinical spectrum of the disease proved to be extremely broad, ranging from asymptomatic infections and mild upper respiratory tract illnesses to severe viral pneumonia [1, 2]. Thromboembolic complications were also observed in numerous cases, which caused multi-organ failure in a specific patient group. The large number of patients with rapidly deteriorating conditions placed a significant strain on intensive care units, while the available treatment options were limited. Patient care therefore was presented novel, unknown challenges.

During the first waves of the pandemic, healthcare professionals had to contend not only with the clinical challenges of patient care but also with shortages of personal protective equipment, limited resources, and constantly changing professional guidelines. Doctors and nurses had to make decisions such as prioritizing the allocation of resources and optimizing care for critically ill patients. The uncertainty was further exacerbated by the fact that the

infection posed a significant risk of illness and death even among healthcare workers. In 2021, the emergence and spread of the Delta variant of the virus (SARS-CoV-2 Delta B.1.617.2) caused the most severe cases of the pandemic among the unvaccinated part of the population.

Many fundamental questions remained unanswered such as the virus's transmission mechanism, the natural course of infection, the role of risk factors, and the optimal treatment strategy for the disease. It was also not precisely known which factors influence the severity of the disease or what immunological mechanisms might underline clinical deterioration. Clinical decision-making was often based on limited evidence, case reports, or experience gained from previous, already known viral infections.

1.2. Characteristics and treatment options for COVID-19-associated ARDS

Hypoxemic respiratory failure developed in approximately 5–7% of patients, which in many cases met the criteria for acute respiratory distress syndrome (ARDS). Similar to classic ARDS, diffuse alveolar membrane damage was observed here as well, along with inflammatory cellular infiltration of the alveolar-capillary spaces, through which fluid leaked into the alveoli, causing edema. As a result, lung compliance decreased, gas exchange deteriorated severely, and hypoxemia developed. At the same time, in addition to classic ARDS, features such as micro thrombosis, significant vascular involvement, and ventilation-perfusion mismatch were also observed. A significant proportion of these critically ill COVID-19 patients exhibited rapidly progressive hypoxemia, which often proved refractory to conventional ventilatory support methods. Physicians working in intensive care units had to develop treatment strategies while the pathophysiology of the disease was still only partially understood.

In the early stages, the optimal timing of invasive mechanical ventilation, the role of non-invasive ventilation, the use of prone positioning and other ventilatory support techniques were strongly debated. Finally, lung-protective ventilation became the guiding principle with the main goal of preventing further lung injury. Using low tidal volumes and positive end-

expiratory pressure (PEEP) aided the prevention of lung collapse, while avoiding overdistension and further damage to lung tissue.

In severe ARDS, when conventional mechanical ventilation is unable to maintain adequate oxygenation and/or CO_2 elimination, the use of veno-venous extracorporeal membrane oxygenation (VV-ECMO) can be life-saving by support gas exchange and minimizing ventilator-induced lung injury [3]. ECMO technology allows for lung unloading and the maintenance of oxygenation, however its use requires significant resources. At the peak of the pandemic, high-level cardiovascular centers began using VV-ECMO support for critically ill COVID-19 patients with refractory hypoxemia [4, 5, 6].

In deeper datail, VV-ECMO is an intensive care life-support procedure - an extracorporeal circulation system that functions as an artificial lung - which temporarily replaces the gas exchange function of the lungs in cases of severe, reversible respiratory failure. It oxygenates the circulating blood volume outside the body and removes carbon dioxide from it (oxygenator), while the heart's own pumping function is maintained. During this process the blood is drained from the body through a central vein (such as the right femoral vein); this is the inflow (drainage) cannula. Gas exchange occurs as the blood passes through the membrane oxygenator. The oxygenated blood is returned to the patient via another central vein (e.g., the right internal jugular vein); this is the outflow (return) cannula.

ECMO support serves multiple purposes: ensuring oxygenation, relieving the burden on the lungs, reducing ventilator-induced lung injury, and allowing time for lung regeneration. One of the greatest clinical challenges of ECMO-supported therapy is appropriate patient selection. Classic prognostic factors - such as age, comorbidities, duration of mechanical ventilation, or organ failure - cannot fully explain the varying outcomes among patients [7, 8].

1.3. The cardiovascular and microvascular effects of COVID-19

As the pandemic progressed, it became increasingly clear that COVID-19 is not merely a respiratory disease. At the peak of the pandemic, it became known that one of the hallmarks of COVID-19 are endothelial dysfunction and immunothrombosis, which can lead to

capillary damage and the formation of microthrombi. These can result in local ischemia. The infection can trigger a significant systemic inflammatory response, which may be associated with endothelial damage, hypercoagulability, and microcirculatory disturbances. Consequently, patients may develop an increased risk of thromboembolism, cardiovascular complications, acute kidney injury, neurological abnormalities, and multi-organ failure. At the onset of the pandemic, myocarditis was suspected based on subjective symptoms (palpitations, tachypnea, exertional dyspnea) and elevated troponin levels; however, direct viral myocarditis proved to be much rarer.

SARS-CoV-2 is also capable of entering certain cardiac muscle cells via the ACE2 receptor. During autopsies, histological examinations showed that true, classic lymphocytic myocarditis is relatively rare, while inflammatory cellular infiltration of the myocardium is common. In addition, microvascular abnormalities and endothelial damage have been described. This is an important distinction. COVID-19 often causes myocardial injury, but not all myocardial injury constitutes true myocarditis. The question has rightly been raised as to whether this has long-term cardiac consequences.

1.4. SARS-CoV-2 and the immune system

Based on our early clinical observations, we hypothesized that the pathophysiology of severe COVID-19 is not driven solely by the virus's direct cytopathic effects. It soon became clear that clinical outcomes are significantly influenced not only by viral replication but also by the host's immune response. Researchers mapped the virus's biological properties with unprecedented speed [9]. In the early stages of infection, the innate immune system is activated, and the well-known antiviral response develops, the primary goal of which is to eliminate the virus and limit the spread of the infection [10, 11]. Research in recent years has shown that dysregulation of the innate and adaptive immune systems plays a decisive role in disease progression and clinical outcome [12, 13]. SARS-CoV-2 primarily infects respiratory epithelial cells; the so-called Spike (S) protein on the virus's surface binds to the angiotensin-converting enzyme 2 (ACE2) receptor on the surface of host cells. The virus's Spike protein is cleaved by a host cell enzyme, most commonly TMPRSS2, which allows the virus to fuse with the cell membrane, thereby allowing the viral RNA to enter the cytoplasm. The virus's

nucleic acids are recognized by various pattern recognition receptors. During the innate immune response, whose primary goal is to eliminate the virus, the activation of these receptors leads to the production of interferon-I and interferon-III, which play a key role in early antiviral defense [14]. However, several studies have shown that in severe cases of COVID-19, the interferon response is delayed or insufficient [15, 16]. As a result, viral replication increases, and the infected cell effectively becomes a “virus factory,” inducing a more intense, excessive inflammatory response. This has also been investigated and confirmed by numerous studies [17, 18]. In the early stages of the pandemic, the term “cytokine storm” was widely used to describe these severe cases. Although it is now known that the inflammatory response to COVID-19 differs from the classic cytokine storm, the significant role of inflammatory mediators is indisputable. A few days after infection, the specific immune response begins. During the adaptive immune response, B cells produce antibodies against the Spike protein, neutralizing the virus. CD4+ T cells coordinate the immune response [19]. CD8+ T cells destroy infected cells [20]. The cellular immune response is key to controlling the virus [21].

Once the SARS-CoV-2 virus enters host cells, it replicates, causing cell damage and triggering an immune response. An appropriate immune response helps eliminate the virus, but in some cases, an excessive or unregulated immune response can lead to severe tissue damage and multi-organ failure.

2. Objectives

Clinical observations have continuously shaped therapeutic approaches and opened up new avenues of research into the immunological and pathophysiological basis of the disease. A growing body of evidence suggested that the host's immune response plays a decisive role in the course and outcome of the disease. We hypothesized that the immune response elicited by SARS-CoV-2 infection may have significant prognostic value for the survival of critically ill patients. At the same time, the prognostic significance of this factor in critically ill patients receiving ECMO support remains unclear.

During the pandemic, identifying patients most likely to benefit from VV-ECMO treatment posed a particular challenge for intensive care specialists. The determination of optimal indications for ECMO use, its timing, and expected clinical outcomes has been based on limited evidence; therefore, it has become necessary to identify prognostic factors that can aid in clinical decision-making.

In addition to short-term survival, another important clinical aspect is the long-term outcome of patients recovering from a critical condition. It is well known that a significant proportion of patients who survive intensive care develop post-intensive care syndrome (PICS), which can result in persistent physical, psychological, and cognitive consequences [22]. In addition, specific organ abnormalities - particularly pulmonary and cardiovascular involvement - as well as prolonged immunological changes have been observed following COVID-19. However, it is not yet fully understood to what extent severe COVID-19 infection and the associated VV-ECMO support affect pulmonary structural and functional regeneration, the long-term consequences of myocardial involvement, and patients' quality of life.

Based on the above, our study had two objectives:

1. To investigate the prognostic significance of the SARS-CoV-2-specific immune response, with particular emphasis on T-cell immunity, among critically ill COVID-19 patients receiving VV-ECMO treatment.
2. A comprehensive assessment of the long-term clinical, functional, immunological, and quality-of-life outcomes of patients who survive VV-ECMO treatment, with particular emphasis on pulmonary and cardiovascular sequelae and the incidence of post-intensive care syndrome.

3. Methods

3.1. Study design and patient population

Our study consisted of two complementary components:

• a prospective, single-center observational study that evaluated the relationship between the immune response and survival during the acute phase,

• and a prospective longitudinal follow-up study that analyzed the long-term (24-month) outcomes of surviving patients. A detailed assessment has been conducted of the patients' cardiological, pulmonary, immunological, physical, and mental status, as well as their quality of life.

Patients were treated in the Intensive Care Unit of the Városmajor Clinic of Cardiology and Vascular Medicine at Semmelweis University between March 1 and December 31, 2021.

3.2. Inclusion criteria

After considering the multitude of complexity of specific influential factors, the following inclusion criteria have been set:

- Age 18 years or older
- SARS-CoV-2 infection confirmed by real-time PCR
- Received VV-ECMO support due to COVID-19-related refractory ARDS
- Patients classified as severe (D10) according to the World Health Organization (WHO) criteria of May 27, 2020 [23]
- Patients who had not yet been vaccinated against SARS-CoV-2 at the time of infection

3.3. Clinical care

Patients were treated in accordance with the updated recommendations of the ELSO (Extracorporeal Life Support Organization) (COVID-19 Interim Guidelines) [24]. Patients with PCR-confirmed COVID-19-refractory ARDS who had no other documented organ

failure outside the lungs were referred to our tertiary and regional ECMO center. At the primary care facility, all patients received:

- antiviral treatment (remdesivir 100 mg daily for 5 days),
- glucocorticoids (dexamethasone 0.1 mg/kg daily for 3 days, with a maximum daily dose of 8 mg, which was then gradually tapered over the next 4 days),
- immunomodulatory therapy as needed,
- and comprehensive intensive care.

Upon arrival, all patients underwent urgent cannulation, and ECMO support was initiated. The preferred cannulation strategy involved the use of the right femoral vein (inflow, long cannula) and the right internal jugular vein (outflow, short cannula) for blood withdrawal and return, respectively. In all cases, we used a CytoSorb absorber, which can reduce circulating pro- and anti-inflammatory cytokine levels, potentially improving the course and outcome of the disease [25].

Lung-protective ventilation has been used in all cases. (VT ≈ 4.0 mL/kg, PEEP ≥ 10, Pplat ≤ 25 cm H_2O, RR = 4–15/min or spontaneous breathing, FiO_2 = 30%–50%)

Prealbumin, IgA, IgG, and IgM levels were determined from blood samples taken at the time of ECMO cannulation. If the IgM level was <0.4 g/L, we initiated immunomodulatory therapy and administered Pentaglobin (12% IgM, 12% IgA, and 76% IgG). These patients received an initial bolus dose at a rate of up to 0.6 mL (30 mg)/kg/hour for 6 hours, followed by a continuous maintenance dose at a rate of 0.2 mL (10 mg)/kg/hour for 72 hours (total dose ≥0.9 g/kg).

Upon admission to our ward, we collected the referring laboratory test results, identified risk factors, and recorded anthropometric data. Immunological tests and SARS-CoV-2 PCR testing were performed on blood samples taken during ECMO cannulation, which confirmed the viral load in all cases. Respiratory samples were collected only in later stages from patients who required therapeutic bronchoscopy. PCR tests performed on samples obtained during bronchoalveolar lavage (BAL) were positive in all cases.

All patients underwent a comprehensive initial clinical evaluation, which included a full laboratory workup, transesophageal echocardiography, chest CT, and abdominal ultrasound. If the PaO2/FiO2 ratio deteriorated after ECMO initiation, we performed bronchoscopy and collected microbiological samples from the lower airways. Once hemodynamic stability was achieved, patients were re-evaluated every 24 hours according to the same protocol until discharge from our department.

3.4. Immunological examinations

Laboratory samples were collected from peripheral venous blood:

- during the acute phase (at the time of ECMO cannulation)
- as well as at long-term follow-up (at 6, 12, 18, 24, and 30 months)

Table 1 provides detailed documentation of the markers of humoral and cellular immune responses measured in our study.

Test name	Test target	Measured variables and cut off	Measuring device	Measuring principle/ method
RocheElecsys ELISA test (Roche Diagnostics International Ltd, Switzerland)	Antibodies specific to SARS-CoV-2 spike (S) protein receptor binding domain (RBD) in human serum and plasma	IgG, IgA, IgM against SARS CoV 2 (quantitative) Limit of quantification: 0.4 U/ml. <0.8 U/ml – negative >0.8 U/ml – positive	Elecsys Anti-SARS-CoV-2 S immunoassay (Roche Diagnostics International Ltd, Switzerland) on a Cobas e6000 instrument	Double-antigen sandwich principle using electrochemiluminescence for quantitative determiation of antibodies.
Euroimmun ELISA test	Humoral immunresponse – antibodies produced against different antigens of the virus: nucleocapsid/spike proteins	Anti-SARS-CoV-2 QuantiVac ELISA (IgG): The results are given in relative units (RU/ml). <8 RU/ml – negative 8 – 11 RU/ml – borderline >11 RU/ml – positive Upper limit of quantification: 120 RU Anti-SARS-CoV-2 ELISA (IgA): The results are given in ratio (extinction of the sample/extinction of calibrator). <0.8 – negative 0.8 – 1.1 – borderline >1.1 – positive Anti-SARS-CoV-2 NCP ELISA (IgG and IgM): The results are given in ratio (extinction of the sample/extinction of calibrator). <0.8 – negative 0.8 – 1.1 – borderline >1.1 – positive	Sample dilution was performed manually, further steps were carried out automatically using an Elite Lite (DAS, Italy) device.	Enzyme immunoassay provides semiquantitative in vitro determination of human antibodies of different immunoglobulin classes.
QuantiFERON SARS-CoV-2 ELISA – IGRA (Interferon-Gamma Release Assays) test	Antigen tubes targetted to different cellular markers. Blood collection tubes coated with specific SARS-CoV-2 peptides pool from spike antigen (S1 S2 RDB). Blood collection tubes coated	QF Ag1 stimulates CD4+ T cells QF-Ag2 stimulates both CD4+ CD8+ T cells with S1/S2 RBD antigen QF-Ag3 stimulates both CD4+ CD8+ T cells with spike and additional peptides, including nucleoprotein Results:	QuantiFERON ELISA (QIAGEN Group)	Based on the production of interferon gamma (IFN-γ) by lymphocytes in peripheral blood. The QF Ag1 stimulates CD4+ T cells, QF-Ag2 stimulates both CD4+ CD8+ T cells with S1/S2 RBD antigen, while QF-Ag3 stimulates both CD4+ CD8+ T cells with spike and additional peptides, including nucleoprotein. After 16-24 hours of incubation at 37°C, the IFN-γ produced

	with specific SARS-CoV-2 peptides pool from spike and additional peptides issued (N (nucleocapsid) and M (M protein) domains) from the full genome of the SARS-CoV-2 virus.	>0.15 – 0.2 IU/ml – positive		during the cell activation was measured from the separated plasma samples by QuantiFERON ELISA
Functional flow cytometry	Virus specific T cells were detected by functional flow cytometry based on IFN-γ secretion.	Cut-off value was set as 0.02% IFNγ+ T cells (minimum 50 events).	Peptivator SARS-CoV-2 Protein S1, S and N (Miltényi Biotec) #130-127-585 #130-127-586 #130-127-583 Cytostim #130-092-172 10ul IFNγ-PE and 10ul CD3-VioBlue /CD4APC/ CD8FITC/ CD14-Per-CP/ CD20-PerCP Miltenyi Biotec, Rapid Cytokine Inspector CD4/CD8 Kit #130-097-343 Rapid Cytokine Inspector Anti-IFNγ-PE # 130-097-600 Cytoflex flow cytometer (Beckman Coulter)	Human peripheral blood mononuclear cells isolated by density gradient centrifugation and mononuclear cells were stimulated with 6nmol Peptivator SARS-CoV-2 Protein S1, S for two hours at 37ºC and 5% CO2, then incubation continued in the presence of Brefeldin-A for 4 hours at 37ºC and 5% CO2. Unstimulated samples without Peptivators were used as negative controls, and samples stimulated with Cytostim served as positive controls. For surface and intracellular staining cells were incubated with a staining mix of antibody cocktail for 10 minutes at room temperature, then Inside Fix and Inside Perm reagents were used for cell fixation and permeabilization. After cell wash and centrifugation, 500 000 cells were acquired from each sample using flow cytometer. Analysis was performed according to Miltenyi Biotec gating strategy: Doublets (FSC-H/FSC-A), CD19+ B cells and CD14+ monocytes were excluded. T cells were identified by CD3 positivity and scatter characteristics (FSC/SSC). Ratio of INF-γ positive virus specific T cells was determined within the CD4+ and CD8+ T cell population separately, compared to negative control.

Table 1 Measures of the SARS-CoV-2 specific immune response

3.4.1. Humoral immune response

Among the markers of the humoral immune response, we examined the following:

- Measurement of SARS-CoV-2-specific IgG, IgA, and IgM antibodies (Roche Elecsys test)

We obtained comprehensive information on the humoral immune response against SARS-CoV-2 by measuring IgG, IgA, and IgM antibody levels that specifically bind to the receptor-binding domain (RBD) of the SARS-CoV-2 Spike (S) protein, using an electrochemiluminescent method based on the double-antigen sandwich principle [26].

In addition, we examined antibodies produced against various viral antigens, including the nucleocapsid and spike proteins, using specific enzyme-linked immunosorbent assay (ELISA) tests that assess the presence of different antibody isotypes [27]. These were as follows:

- Human antibodies belonging to the IgG and IgA immunoglobulin classes against the S1 domain of the SARS-CoV-2 spike protein (Euroimmun ELISA test)

- Human antibodies of the IgG and IgM immunoglobulin classes against the SARS-CoV-2 modified nucleocapsid protein (NCP) antigen in serum or plasma (Euroimmun ELISA test)

3.4.2. Cellular immune response

We also examined our patients' cellular immune responses. To do this, we used interferon-gamma release assays (IGRA) and flow cytometry.

- Interferon-gamma release assay (QuantiFERON)

The QuantiFERON (QF) SARS-CoV-2 ELISA test is based on the amount of interferon-gamma (IFN-γ) produced by lymphocytes in peripheral blood. The sample tubes consist of three antigen tubes: Ag1, Ag2, and Ag3. These contain a combination of SARS-CoV-2-specific antigens to stimulate T-lymphocytes. The Ag1 tube, a blood collection tube coated with a specific peptide mixture derived from the SARS-CoV-2 spike antigen, stimulates (CD4+) T-lymphocytes. The Ag2 tube, also a blood collection tube coated with a specific

peptide mixture derived from the spike antigen (S1, S2, RDB), stimulates both (CD4+) and (CD8+) T lymphocytes. The Ag3 tube contains a specific SARS-CoV-2 peptide mixture (spike, nucleocapsid, M domains) and stimulates both (CD4+) and (CD8+) T lymphocytes. After 16–24 hours of incubation at 37 °C, we measured the IFN-γ produced during cell activation from the isolated plasma samples [28]. For the sake of clarity in our study, we designated the results of Ag1 quantification as "QF CD4+", those of Ag2 as "QF CD4+ and 8+", and those of Ag3 as "QF whole genome".

- Flow cytometric T-cell analysis (CD4+, CD8+)

Virus-specific T-cells were detected by functional flow cytometry based on IFN-γ secretion. Both the peptide stimulators of the spike (S1) and nucleocapsid (NC) antigens were used to stimulate the T-cells. T-cell activation, staining, and analysis were performed according to Miltenyi Biotec protocols, adapted to local equipment and personnel. The proportion of IFN-γ-positive virus-specific T cells was determined separately within the CD4+ and CD8+ T-cell populations, relative to a negative control. Figure 1 shows the flow cytometric gating strategy diagram. The results are referred to in the study as "Flow S1 CD4+," "Flow S1 CD8+," "Flow NC CD4+," and "Flow NC CD8+."

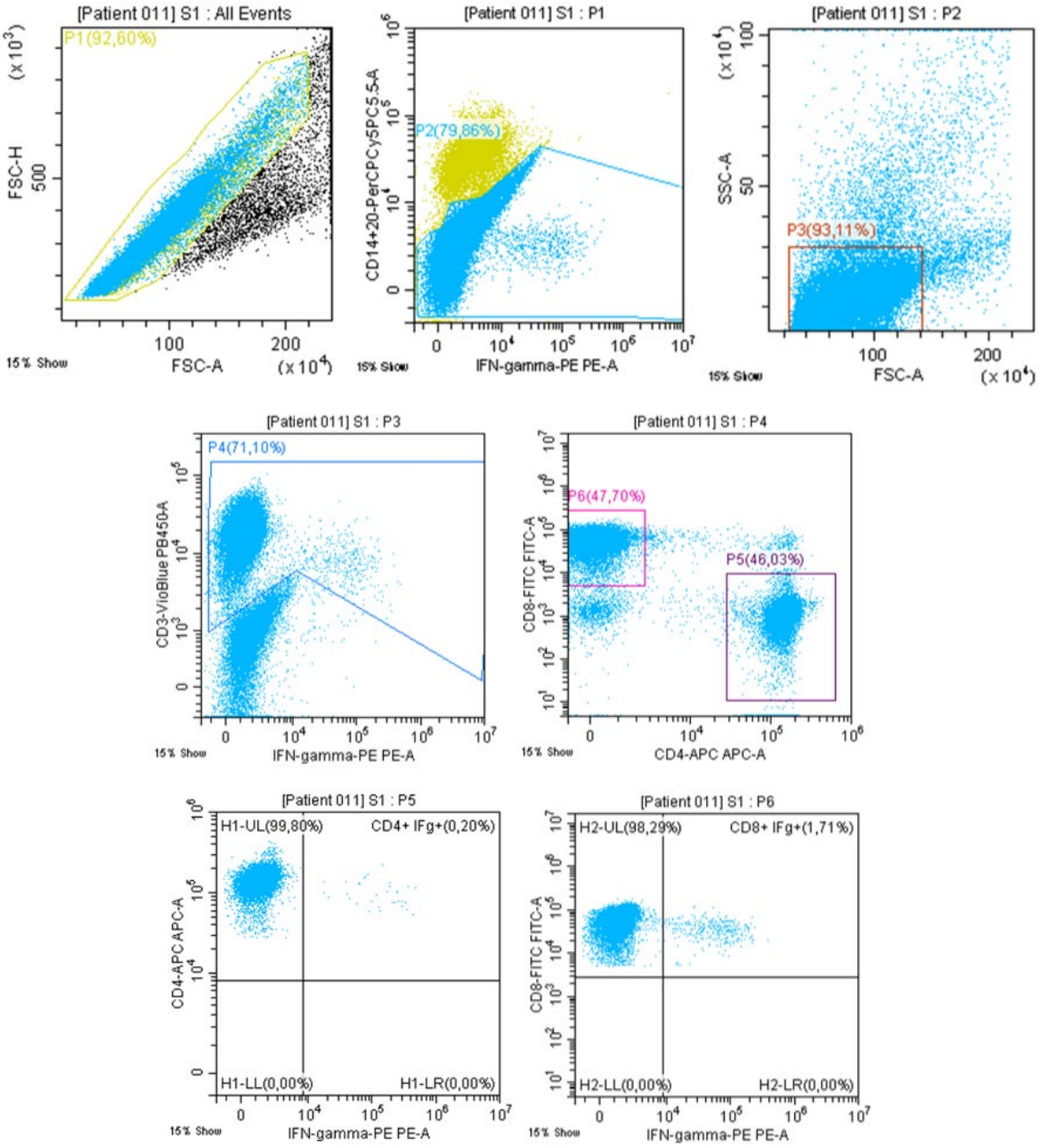

Figure 1 High T-cell activity as detected by flow cytometry. *Sequential gating strategy used by Milteny. Patient sample containing SARS-CoV-2-specific T cells (VST-positive sample). P1: Single cells derived from peripheral blood mononuclear cells (PBMCs). P2: CD20-negative and CD14-negative cells. (Exclusion of B cells and monocytes). P3: Lymphocytes with low FSC/SSC characteristics. P4: CD3+ T cells. P5: CD4+ T cells, P6: CD8+ T cells. Proportion of IFN-γ-*

positive T cells: 0.20% and 1.71% virus-specific T cells were identified in the CD4+ and CD8+ T cell populations.

3.5. Long-term follow-up

Patients were continuously monitored even after their discharge from the intensive care unit. They participated in a comprehensive follow-up study for 30 months following their COVID-19 diagnosis:

3.5.1. Pulmonary examinations

Chest CT

During admission to the emergency department, a baseline native chest CT scan was performed on all patients as part of the routine imaging protocol for patients admitted to the hospital with severe pneumonia during the COVID-19 pandemic. The baseline and follow-up CT scans were interpreted at our institute by a board-certified radiologist specializing in cardiothoracic imaging. The CT scans were evaluated using syngo.via software (Siemens Healthineers AG, Forchheim, Germany).

- Pulmonary function tests

Forced vital capacity (FVC) and forced expiratory volume were measured in the first second (FEV1) according to standard clinical protocols [29], calculated the ratio of the two (FEV1/FVC), measured the lung carbon monoxide diffusion capacity (DLco) and its value corrected for alveolar volume (Klco) [30].

- 6-Minute Walk Test (6MWT)

The 6-minute walk test is a simple functional exercise test that measures the distance a patient can walk on a flat surface at their own pace over 6 minutes, thereby providing a good indication of exercise capacity in patients with heart and lung diseases.

3.5.2. Cardiologycal examinations

During our cardiological follow-up, the following diagnostic tests were performed :

- Laboratory tests (hs-troponin T, NT-proBNP)
- Standard 12-lead ECG
- Echocardiography

During follow-up, transthoracic echocardiography (GE Vivid E95, equipped with a 4Vc-D matrix-array transducer; GE Healthcare, Horten, Norway) was carried out. In accordance with current guidelines, a standard imaging protocol was implemented, consisting of loops containing parasternal, apical, and subxiphoid views [31].

- Cardiac MRI

Cardiac MRI images were acquired on a 1.5 T MRI scanner (Magnetom Aera; Siemens Healthcare, Erlangen, Germany). The cardiac MRI protocol included cine sequences, T2 mapping, T1 mapping, and late gadolinium enhancement (LGE) imaging. Examinations in the control group were performed without contrast agent administration. Cardiac MRI images were analyzed by a blinded observer using Medis Suite software (Medis Medical Imaging Software, Netherlands). We calculated the volume, function, and mass of the left (LV) and right ventricles (RV). Native T1 and T2 relaxation times of the myocardium were measured on the basal and mid-ventricular SA slices. We measured global strain, including LV global longitudinal strain (GLS), global circumferential strain (GCS), RV longitudinal strain, and free wall strain.

3.5.3. Immunology

The measurements described in Section 3.4 at 6, 12, 18, 24, and 30 months following SARS-CoV-2 infection were repeated. Samples were obtained from peripheral venous blood. We examined the:

- dynamics of the antibody (humoral) and
- T-cell (cellular) response dynamics.

3.5.4. Psychological assessments

Validated questionnaires have been distributed to assess participants' mental health status and quality of life. The most common psychological complaints reported by our patients were:

- depression
- anxiety
- post-traumatic stress disorder (PTSD)

As measure of depressive symptoms the Beck Depression Inventory was chosen [32]. Trait anxiety was assessed using the Spielberger Trait Anxiety Inventory [32]. Symptoms of post-traumatic stress syndrome were assessed using the Post-Traumatic Diagnostic Scale. Participants' (positive or negative) affectivity was examined using the Positive and Negative Affectivity Test [33].

3.5.5. Quality of life

Mental and physical quality of life were assessed using the 36-item Short Form Health Survey (SF-36 questionnaire) [34]. Life satisfaction was assessed using a 1-item life satisfaction scale [35]. We recorded data on overall quality of life, including the ED-5Q-3 L score and the visual analog scale (VAS) score [36]. To assess sleep habits and sleep-related symptoms, participants completed questionnaires, specifically the Pittsburgh Sleep Quality Index (PSQI) [37], the Epworth Sleepiness Scale (ESS) [38], and the Fatigue Severity Scale (FSS) [39].

- subjective quality of life
- sleep and
- fatigue scales

3.6. Control groups

To evaluate the cardiac MRI data, a control group of 15 healthy individuals who had undergone MRI examinations prior to the COVID-19 pandemic was selected from our clinic's database. The control group was matched to the patients who received ECMO support in terms of age and sex.

For the immunological studies, a control group of 10 individuals were selected, matched for age and sex. The control group consisted of patients who were infected with the virus (SARS-CoV-2 Delta B.1.617.2) at the same time as the ECMO-treated group and who were also unvaccinated at the time of infection. The control group did not require hospitalization, medication, or supplemental oxygen therapy; they recovered at home. According to the WHO criteria of May 27, 2020 (D1), they were classified as having mild illness; no viral pneumonia or hypoxia was detected in the context of their upper respiratory symptoms. During follow-up, members of the control group received the same RNA-based vaccine as members of the ECMO-supported group (BNT162b2), at the same time (6–7 months) after infection.

3.7. Statistical analysis

Statistical analyses were performed using MedCalc software (version 17.9.5), R (version 4.3.0), and GraphPad. Variables with a missing value rate greater than 40% were excluded from further analysis. Correlation analysis was performed using Spearman's rho test. Measurements taken at different time points from the same individual were compared using the paired-sample version of the Wilcoxon signed-rank test. Comparisons between two groups at the same time points were performed using the Mann-Whitney U test. Survival analysis was performed using the Survminer package, and p-values for differences between Kaplan–Meier curves were calculated using the log-rank test. Optimal cut-off values for predicting patient survival were calculated based on the measured immunological characteristics using the cut-point R package. ROC analysis was performed using the plotROC library. The effects of clinical variables, including age, sex, comorbidities, time on ECMO support, and the immune response to SARS-CoV-2, on patient survival were evaluated using Cox proportional hazards models. Variables that showed a positive association in the univariate test were selected for multivariate analysis. To reconstruct the dynamic changes in the immune response, we calculated the median values for each cohort at each time point. Confidence intervals around the medians were calculated using the DescTools package and the bootstrap method. Differences were considered statistically significant at $p < 0.05$.

4. Results

4.1. Acute outcome

Of the 35 critically ill patients on ECMO included in the study:

- 9 patients (26%) survived hospitalization,
- 26 patients (74%) passed away.

The baseline characteristics of the study population (demographic data, medical history, comorbidities) are summarized in Table 2.

	All patients (n=35)	**Survivors (n=9)**	**Deceased (n=26)**	**P-value**
Male (%)	62.9% (22/35)	66.7% (6/9)	61.5% (16/26)	0.787
Female (%)	37.1% (13/35)	33.3% (3/9)	38.5% (10/26)	
Age (years) median (IQR)	37 (32-49)	34 (28-42)	39 (32-49)	0.250
Body Mass Index (kg/m^2)	29.5 (27.8-34.5)	29.2 (27-30.1)	30.9 (27.8-37)	0.070
Obesity	48.6% (17/35)	22.2% (2/9)	57.7% (15/26)	0.071
Hypertension	28.6% (10/35)	22.2% (2/9)	30.8% (8/26)	0.630
Diabetes	11.4% (4/35)	22.2% (2/9)	7.7% (2/26)	0.245
Smoking	8.6% (3/35)	0% (0/9)	11.5% (3/26)	0.294
Atrial fibrillation	2.9% (1/35)	0% (0/9)	3.8% (1/26)	0.556
Myocardial infarction	2.9% (1/35)	11.1% (1/9)	0% (0/26)	0.089
Chronic Obstructive Pulmonary Diseases	5.7% (2/35)	0% (0/9)	7.7% (2/26)	0.398

Hypothyroidism	5.7% (2/35)	0% (0/9)	7.7% (2/26)	0.398
Hashimoto's disease	2.9% (1/35)	0% (0/9)	3.8% (1/26)	0.556
Arthritis	2.9% (1/35)	0% (0/9)	3.8% (1/26)	0.556

Table 2 Basic demographic and clinical characteristics, as well as medical history, observed at the time of admission to the intensive care unit. *Categorical variables are presented as percentages (n/total n), and continuous variables as medians (interquartile range).*

Clinical characteristics related to ECMO and organ function assessed during the first examination following cannulation are summarized in Table 3.

	All patients (n=35)	**Survivors (n=9)**	**Deceased (n=26)**	**P-value**
Time from onset of symptoms to ECMO cannulation (days)	10 (7-14)	12 (9-14)	9.5 (7-14)	0.257
Duration of ventilation [IMV to ECMO] (days)	2 (1-5)	3 (1-5)	2 (1-3)	0.701
Duration of ECMO support (days)	13 (9-19)	12 (11-23)	14 (9-19)	0.763
Length of hospital stay (days)	20 (14-28)	46 (43-74)	17 (11-20)	<0.001

Renal failure (CRRT)	45.7% (16/35)	22.2% (2/9)	54.0% (14/26)	0.106
Right-sided heart failure	48.6% (17/35)	11.1% (1/9)	61.5% (16/26)	0.01
Liver failure	14.3% (5/35)	0% (0/9)	19% (5/26)	0.161
Multiple organ failure (MOF)	80% (28/35)	22.2% (2/9)	100% (26/26)	<0.001
Bacterial superinfection	48.6% (17/35)	55.6% (5/9)	46.2% (12/26)	0.632
Vasopressor	100% (35/35)	100% (9/9)	100% (26/26)	0.004
Inotropic	48.6% (17/35)	11.1% (1/9)	61.5% (16/26)	0.01
Human normal IgG	37.1% (13/35)	55.6% (5/9)	30.8% (8/26)	0.191
Antibiotics	48.6% (17/35)	55.6% (5/9)	46.2% (12/26)	0.632
White blood cells (g/L)	12.61 (9.92 - 13.89)	11.3 (6.1-13.9)	12.9 (9.3-16.9)	0.227
Lymphocyte count	0.93 (0.70 -1.36)	0.7 (0.5-1.4)	1.1 (0.7-1.5)	0.235
Lymphocyte percentage (%)	9.2(7.02 -10.3)	9.2 (4.6-11.9)	9.2 (6-11)	0.88
CRP (mg/L)	113.9 (38.9-216.4)	66.2 (14.2-110.1)	146 (42-226.8)	0.07
LDH (U/L)	661 (506-960)	625 (472.5-976.3)	676 (498-946)	0.777

Table 3: Characteristics during hospitalization and ECMO support.*Comparison of surviving and deceased participants. Categorical variables are presented as percentages (n/total n), and continuous*

variables as medians (interquartile range). Abbreviations: CRRT = continuous renal replacement therapy, IMV = invasive mechanical ventilation

Human normal immunoglobulin: Pentaglobin (Biotest Pharma GmbH) ATC: J06B A02 (IgG, IgM, IgA) Antibiotics: targeted therapy based on microbiological evaluation

Between survivors and non-survivors there was no significant difference in either demographic data or most risk factors. Right ventricular failure occurred more frequently in non-survivors.

Long-term follow-up studies were conducted with the 9 surviving patients

4.2. Immune response and survival

Immunological tests performed on blood samples obtained during ECMO cannulation confirmed ongoing SARS-CoV-2 infection in all patients. There were no differences between the two groups in white blood cell (WBC) count, lymphocyte count and proportion, or baseline levels of inflammatory markers (neutrophil count, CRP, and LDH).

Immune responses measured before the initiation of ECMO treatment in survivors and deceased patients were compared (Figures 2 and 3), showing an enhanced humoral immune response in both groups. The humoral immune response did not differ between survivors and deceased patients. However, the T-cell immune response was significantly higher in survivors.

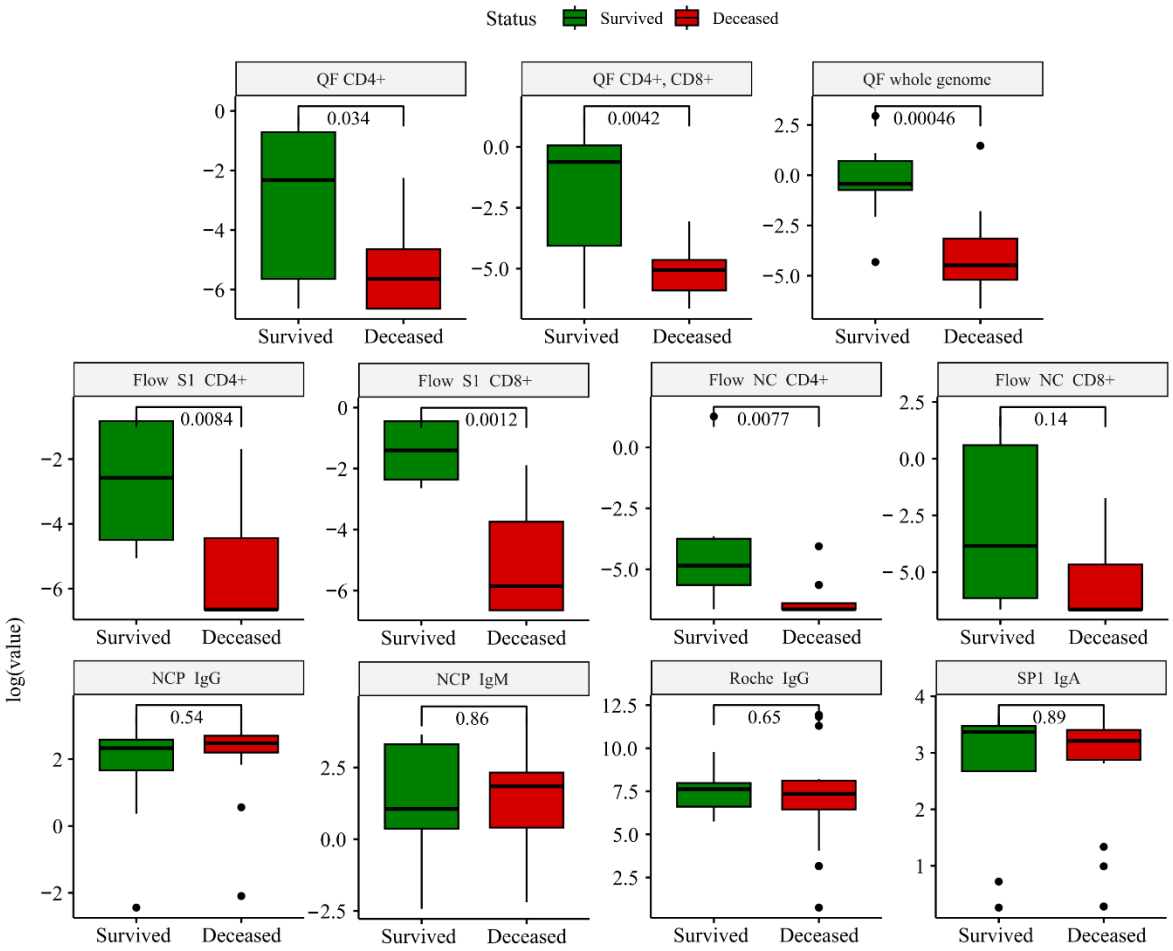

Figure 2 Comparison of SARS-CoV-2 immune markers during the acute phase of the disease prior to the initiation of ECMO support. *Box plots illustrating the comparison between surviving (green) and deceased (red) patients. The comparison was performed using the Wilcoxon test, and the immune markers were plotted on a logarithmic scale to account for data skewness.*

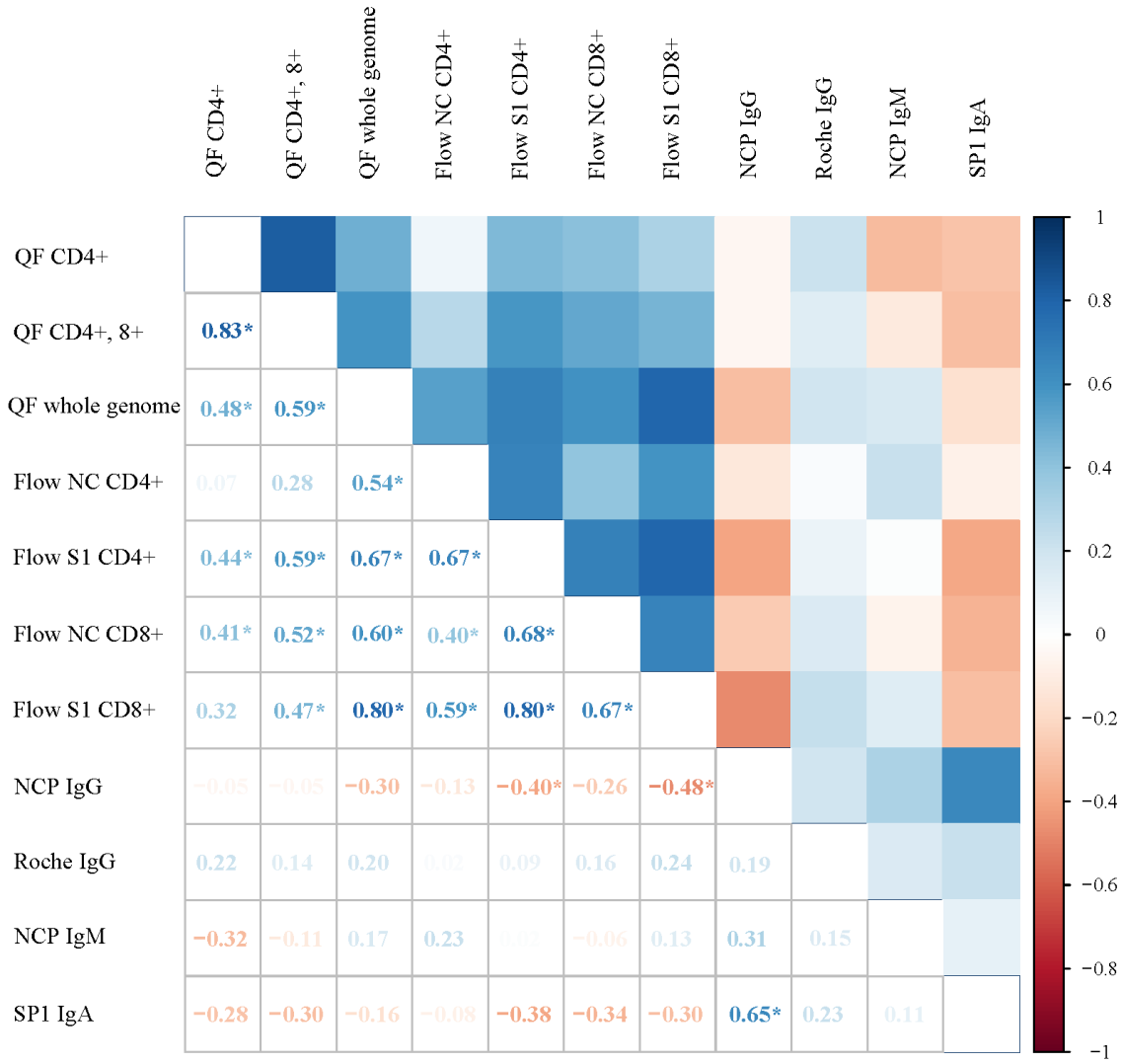

Figure 3 The correlation matrix shows the relationships among markers of the SARS-CoV-2 immune response in patients with severe COVID-19. *The color-coded squares in the upper part of the figure illustrate the strength of the correlation between the immunomarkers, while the lower part shows the Rho values calculated based on Spearman's rank correlation. Each square represents the result of a single correlation: positive values indicate a positive correlation, while negative values indicate a negative correlation. The asterisk (*) denotes significant correlations.*

A higher probability of survival has been observed in patients who had markers indicating a higher T-cell immune response, as detected by both QuantiFeron (QF) and flow cytometry (Flow) tests. (Flow S1 CD8+ ≥0.15%, Flow S1 CD4+ ≥ 0.02%, QF CD4+ ≥ 0.07, QF whole

genome ≥ 0.59) (Figure 4). In a univariate Cox proportional hazards regression analysis, BMI, right ventricular (RV) failure, QF total T-cell count, and Flow S1 CD8+ T-cell count were associated with mortality. We found that an increased T-cell immune response showed a significant negative association with mortality, independent of BMI and right ventricular (RV) failure (Table 4).

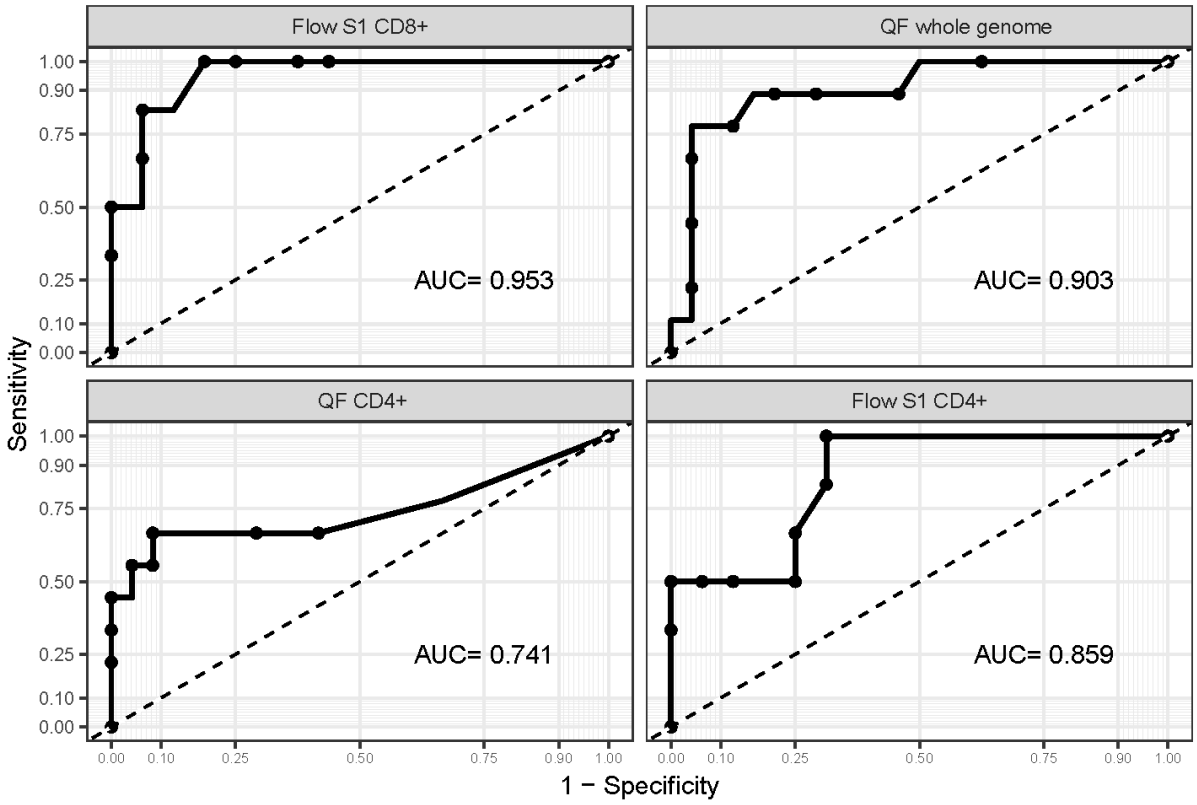

Figure 4 Markers of the cellular immune response using ROC curves. *The ideal cutoff values were plotted for certain markers of the cellular immune response on ROC curves. The AUC values suggest that the whole-genome levels of Flow S1 CD8+ and QF measured at the start of ECMO treatment can help identify at-risk patients with a high degree of accuracy.*

	Beta	HR (95% CI for HR)	Wald.test	p-value
Body Mass Index (BMI)	0.002	1.00 (0.89-1.12)	0.001	0.97
Right-sided heart failure	-0.06	0.9 (0.31-2.84)	0.01	0.92
Flow S1 CD 8+	-8.07	0.0003 (0.00-0.85)	4.00	0.04*

Table 4A Multivariate Cox proportional hazard regression model 1.

Asterix (*) shows significant results.

	Beta	HR (95% CI for HR)	Wald.test	p-value
Body Mass Index (BMI)	0.04	1.04 (0.97 -1.13)	1.24	0.27
Right-sided heart failure	0.43	1.50 (0.66 -3.61)	1.01	0.32
QF whole genome	-1.07	0.34 (0.09 -1.27)	2.55	0.11

Table 4B Multivariate Cox proportional hazard regression model 2.

Table 4A and 4B Results from the two multivariate Cox proportional hazard regression models. *Both tables present a separate model. Each model was developed based on the results of univariate models and biological plausibility.*

Summarizing the immunological results of the acute phase (Figure 5):

- All patients in whom no CD4+ and CD8+ T-lymphocyte response was detectable by functional flow cytometry and the QantiFeron test at the start of ECMO support died. (The absence of a T-cell immune response resulted in 100% mortality.)
- Higher CD4+ and CD8+ activity was associated with better survival.
- T-cell activity was an independent predictor of survival (regardless of BMI and RV failure).

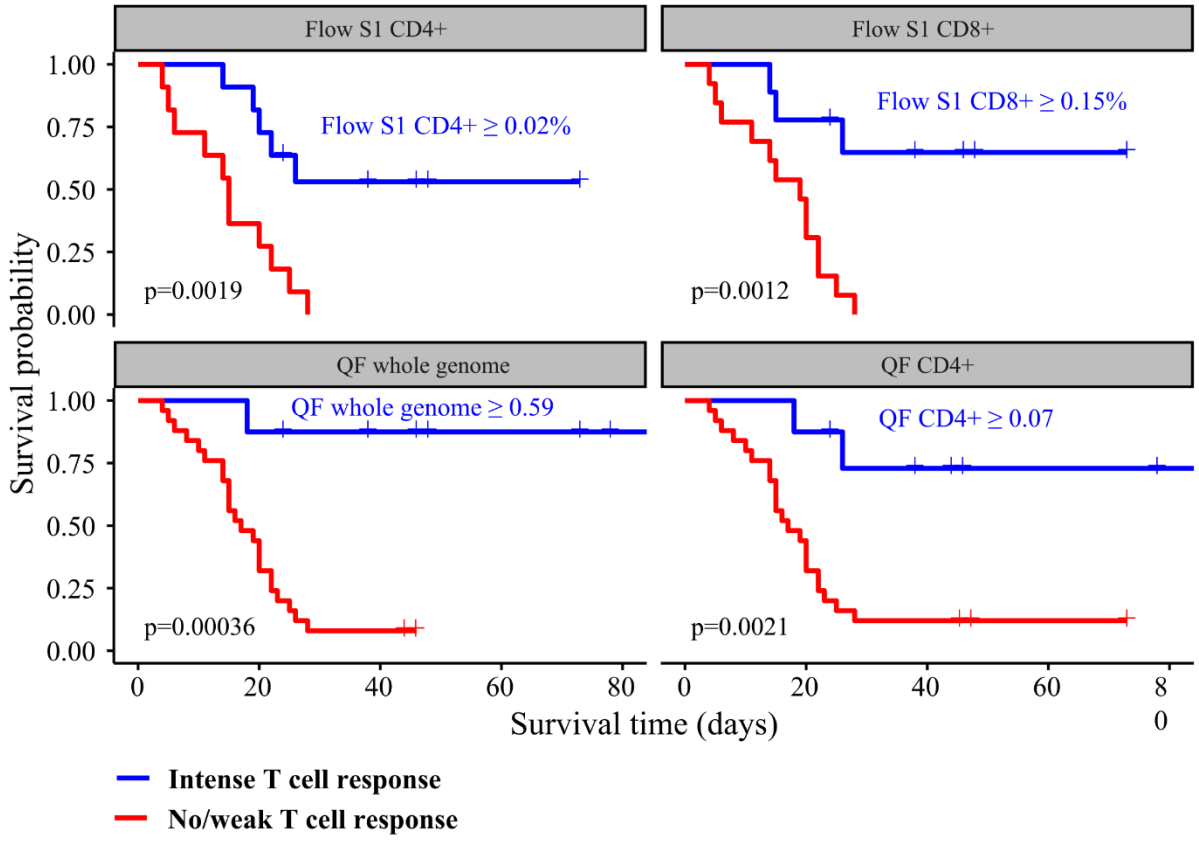

Figure 5 Kaplan-Meier curves showing the associations between T cell response intensity and all-cause mortality among critically ill COVID-19 patients. *Kaplan–Meier curves; patients were grouped according to the intensity of their immune response.*

At the 6-month mark of our follow-up study, we detected SARS-CoV-2-specific antibodies and T-cell activity in the peripheral blood samples of all 9 surviving patients and all members of the control group. There was no significant difference between the two groups in the

expression of markers of the cellular immune response. The humoral (IgG) immune response was significantly stronger in the control group. (Figure 6)

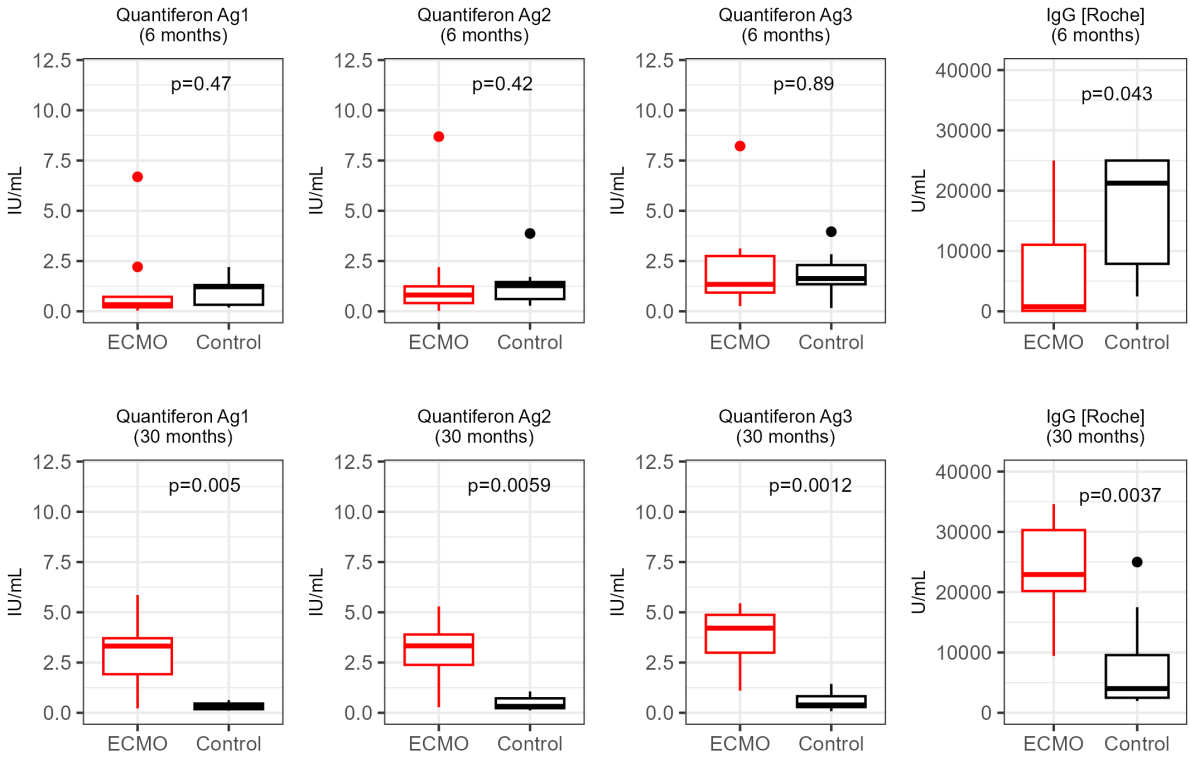

Figure 6 Differences in immune parameters between severe and less patients with severe COVID-19 at the initial and final follow-ups. *Both cellular (Quantiferon) and humoral (IgG) immune responses against SARS-CoV-2 were measured in patients requiring ECMO treatment and in a control group of patients with less severe COVID-19. Immune reactivity at the first follow-up visit (6 months), as well as at the last available visit (30 months), was compared between the two groups using Wilcoxon signed rank test. The raw p values are shown before any correction for multiple hypotheses.*

Subsequently (at the end of the 6th month post-infection), all members of both groups received an RNA-based SARS-CoV-2 vaccine (BNT162b2). Two years after COVID-19, we performed the immunological measurements again. Based on the results obtained, we found that both the humoral and cellular immune responses were significantly higher in the ECMO group as Figure 7 shows.

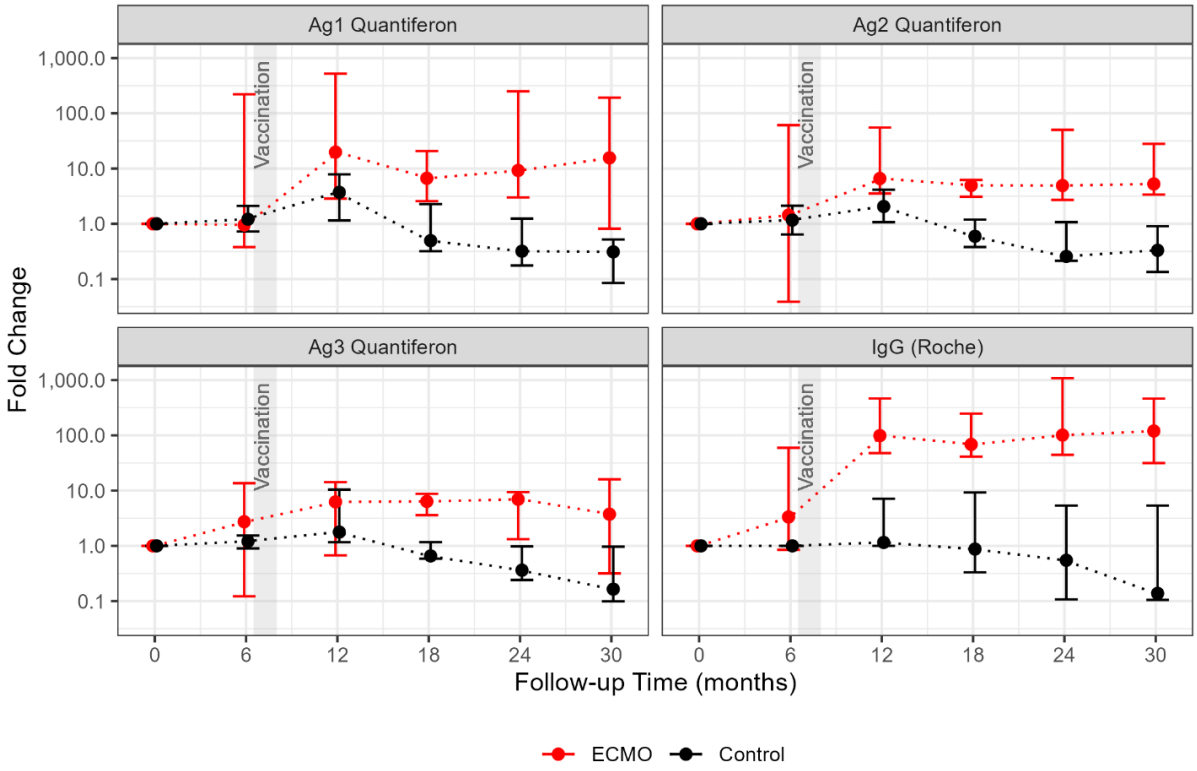

Figure 7 Dynamics of the anti-SARS-CoV-2 immune response in patients requiring ECMO compared to patients with less severe infection.

The median changes were plotted in the odds ratio measured during follow-up relative to baseline values (the first available data point for each patient) and the 95% confidence intervals calculated using the bootstrap method as error bars. The colors correspond to the patient cohorts: patients requiring ECMO (in red) and the control group (in black).

Summarizing the immunological results of the long-term follow-up:

- T-cell activity is stronger in the ECMO group than in the control group.
- Both humoral and cellular immune responses persisted in the ECMO group. Two years after the onset of COVID-19, we still observed a strong immune response.

4.3. Pulmonary outcome

The evaluation of chest CT images obtained at hospital admission and during follow-up is summarized in Table 5.

	Baseline (hospital admission)	**6th month**	**24th month**
GGO (n,%)	8 (89%)	2 (22%)	2 (22%)
Consolidation (n,%)	8 (89%)	0 (0%)	0 (0%)
Lymphadenopathy (n,%)	4 (45%)	0 (0%)	0 (0%)
Traction bronchiectasis (n,%)	1 (11%)	3 (33%)	2 (22%)
Residual bundles (n,%)	2 (22%)	8 (89%)	8 (89%)

Table 5 CT findings at baseline and during follow-up.

Abbreviation GGO: ground-glass opacity

At the time of ECMO cannulation, more than 75% of the lung was involved on native CT scans in 6 patients (66.7%). Initially, ground-glass opacities (GGOs) and consolidation were present in 8 patients (89%) (Figure 8). The presence of GGOs decreased to 22% during follow-up examinations. We did not observe consolidation on the 6-month follow-up CT scans in any of the patients. Reactive lymphadenopathy was detected in 4 patients (45%) during the baseline examination, but was no longer present at the 6-month follow-up. The presence of residual fibrotic banding, which was initially described in two patients (22%), increased to 8 patients (89%) by the 6-month mark and persisted throughout the 24-month follow-up.

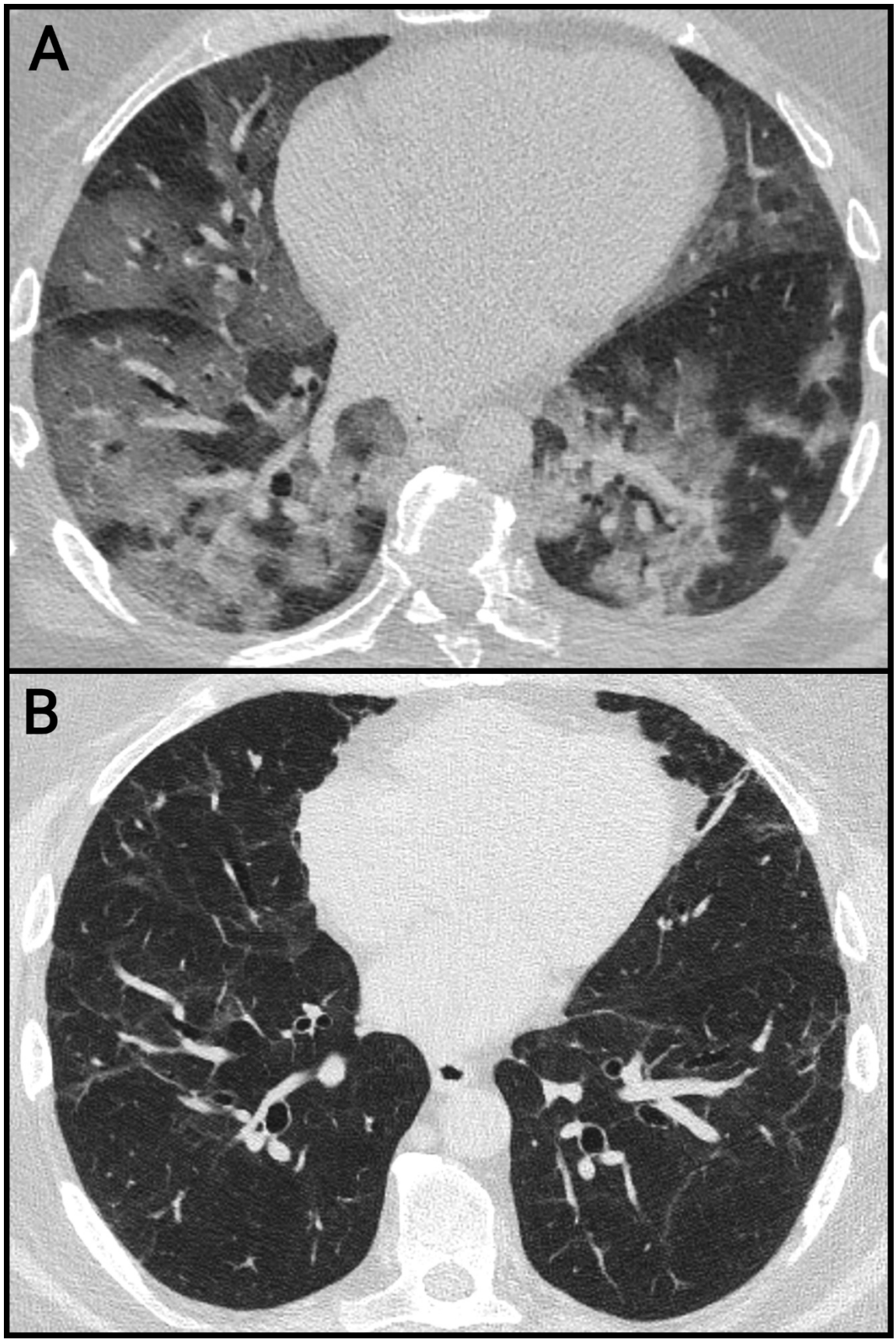
A
B

Figure 8 Lung changes on baseline and follow-up chest CT images. *The baseline examination (A) shows extensive, patchy, and confluent opacities. The 24-month follow-up examination (B) shows residual band-like lesions and mild traction bronchiectasis.*

Due to the patients' critical condition at admission, it was not possible to perform pulmonary function tests (PFTs). PFTs performed after 2 years showed mild restrictive respiratory disorders in three patients, mild obstructive respiratory disorders in two patients, and combined respiratory disorders in one patient. In three patients, lung function values were within the normal range. The results of the pulmonary function tests are summarized in Table 6.

	COVID-19 ECMO group (N=9) mean ± standard deviation
Time elapsed since infection (days)	968±155
FEV1 (L)	3.3±0.6
FEV1 (%)	82.7±10.8
FVC (L)	3.9±0.7
FVC (%)	81.3±10.9
FEV1/FVC (%)	82.7±5.6
TLC (L)	6.0±2.1
TLC (%)	90.1±16.9
RV (L)	1.84±0.73
RV (%)	104.4±28.7
DLco (mmol/min/kPa)	12.7±4.9
DLco (%)	127.7±27.5
KLco (mmol/min/kPa)	2.2±0.8
KLco (%)	132.1±19.0
6MWT (m)	506±60
HR at start (/min)	83±5
HR at end (/min)	117±14

Spo2 at start (%)	98±1
Spo2 at end (%)	94±3
Desaturation >5% (N %)	2 (22)

Table 6 The PFT results of COVID-19 ECMO patients at their last post-COVID visit.

Abbreviations: FEV1: forced expiratory volume in the first second, FVC: forced vital capacity, DLco: diffusing capacity of the lungs for carbon monoxide, KLco: carbon monoxide transfer coefficient, 6MWT: 6-minute walk test, HR: heart rate

Summarizing the results of our pulmonological examinations, by the end of the second year of recovery:

- fibrotic changes were common (89%),
- lung function had mostly normalized.

4.4 Cardiological outcomes

Both at the time of acute hospital admission and during follow-up, standard 12-lead ECGs showed no clinically relevant abnormalities; all patients maintained sinus rhythm, with a mean heart rate of 71 ± 10 beats per minute; one patient's ECG showed right Tawara bundle branch block, and another patient had incomplete right bundle branch block.

Echocardiography performed during the acute phase revealed a mildly increased right ventricular end-diastolic diameter (RVEDD), which normalized during follow-up (baseline RVEDD: 37.6 ± 2.9 mm, follow-up RVEDD: 35.6 ± 2.2 mm, $p < 0.05$). Left and right ventricular function (LV-EF and RV-EF) remained within the normal range throughout. Furthermore, no significant valvular abnormalities were detected. (Table 7)

Laboratory results	
Baseline hs-troponin T (ng/L) median (IQR)	11 (6.9 to 21)
Peak hs-troponin T (ng/L) median (IQR)	20 (14.5 to 68.3)
Baseline NT-proBNP (ng/L) median (IQR)	235 (149 to 299)
Peak NT-proBNP (ng/L) median (IQR)	304 (227 to 629)
Echocardiography	
LVEDD (mm) median (IQR)	47 (44 to 51)
LVEF (%) median (IQR)	58 (55 to 61)
RVEDD (mm) median (IQR)	37 (36 to 40)
TAPSE (mm) median (IQR)	23 (22 to 25)
RVFAC (%) median (IQR)	40 (37 to 43)

Table 7 Cardiological characteristics of the acute phase.

Abbreviations: LVEDD: left ventricular end-diastolic diameter, LVEF: left ventricular ejection fraction, RVEDD: right ventricular end-diastolic diameter, TAPSE: tricuspid annular plane systolic excursion. It characterizes the right ventricle's ability to contract (systolic function), RVFAC: right ventricular fractional area change

During both the 6-month and 24-month follow-up examinations, the cardiac MRI parameters of all our patients were within the normal range, including ventricular volume, function, deformation parameters, and mapping values. Only one patient, who had a history of myocardial infarction prior to COVID-19, showed abnormal contrast enhancement, the pattern of which was consistent with the myocardial infarction in the patient's medical history. No patient showed contrast enhancement or mapping abnormalities suggestive of COVID-19-related myocardial damage. (Table 8)

	Control group (N=15)		COVID-19 ECMO group (N=9)		
	median	**IQR**	**median**	**IQR**	**p-value**
Age (years)	37.3	33.5 to 43.2	37.7	32.3 to 47.5	0.83
LVEDVi (ml/m²)	87	81 to 96.25	72	66.75 to 84.75	0.02
LVESVi (ml/m²)	34	33 to 38.75	27	25.5 to 32.25	0.01
LVSVi (ml/m²)	53	49 to 54.75	46	41 to 53.25	0.11
LVEF (%)	60	56.25 to 62.5	63	61.5 to 64.25	0.10
LVMi (g/m²)	49	45 to 52	42	39 to 54	0.42
LV GCS (%)	-29.8	-31.4 to -28.4	-33	-36.5 to -29.6	0.17
LV GLS (%)	-20.1	-24.5 to -19.4	-22	-22.9 to -20.7	0.70
RVEDVi (ml/m²)	89	85.5 to 99	80	70.75 to 87.25	0.04
RVESVi (ml/m²)	41	36.5 to 42.75	35	30.75 to 38.5	0.02
RVSVi (ml/m²)	48	47 to 54.25	46	40 to 51.75	0.36
RVEF (%)	55	53.25 to 58	57	53.75 to 60	0.51
RV free wall LS (%)	-34.3	-39.5 to -33.9	-35.5	-37.1 to -31.8	0.88
RV GLS (%)	-26.7	-30.8 to -25.7	-28.3	-31.1 to -25.8	0.64
T1 basis (ms)	968	956 to 996	972	962 to 984	0.61
T1 mid (ms)	960	927 to 981	951	937 to 976	0.52
T2 basis (ms)	45	44 to 45	44	44 to 46	0.78
T2 mid (ms)	46	45 to 47	44	43 to 46	0.06

Table 8 Cardiac MR parameters of healthy controls and patients after COVID-19 diagnosis and ECMO support.

Abbreviations: LVEDVi: left ventricular end-diastolic volume index; LVESVi: left ventricular end-systolic volume index; LVSVi: left ventricular stroke volume index; LVEF: left ventricular ejection fraction; LVMi: left ventricular mass index; LVGCS: left ventricular global circular strain; LVGLS: left ventricular global longitudinal strain; R: right (Right)

In blood samples taken at the time of ECMO cannulation, hs-troponin T levels were mildly elevated in 7 patients and not elevated in 2 patients. NT-proBNP levels were significantly higher in one patient and only moderately elevated in the remaining patients. (Table 7)

During follow-up, normal hs-troponin T and NT-proBNP levels were measured in all patients.

Summarizing the results of our cardiological examinations, by the end of the second year of recovery:

- we observed complete regression of the initial right ventricular dilation,
- and long-term normal cardiac function was demonstrated.

4.5. Outcomes regarding psychology and quality-of-life

Upon analyzing the correlation between the number of days spent on ECMO (mean ± SD: 15.9 ± 7.8) and the responses to the psychological questionnaires (M6), we found a positive, significant association with depression ($r = 0.727$; $p<0.05$), anxiety ($r = 0.848$; $p<0.01$), and post-traumatic stress ($r = 0.834$; $p<0.01$). At the same time, we found a strong negative correlation with positive affect ($r = -0.868$; $p < 0.01$). Table 9 presents the comparative results of the psychological tests for the two data collection points.

	BDI	**STAI**	**PDS**	**SL**	**PANAS positive**	**PANAS negative**	**SF-36 physical health**	**SF-36 mental health**
Z	-0.705[b]	-0.981[c]	-0.780[c]	-1.089[b]	-1.423[b]	-0.070	-0.422[b]	-0.059[c]
p	0.481	0.326	0.436	0.436	0.155	0.944	0.673	0.953

Table 9 Comparative analysis of the two measurements of the psychological test (M6–M24).

Abbreviations: b: Based on negative ranks; c: Based on positive ranks; Z = z-value

BDI = Beck Depression Inventory (consists of 21 items, each assessing a symptom associated with depression); STAI = State-Trait Anxiety Inventory; PDS = Post-traumatic Diagnostic Scale (for assessing symptoms of post-traumatic stress disorder); SL = Satisfaction with Life (refers to life satisfaction); PANAS = Positive and

Negative Affect Schedule (a scale that measures the extent to which a person experiences positive and negative emotions); SF-36 = 36-Item Short Form Health Survey (a questionnaire measuring general health status and quality of life).

Patients reported a significant improvement in their subjective health status at the end of month 24 compared to month 6 (subjective health status at month 6 vs. month 24: median 50% vs. 80%, $p<0.01$). However, the majority of patients still reported numerous physical and psychological symptoms 24 months after COVID-19 infection. Notable among these were fatigue and a significant decrease in physical activity. Consistent with these findings, the average BMI increased from 29 to 35, meaning that 22% of patients were overweight at the time of infection, while by the end of the second year, 89% were overweight. (Table 10)

	6th month	24th month
Health status (%) median (IQR)	50 (40 - 90)	80 (60 - 95)
Returning to Work:		
- does not work (n, %)	4 (44%)	3 (33%)
- carries out different job (n, %)	2 (22%)	3 (33%)
- retained their previous job (n, %)	3 (33%)	3 (33%)
Sport (h/week) median (IQR)	5 (2.5 - 6.25)	0 (0 - 2.25)
Energy level:		
- very low (n, %)	1 (11%)	0 (0%)
- low (n, %)	2 (22%)	2 (22%)
- intermediate (n, %)	6 (67%)	5 (56%)
- high (n, %)	0 (0%)	2 (22%)
Fatigue:		
- light (n, %)	0 (0%)	2 (22%)
- moderate (n, %)	3 (33%)	4 (44%)
- strong (n, %)	6 (67%)	3 (33%)
Muscle pain (n, %)	5 (56%)	3 (33%)

Joint pain (n, %)	7 (78%)	7 (78%)
Palpitations (n, %)	9 (100%)	5 (56%)
Light coughing (n, %)	4 (44%)	0 (0%)
Psychological consultation (n, %)	5 (56%)	3 (33%)
Psychotherapy (n, %)	9 (100%)	1 (11%)
Tension (behavioural):		
- rarely (n, %)	3 (33%)	7 (78%)
- moderately (n, %)	3 (33%)	0 (0%)
- often (n, %)	3 (33%)	2 (22%)
Concentration problems (n, %)	9 (100%)	5 (56%)
Irritability (n, %)	6 (67%)	5 (56%)
Hysterical reactions (n, %)	5 (56%)	5 (56%)
Impatience (n, %)	6 (67%)	6 (67%)
Mood swings (n, %)	6 (67%)	5 (56%)
Sexual activity:		
- complete absence (n, %)	2 (22%)	2 (22%)
- decreased significantly (n, %)	2 (22%)	2 (22%)
- decreased (n, %)	3 (33%)	3 (33%)
- did not change (n, %)	2 (22%)	2 (22%)
Sleep disorder (n, %)	4 (44%)	4 (44%)
Hair loss (n, %)	5 (56%)	5 (56%)
Loss of smell (n, %)	2 (22%)	2 (22%)
Loss of taste (n, %)	2 (22%)	2 (22%)
Menstrual irregularities (n, %)	3 (100% in women)	3 (100% in women)
Numbness in the fingers (n, %)	2 (22%)	2 (22%)
Limited quality of life:		
- none (n, %)	0 (0%)	2 (22%)
- light (n, %)	2 (22%)	1 (11%)
- moderate (n, %)	1 (11%)	1 (11%)

- severe (n, %)	6 (67%)	5 (56%)

Table 10 Subjective health status of ECMO survivors.

The results of the QoL questionnaires administered at month 24 are presented in Table 11. The sleep-related questionnaires showed higher-than-normal daytime sleepiness scores on the ESS (Epworth Sleepiness Scale) and elevated scores on the FSS (Fatigue Severity Scale), suggesting that patients experienced fatigue and sleepiness in their daily lives. The VAS score was lower than the Hungarian age- and gender-adjusted mean values in 5 patients.

	COVID-19 ECMO group (N=9) (median; [range])
Visual Analog Scale (VAS)	75 [30-90]
Epworth Sleepiness Scale (ESS)	8 [3-10]
Fatigue Severity Scale (FSS)	40 [19-58]

Table 11 Results of the QoL VAS and sleep questionnaires > 2 years after COVID-19.

Overall, the analysis of these questionnaires suggested that, more than two years after COVID-19, the quality of life of former ECMO patients remained poor in certain areas.

Our 2-year follow-up revealed that our patients who survived severe COVID-19:

- experienced an improvement in quality of life, but it did not return to normal,
- continued to experience fatigue and reduced activity,
- and the presence of depression, anxiety, and PTSD was not negligible.

5. Discussion

Our current study combines an analysis of the acute prognostic role of the SARS-CoV-2-specific immune response with an assessment of the long-term outcomes of survivors who received VV-ECMO support. Based on our findings, the cellular immune response is a key determinant of survival in critically ill COVID-19 patients. The strength of the T-cell immune response is an independent predictor of survival, which has significant clinical relevance. In contrast, the humoral response showed no prognostic value, suggesting that cellular immunity plays a more dominant role in viral elimination and disease control. Long-term follow-up revealed complex, multisystemic abnormalities that are partly reversible and partly persistent. The results indicate that while organ functions recover to a significant extent, patients' quality of life may be permanently impaired.

5.1. The prognostic significance of the cellular immune response

The most important finding of our study is that SARS-CoV-2-specific T-cell activity is significantly associated with survival in patients with COVID-19. The observed association was independent of clinical risk factors, including body mass index and right ventricular dysfunction, suggesting that cellular immunity is not merely an epiphenomenon but potentially plays a causal role in disease outcome.

Our findings are consistent with observations suggesting that the severe course of COVID-19 is determined not merely by the cytopathic effect of the virus, but by the quality of the immune response [40,41]. In the absence or exhaustion of the T-cell immune response, viral elimination may become insufficient, leading to persistent viremia and an enhanced inflammatory response [42,43]. In contrast, effective T-cell immunity may facilitate viral control, mitigating systemic inflammation and organ damage [11].

Our observations are also consistent with studies emphasizing the protective role of SARS-CoV-2-specific T cells. Moderbacher and colleagues [14] demonstrated that a coordinated adaptive immune response, particularly CD4+ and CD8+ T-cell activity, is associated with a milder disease outcome, whereas an inadequate cellular response is associated with a more severe clinical course. Similarly, the review articles by Sette and Crotty [11] have highlighted

that T-cell immunity is one of the most important components of the immune response against SARS-CoV-2, the significance of which is particularly evident in critically ill patients.

It is worth noting that during the acute phase, the humoral immune response did not show significant differences between survivors and non-survivors. This supports the concept that in severe COVID-19, the antibody response alone is not sufficient to determine disease outcome [35, 41]. The clinical significance of the dissociation between humoral and cellular immunity may be particularly important when designing immunotherapeutic strategies.

5.2. Clinical implications: patient selection and risk stratification

The present findings have direct clinical relevance for determining the indication for ECMO. Measuring the T-cell immune response prior to cannulation may serve as a potentially new, objective biomarker for prognostic stratification of patients. This is particularly important in the case of a resource-intensive intervention such as ECMO, where appropriate patient selection is crucial. Integrating the cellular immune response into decision-making may enable: the identification of patients with a better expected outcome, the avoidance of unnecessary or disproportionate therapeutic burden, the development of personalized therapeutic strategies, and the rationalization of costs. Furthermore, the T-cell immune response also emerges as a potential therapeutic target. Although intervention options in this area are currently limited, immunomodulatory or cell therapy approaches may be considered in the future.

5.3. Long-term pulmonary and cardiac outcomes

The findings observed during long-term follow-up provide important insights into the recovery processes of survivors of severe, critical COVID-19. Consistent with the findings of Chommeloux and Mendrick [44, 45], the pulmonary function test results in our study group were nearly normal, showing only a mild decline and a restrictive pattern. Based on pulmonological examinations, structural abnormalities - particularly fibrotic changes - remained common, while functional parameters had largely normalized [44, 46, 47].

Similar results were reported by Huang et al. and Wu et al., [48, 49, 50] who pointed out that there is often only a weak correlation between the persistence of radiological abnormalities

and clinical function. This discrepancy between CT and PFT findings was also described by the research group led by Colombi and Han, who likewise found no strong correlation between quantitative CT parameters and the results of pulmonary function tests [51, 52]. This discrepancy suggests that radiological abnormalities do not necessarily correlate with clinical function, and that the lungs possess significant adaptive capacity even after severe damage. However, it cannot be ruled out that these structural abnormalities may have clinical relevance in the long-term, particularly in the presence of exercise or additional lung-damaging factors. Therefore, we consider further follow-up of this patient group to be important.

The reversibility observed in cardiac involvement is also encouraging. The regression of right ventricular dilation and normal MRI parameters suggest that a significant proportion of the cardiac abnormalities associated with COVID-19 are functional and potentially reversible. According to initial data published by Puntmann et al. at the start of the pandemic, there was an alarmingly high prevalence of cardiac MRI abnormalities suggestive of myocarditis [53].

However, this high rate was not confirmed by subsequent studies [54]. Our findings are consistent with the observations of Tsampasian, as well as Vágó et al., who reported that some of the myocardial abnormalities initially detected regress over time, and that the incidence of permanent structural damage may be lower than was assumed in the early stages of the pandemic [55, 56]. The results suggest that early studies with small sample sizes (e.g., Puntmann et al.) likely overestimated the prevalence of persistent myocardial involvement.

5.4. Long-lasting immune response and immunological memory

Long-term immunological outcomes indicate that patients who have had severe COVID-19 develop a long-lasting and robust immune response, which is detectable in both humoral and cellular components. It is particularly noteworthy that the T-cell immune response was stronger and more persistent in patients who received ECMO support than in those who had a mild course of infection.

This phenomenon is likely explained by the intensity and duration of antigen exposure. The high viral load and prolonged immune activation during severe infection may contribute to the development of long-term immune memory. However, it is not entirely clear to what extent this enhanced immune response contributes to late symptoms or immune-mediated damage. Understanding this requires further investigation.

5.5. Psychological consequences and quality of life

One of the most important aspects of this study - and one that is often clinically underestimated - is the exploration of long-term psychological consequences and their impact on quality of life. Our findings indicate that the duration of ECMO treatment is closely associated with symptoms of depression, anxiety, and post-traumatic stress.

This is consistent with the long-established concept of post-intensive care syndrome (PICS), according to which a significant proportion of patients recovering from a critical illness suffer from persistent mental and physical impairments. Needham et al. and Inoue et al. [22,57] emphasized that PICS is one of the most important determinants of the long-term consequences of critical illness, significantly influencing patients' functional status and quality of life. Although quality of life improved over time, a significant proportion of patients continued to report depression, anxiety, marked fatigue, reduced physical activity, and sleep disturbances.

Our findings are consistent with the observations of Hodgson et al. and Herridge et al., [58, 59] who noted that the functional and psychological recovery of patients recovering from a critical illness is a protracted process, and that some symptoms may persist even years after the acute event. Long-term cohort studies examining COVID-19 survivors, including the results of a one-year and then a two-year follow-up study by Huang et al., [48, 49] have also highlighted that fatigue, sleep disturbances, anxiety, and depression are among the most common persistent symptoms.

These findings underscore that survival alone cannot be considered a full recovery, and that rehabilitation strategies must also address psychological and social dimensions. The

psychological consequences are particularly significant and underscore the need for multidisciplinary rehabilitation.

5.6. Limitations and future outlook

This study has several limitations. The relatively small sample size and single-center design limit the generalizability of the results. In addition, the homogeneity of the patient population (e.g., unvaccinated status) may affect the ability to extrapolate the results to the current, vaccinated population.

Future research should involve larger, multicenter cohorts and should examine:

- the dynamics of the T-cell immune response to different viral variants,
- the relationship between the immune response and long-term symptoms,
- and the effectiveness of targeted immunomodulatory interventions.

6. Conclusions

This study provides a comprehensive overview of the immunological and clinical outcomes of critically ill COVID-19 patients during and after VV-ECMO support.

Key findings:

1. The SARS-CoV-2-specific cellular immune response is an independent and strong predictor of survival in COVID-19 patients who received ECMO support.

2. The humoral immune response alone is not suitable as a prognostic marker in this patient population.

3. Organ function in survivors recovers to a significant degree, although structural abnormalities may persist.

4. A sustained immune response indicates the development of robust immune memory, which is particularly pronounced in patients who have had severe infections.

5. Long-term outcomes are significantly influenced by psychological and quality-of-life factors, which require a multidisciplinary approach.

The clinical significance of our findings lies in the fact that they open up new perspectives for refining the indications for ECMO treatment and highlight the importance of long-term follow-up and structured rehabilitation. Integrating the cellular immune response into clinical decision-making may contribute to the development of personalized intensive care strategies in the future.

The COVID-19 pandemic has demonstrated the extraordinary adaptability of medicine and international scientific collaboration. Following the genetic identification of the virus, researchers developed diagnostic methods and evaluated potential therapies in record time. The experience gained during the pandemic has contributed significantly to advances in the care of critically ill patients and has yielded numerous scientific findings that will shape the practice of intensive care and the treatment of infectious diseases for years to come.

7. Summary

In our study, we investigated the immunological characteristics and long-term clinical outcomes of patients receiving veno-venous extracorporeal membrane oxygenation (VV-ECMO) due to critical COVID-19-associated ARDS. Our research had two objectives: first, to determine the prognostic significance of the SARS-CoV-2-specific immune response; and second, to conduct long-term, multi-organ system follow-up of patients who survived VV-ECMO support.

Based on our results, the intensity of the humoral immune response did not differ between survivors and deceased patients, while the SARS-CoV-2-specific T-cell immune response was significantly stronger in survivors. The absence of functional T-cell activity was associated with a fatal outcome in all cases, whereas higher CD4+ and CD8+ T-cell activity was associated with better survival. Our study confirmed that the cellular immune response may be an independent prognostic factor, unrelated to body mass index and right ventricular failure, in critically ill COVID-19 patients. During long-term follow-up, we found that the SARS-CoV-2-specific immune response persisted in surviving patients and remained detectable even two years after infection. In patients treated with VV-ECMO, the cellular immune response was stronger than in the control group, which had experienced a mild course of infection. Pulmonary examinations confirmed significant structural regeneration; however, residual findings and mild functional abnormalities persisted in most patients. During cardiological follow-up, we found no abnormalities suggestive of progressive myocardial damage. The comprehensive follow-up study also highlighted that, in addition to physical recovery, assessing the psychological and quality-of-life consequences is equally important.

Our findings contribute to a better understanding of the immunopathomechanism of severe COVID-19 and suggest that assessing the SARS-CoV-2-specific T-cell immune response may help in evaluating the prognosis of patients requiring VV-ECMO treatment in the future. Our study also confirmed that the majority of patients recovering from critical COVID-19 show favorable long-term organ regeneration; however, full rehabilitation requires a multidisciplinary approach and long-term care.

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9. Bibliography of the Candidate's Publications

9.1. Publications related to the PhD thesis

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