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ORIGINAL RESEARCH PAPER

Adaptive immune system in severe COVID-19 patients in the first week of illness: A pilot study

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ABSTRACT

Introduction: The presentation of the course of COVID-19-related T-cell responses in the first week of the disease may be a more specific period for adaptive immune response assessment. This study aimed to clarify the relationship between changes in peripheral blood lymphocyte counts and death in patients with COVID-19 pneumonia. *Methods:* Thirty-three patients (14 females and 19 males) admitted for severe and desaturated COVID-19 pneumonia confirmed by polymerase chain reaction were included. Lymphocyte subsets and $CD4^+/CD8^+$ and $CD16^+/CD56^+$ rates were measured using flow cytometry from peripheral blood at admission and on the day of death or hospital discharge. *Results:* Twenty-eight patients survived and five died. On the day of admission, the $CD4^+$ cell count was significantly higher and the saturation of O_2 was significantly lower in the deceased patients compared to the survivors ($P < 0.05$). The $CD16^+/CD56^+$ rate was significantly lower on the day of death in the deceased patients than in discharge day for the survivors ($P = 0.013$). *Conclusion:* $CD4^+$ lymphocyte percentages and O2 saturation in samples taken on the day of admission to the hospital and CD16+/CD56+ ratios taken at the time of discharge from the hospital were found to be associated with the mortality in patients with severe COVID-19.

KEYWORDS

COVID-19, $CD4^+$ T-cells, $CD8^+$ T-cells, lymphocyte subgroups

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic is a major public health concern worldwide [1]. Innate and adaptive immunity develops against the virus, and the severity of the disease affects the prognosis. The first innate immune response is the type 1 interferon response, mediated by toll-like receptors after viral cell entry [2–4].

Local neutrophilic activity triggered by inflammation, cell death, and T helper-1 mediated natural killer cell activation is the first element of the innate immune response. Based on serum samples taken during the recovery period, it is apparent that the subsequent development of T- and B-cell responses contribute substantially to the prognosis of the disease [5]. Evaluation of the adaptive immune response in recovered patients, observation of changes in the active disease process, and evaluation of improved cross-immunity against other possible coronaviruses have been studied [6].

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T lymphocyte depletion in patients also plays a role in pro-inflammatory processes such as secondary bacterial infections and thromboembolic events, which may be the determinants of disease prognosis and are usually present in at least the second week of the disease. Therefore, the presentation of the course of COVID-19-related T-cell responses in the first week of the disease may be considered a more specific period for adaptive immune response assessment. In the present study, we examined the T-cell response and the association between disease course and mortality in patients with severe pneumonia and desaturated COVID-19 who presented in the first week of their illness.

METHODS

This was a prospective, observational, pilot study. This study was conducted at Prof. Dr. Süleyman Yalçın City Hospital, Istanbul, Turkey, between June 2020 and February 2021. Thirty-three desaturated and symptomatic patients with severe COVID-19 pneumonia, confirmed by real-time polymerase chain reaction (PCR), admitted to our hospital were included in the study. The flow diagram of the study is shown in Fig. 1. All the patients included in the study were unvaccinated and had contracted COVID-19 for the first time. The pandemic started in our country in March 2020. The symptoms of the patients started within the last 2–4 days. In addition, the patients stated that they had not had any upper respiratory tract infections in the previous 3 months. As the study was conducted at the beginning of the pandemic, patients were considered sick for the first time.

Inclusion criteria

Patients with abnormal findings detected on lung radiography and computed tomography of the thorax and O_2 saturation (SO_2) $\leq$ 93% were classified as severe cases and included in the study [7].

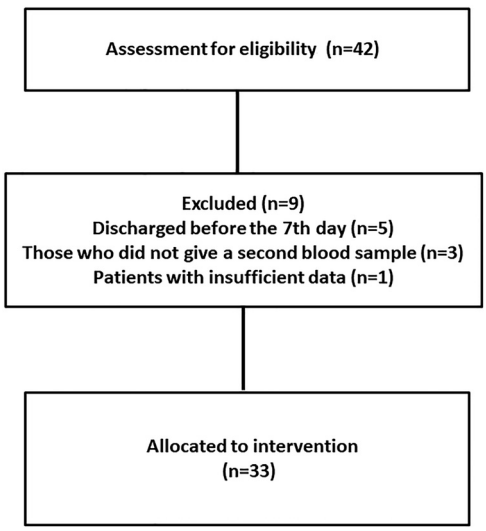

Fig. 1. Study flow diagram

Exclusion criteria

Patients with autoimmune disease, hematological malignancy, solid organ malignancy, immunocompromised status, or history of immunosuppression therapy were excluded.

Patients requiring intensive care unit (ICU) support by invasive or noninvasive means were provided with a high-flow nasal cannula (HFNC) or invasive mechanical ventilation support. For non-invasive mechanical ventilation support with HFNC in the ICU, it was determined that a) shortness of breath, b) respiratory rate >23 breaths per minute, and c) respiratory dysfunction was <94% SO2 at 5 L min^{-1} oxygen support with a mask. Five patients who died were intubated and connected to mechanical ventilation. In contrast, 28 patients who survived received HFNC therapy alone. Antiviral treatment with 400 100 mg^{-1} of lopinavir/ritonavir or favipiravir was administered for five days. Intravenous methylprednisolone (1 mg kg^{-1} day^{-1}) was administered to all patients during desaturation.

Complete blood samples (2 mL) were collected from all patients in ethylenediaminetetraacetic acid tubes on the first day and on the day of death or hospital discharge. The total blood cell counts, rates, and absolute counts of $CD3^+$, $CD4^+$, and $CD8^+$T lymphocytes (cells/μL), $CD19^+$ B lymphocytes, $CD16^+/CD56^+$ natural killer (NK) cells, and anti-HLA-DR were measured. After centrifugation, the erythrocytes were separated using lysis buffer. White blood cells were collected and rinsed with cold phosphate-buffered saline (PBS). Fc receptors on the cell surface were blocked with 2.4G2 (Pharmingen, San Diego, CA, USA) before staining with phycoerythrin-conjugated anti-human $CD4^+$, $CD19^+$, and $CD16^+$ to stain $CD4^+$ T-cells, $CD19^+$ B cells, and $CD16^+/CD56^+$ NK cell antibodies (Pharmingen). Allophycocyanin-conjugated anti-human $CD8^+$ and $CD16^+$ antibodies were used to stain the $CD8^+$ and $CD16^+$ cells, respectively. $CD3^+$ T cells were stained with fluorescein isothiocyanate-conjugated antibodies according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using the Number Cruncher Statistical System program 2007 (Kaysville, Utah, USA). Descriptive statistical methods were used to evaluate the data. The Kolmogorov–Smirnov test, Shapiro–Wilk test, and graphical evaluations were used to test the conformity of the quantitative data to a normal distribution. The Mann–Whitney *U* test was used to compare two data groups without a normal distribution. Fisher's exact test was used to compare quantitative data. The Wilcoxon signed-rank test was used to compare the first and last measurements of the non-normally distributed parameters. The significance level was set at $P < 0.05$.

Ethics statement

Approval was obtained from the ethical committee of Prof. Dr. Süleyman Yalçın City Hospital (code 2020/0323).

All participants provided written informed consent after an explanation was provided about the aims and scope of the study in accordance with the principles of the Declaration of Helsinki.

RESULTS

Minimum co-morbidity was present in 25 patients (75.8%). Only 8 (24.2%) patients did not have any co-morbidity. The patients who died had at least one co-morbidity. Hypertension was found in 17 (51.5%) patients, and diabetes mellitus was found in 6 (18.2%) patients. All five patients who died had at least one co-morbidity, and four of these patients had hypertension. None of the eight patients who did not die had comorbid conditions.

The demographic characteristics, treatment regimens, and statistical comparisons of the scoring systems between the patients who survived and those who died are presented in Table 1. Patients were transferred to the ICU if their condition worsened. Acute Physiology and Chronic Health Evaluation (APACHE) II, APACHE IV, and sequential organ failure assessment (SOFA) scores were calculated at admission. The mortality rates of all three systems were evaluated using receiver operating characteristic curves (ROC). APACHE IV had the best discriminating power among the three scoring systems (APACHE IV ROC AUC 0.81, SOFA ROC AUC 0.73, and APACHE II ROC AUC 0.65).

A comparison of the laboratory findings of the patients who survived and died on the first and last day of hospitalization is shown in Table 1. On the first day of hospitalization, the percentage of CD4+ cells and D-dimer levels were found

Table 1. Evaluation of the demographic characteristics, statistical comparison of scoring systems and initial and final measurement laboratory findings in dead and surviving patients

Parameters		Lived (n = 28)	Died (n = 5)	P
Age (year) median (min-max)		61 (43–72)	70 (60–87)	[a]*0.097*
Gender *n* (%)	Female	12 (42.9)	2 (40.0)	[b]*1.000*
	Male	16 (57.1)	3 (60.0)	
Drugs *n* (%)	Favipravir	2 (7.1)	2 (40.0)	[b]*0.099*
	Lopinavir/Ritonavir	26 (92.9)	3 (60.0)	
CRD *n* (%)	No	24 (85.7)	4 (80.0)	[b]*1.000*
	Yes	4 (14.3)	1 (20.0)	
CHF *n* (%)	No	28 (100)	4 (80.0)	[b]*0.152*
	Yes	0 (0)	1 (20.0)	
DBOSFS (day) (Mean ± SD)		8.46 ± 2.05	9.20 ± 1.64	[c]*0.651*
APACHE II Score (*Mean* ± SD)		16.71 ± 6.710	22.00 ± 9.465	[c]***0.011****
APACHE IV Score (*Mean* ± SD)		65.19 ± 21.712	95.78 ± 29.300	[c]***0.001****
SOFA Score *(Mean* ± SD)		4.58 ± 2.144	7.25 ± 3.471	[c]***0.001****
Initial measurements [Median (min-max)]	Leukocyte (10^9/L)	8.39 (5.21–11.57)	7.71 (3.78–12.08)	*0.670*
	T-cell (10^9/L)	6.9 (3.3–10.2)	2.8 (2.1–18.8)	*0.763*
	$CD4^+$ (%)	33.3 (26.7–40.7)	44.9 (36.1–52.6)	***0.029****
	$CD8^+$ (%)	25.9 (21.4–33.9)	25.5 (15.1–38.5)	*0.821*
	$CD4^+/CD8^+$ ratio	1.3 (0.9–1.6)	1.4 (1.1–3.5)	*0.228*
	$CD9^+$ (%)	15.3 (8.5–22.4)	11.3 (8.3–25.6)	*0.860*
	$CD16^+/CD56^+$ ratio	14.3 (6.9–25.2)	12.9 (6.8–19.9)	*0.670*
	Anti- HLA DR (%)	25.9 (17.0–36.4)	25 (18.2–35.6)	*0.851*
	D-dimers (mg L^{-1})	0.9 (0.4–1.6)	1.9 (1.5–3.8)	***0.017****
	LDH (IU/L)	362 (269.5–429.0)	292 (192.5–420.5)	*0.248*
	SO_2 (%)	88.5 (85–92)	74 (65–83)	***0.012****
Final measurements [Median (min-max)]	Leukocyte (10^9/L)	9.43 (3.22–18.22)	10.9 (5.59–17.45)	*0.613*
	T-cell (10^9/L)	9.25 (0.28–41.30)	6.40 (2.20–41.30)	*0.543*
	$CD4^+$ (%)	36.40 (17.20–77.30)	37.20 (31.19–77.30)	*0.159*
	$CD8^+$ (%)	29.00 (14.30–44.40)	17.50 (14.30–42.83)	*0.468*
	$CD4^+/CD8^+$ ratio	1.26 (0.39–5.41)	2.13 (0.73–5.41)	*0.103*
	$CD9^+$ (%)	17.80 (1.80–36.40)	16.86 (8.60–34.90)	*0.987*
	$CD16^+/CD56^+$ ratio	9.75 (1.80–34.90)	4.93 (2.10–9.90)	***0.013****
	Anti- HLA DR (%)	29.90 (3.28–56.70)	24.42 (9.30–56.70)	*0.819*
	D-dimers (mg L^{-1})	1.54 (0.64–3.70)	2.36 (2.32–3.70)	***0.023****
	LDH (IU/L)	365.00 (152.00–674.00)	479.00 (466.00–536.00)	***0.004****
	SO_2 (%)	95.00 (88.00–100.00)	78.00 (75.00–87.00)	***0.001****

*Mann-Whitney U Test, [a]Mann Whitney *U* Test, [b]Fisher's exact test, [c]Indipendent sample *T* test. *$P < 0.05$, Results were given as the median (min-max). *n* (%) and mean ± SD. SD: Standard deviation, CRD: chronic respiratory diseases, CHF: Chronic heart failure, DBOSFS: Duration between onset of symptoms and first sampling, APACHE: Acute Physiology and Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment. LDH: Lactic dehydrogenase, SO2: Saturation of oxygen.

to be significantly higher in patients who died than in those who survived ($P = 0.029$ and $P = 0.017$, Figure 2A and B). In addition, SO2 levels were significantly lower in the patients who died than the patients who survived ($P = 0.012$, Fig. 2C). On the last day of hospitalization, the CD16/CD56 ratio (Fig. 2D), and SO2 percentage were significantly lower in the patients who died than the patients who survived ($P = 0.013$ and $P = 0.001$, respectively). D-dimer and lactate dehydrogenase levels were higher in the patients who died than the patients who survived ($P = 0.023$ and $P = 0.004$, respectively). Regarding the matching of patients in Table 1, one group consists of only five individuals, and it should be noted that it is quite difficult to demonstrate significance (unsuccessful matching) for the difference. Accordingly, there may have been an undetected bias in the matching of the study groups. Nevertheless, our study provides insights into the reactions of the adaptive immune system during the first week of COVID-19.

The clinical findings and laboratory results of all living and deceased patients in the first and last days of hospitalization are shown in Table 2. A significant decrease in the $CD16^{+}/CD56^{+}$ ratio on the last day of admission compared to the first day was detected among all patients ($P = 0.003$). In addition, there was a significant increase in the partial pressure of oxygen (PaO2) and SO2 levels on the last day compared to the first day among all patients ($P = 0.001$ and $P = 0.004$, respectively). The CD16+/CD56+ ratio was significantly higher and SO2 levels were significantly higher on the first day than on the last day in patients who survived ($P = 0.018$ and $P = 0.001$, respectively). In the deceased patients, the CD16+/CD56+ ratio in the samples taken on the first day was significantly higher than the levels in the blood taken on the last day ($P = 0.049$). In contrast, lactate dehydrogenase levels were higher in samples collected on the last day ($P = 0.033$).

DISCUSSION

This study included 33 patients with severe symptomatic COVID-19 pneumonia and SO2 < 93%. We found that the APACHE IV had the best discriminatory power among the three scoring systems in the general population. Low CD4+ and SO2 levels measured on the first day of admission were associated with mortality. In contrast, the CD16+/CD56+ ratio was found to be significantly lower in the samples taken from patients on the last day of hospitalization. The PaO_2 and SO_2 levels detected on the day of death or hospital discharge improved significantly in those who survived; however, no improvement was detected in those who died.

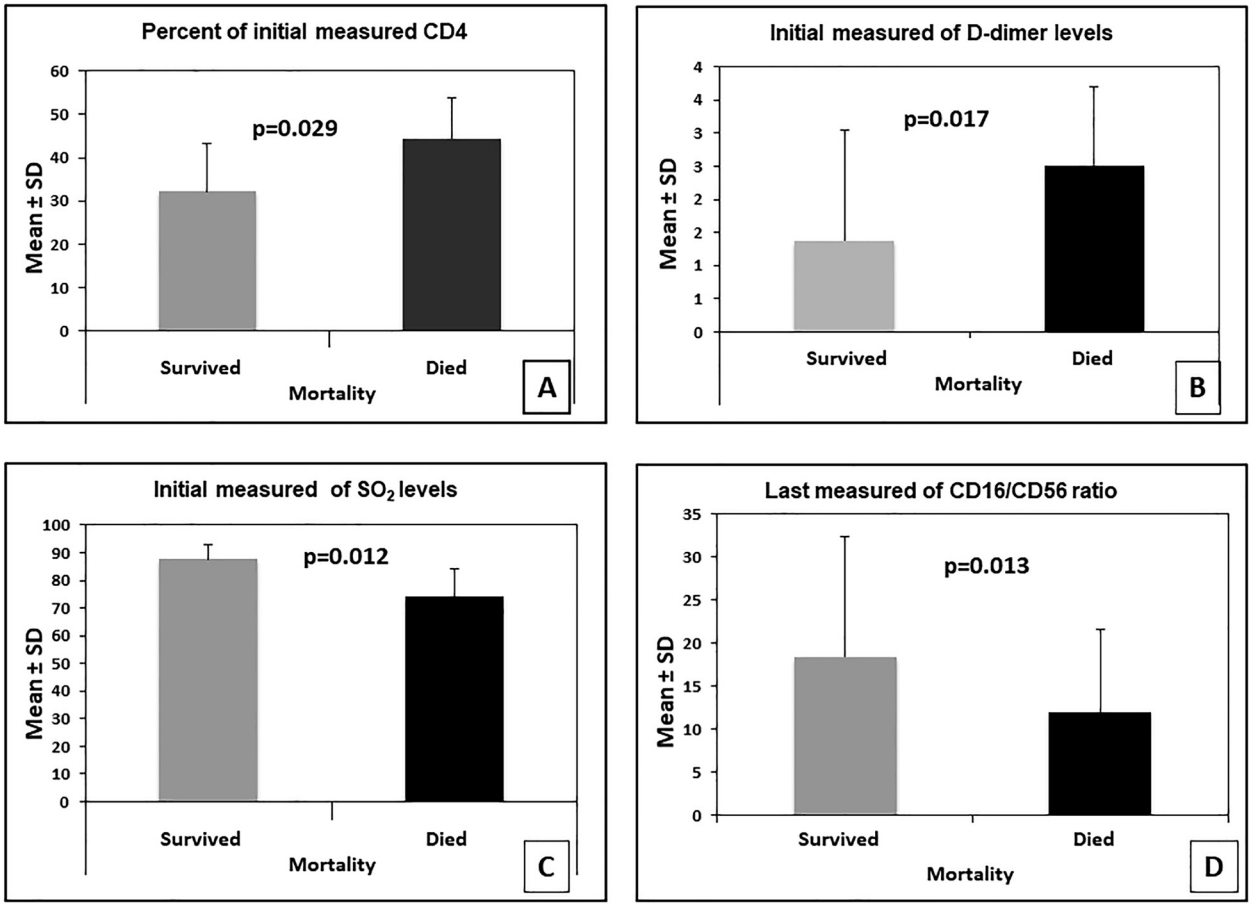

Fig. 2. Distribution of the initially measurement of CD4 levels according to outcome (A). Initially measured D-dimers levels according to outcome (B). Initially measured SO2 levels according to outcome (C). Finally measured $CD16^{+}/CD56^{+}$ ratios according to outcome (D)

Table 2. Initial and final measurements of laboratory parameters of patient groups

Groups	Parameters	Initial measurement Median (min-max)	Last measurement Median (min-max)	*P*
All patients (n = 33)	Leukocyte (10^9/L^{-1})	8.44 (6.11–12.1)	9.53 (6.90–12.21)	*0.600*
	T-cell (10^9/L^{-1})	7.2 (3.1–13.8)	9.1 (3.7–11.8)	*0.367*
	CD4$^+$ (%)	36 (29.6–41.5)	36.4 (30.8–44.7)	*0.300*
	CD8$^+$ (%)	26.6 (21.9–34)	28.7 (19.8–38.1)	*0.333*
	CD4$^+$/CD8$^+$ ratio	1.3 (1–1,6)	1.3 (0.8–2.1)	*0.872*
	CD9$^+$ (%)	15.4 (8.6–24.4)	17.5 (9.2–27.3)	*0.510*
	CD16$^+$/CD56$^+$ ratio	16.2 (7.1–26.9)	9.3 (4.5–17.7)	***0.003****
	Anti-HLA-DR (%)	29.3 (19.1–37)	30.1 (17.4–38.7)	*0.427*
	D-dimers (mg L^{-1})	1.55 (0.18–8.47)	1.94 (0.64–3.70)	*0.197*
	PaO_2 (mmHg)	59.7 (46.0–83.0)	82.1 (40.0–154.0)	***0.001****
	SO2 (%)	88.0 (82.0–92.0)	93.0 (75.0–100.0)	***0.004****
Lived (n = 28)	Leukocyte (10^9/L^{-1})	8.39 (5.21–11.57)	9.43 (3.22–18.22)	*0.306*
	T-cell (10^9/L^{-1})	6.9 (3.3–10.2)	9.25 (0.28–41.30)	*0.287*
	CD4$^+$ (%)	33.3 (26.7–40.7)	36.40 (17.20–77.30)	*0.149*
	CD8$^+$ (%)	25.9 (21.4–33.9)	29.00 (14.30–44.40)	*0.288*
	CD4$^+$/CD8$^+$ ratio	1.3 (0.9–1.6)	1.26 (0.39–5.41)	*0.488*
	CD9$^+$ (%)	15.3 (8.5–22.4)	17.80 (1.80–36.40)	*0.418*
	CD16$^+$/CD56$^+$ ratio	14.3 (6.9–25.2)	9.75 (1.80–34.90)	***0.018****
	Anti- HLA DR (%)	25.9 (17.0–36.4)	29.90 (3.28–56.70)	*0.223*
	D-dimers (mg L^{-1})	0.9 (0.4–1.6)	1.54 (0.64–3.70)	*0.222*
	LDH (IU/L)	362 (269.5–429.0)	365.00 (152.00–674.00)	*0.201*
	SO_2 (mmHg)	88.5 (85–92)	95.00 (88.00–100.00)	***0.001****
Died (n = 5)	Leukocyte (10^9/L^{-1})	7.71 (3.78–12.08)	10.9 (5.59–17.45)	*0.470*
	T-cell (10^9/L^{-1})	2.8 (2.1–18.8)	6.40 (2.20–41.30)	*0.472*
	CD4$^+$ (%)	44.9 (36.1–52.6)	37.20 (31.19–77.30)	*0.898*
	CD8$^+$ (%)	25.5 (15.1–38.5)	17.50 (14.30–42.83)	*0.965*
	CD4$^+$/CD8$^+$ ratio	1.4 (1.1–3.5)	2.13 (0.73–5.41)	*0.798*
	CD9$^+$ (%)	11.3 (8.3–25.6)	16.86 (8.60–34.90)	*0.778*
	CD16$^+$/CD56$^+$ ratio	12.9 (6.8–19.9)	4.93 (2.10–9.90)	***0.049****
	Anti- HLA DR (%)	25 (18.2–35.6)	24.42 (9.30–56.70)	*0.652*
	D-dimers (mg L^{-1})	1.9 (1.5–3.8)	2.36 (2.32–3.70)	*0.719*
	LDH (IU/L)	292 (192.5–420.5)	479.00 (466.00–536.00)	***0.033****
	SO_2 (mmHg)	74 (65–83)	89.00 (80.00–90.00)	*0.426*

Wilcoxon signed-rank test, Mann-Whitney *U* Test, $^*P < 0.05$, Results were given as the median (min-max). PaO2: partial pressure of oxygen, SO2: Saturation of oxygen, LDH: lactate dehydrogenase.

Scoring system variables were analyzed to explore the possible causes of mortality in severely ill COVID-19 patients. In one of the studies, APACHE II was more valuable for mortality estimation, probably because it considered both age and comorbidity [8]. In our study, we used these systems in our patients and obtained significant results. According to our study results, the APACHE IV scoring system provided the most accurate results, followed by the SOFA score, and finally, APACHE II. The patients who died had at least one co-morbidity. The most important comorbidity in the patients who died was hypertension, which was present in four out of the five patients. None of the patients without co-morbidity died.

Immune system dysfunction is the strongest mechanism causing the worsening of the disease [9–12]. Lymphocytes and their subgroups have been studied using flow cytometry and play an important role in the adaptive immune system. In addition, they serve as useful tools for monitoring the sustainability of cellular immunity [13, 14]. There are studies showing the relationship between the severity of COVID-19 and the number of lymphocyte subsets [15–17]. However, few studies have focused on the dynamic and longitudinal changes in these parameters during COVID-19 [18, 19]. Available data suggest that lymphopenia, particularly CD4+ and CD8+ T cells, is common in severe COVID-19 patients [20–23]. In our study, the baseline percentage of CD4$^+$ on day one was lower in patients who survived than in those who died. In contrast, the two groups' CD8+ levels and CD4$^+$/CD8$^+$ ratios were not statistically different. Although the percentage of CD4$^+$ cells increased in the patients who died, the percentage of CD8$^+$ cells did not. This indicates that despite the presence of increased number of antigen-presenting CD4$^+$ T-cells, the absence or low level of cytotoxic T-cells, which kill the virus, results in insufficient viral resistance in these patients. Therefore, it may be considered that intracellular viruses could not be killed in these patients, and the subsequent disease progression resulted in death.

Hypoxia is the primary cause of morbidity and mortality in COVID-19 patients. The mortality rate is higher, especially in patients with lower initial SO_2 levels [24, 25]. Delays

in detecting and correcting hypoxia in these patients are thought to worsen the disease, increase mechanical ventilation, and increase the mortality rate [26–28]. This study found an association between SO_2 levels measured at admission and mortality in patients with severe pneumonia and those with desaturated COVID-19. The SO_2 levels of the five patients who died were significantly lower at admission than those who survived. This result indicates that patients have a poor prognosis with SO_2 levels of <93%, regardless of age and sex.

Peripheral CD3+ T lymphocyte and CD3+ CD4+ T lymphocyte counts, especially CD3+ CD8+ T lymphocytes and CD16+/CD56+ NK cells, were found to be low in patients with severe COVID-19 [29, 30]. The CD16+/CD56+ ratio, measured on the seventh day of hospitalization or on discharge, was significantly lower in patients who died than in those who survived compared to day one. This finding indicated that $CD16^+/CD56^+$ NK cells no longer exhibited virus-killing characteristics. This may cause the proliferation of the virus, worsening of disease conditions, and death.

The present study had several limitations. First, analysis of lymphocyte subgroups is an expensive test conducted in limited laboratories in our country. Therefore, only a small number of patients were included in the study. Second, the patients experienced discomfort from having a blood sample collected twice a day for seven days, and it was challenging to convince some patients to participate. In addition, one of the reasons for limiting our study is that, despite the patient's declaration of initial infection, we were unsure about initial infections or recurrent infections, and concluded only "presumed initial infections" from the evaluation range.

In conclusion, on the day of admission, the percentage of $CD4^+$ lymphocytes and saturation of O_2 were associated with the mortality in patients with severe COVID-19. In addition, the $CD16^+/CD56^+$ ratio in samples obtained on the day of death was associated with mortality in these patients. Analysis of these parameters at the beginning, if possible, will guide patient prognosis, particularly for severe COVID-19. The patients who died had at least one co-morbidity. The most important co-morbidity in the patients who died was hypertension. Despite these results, the small number of patients in our study prevented us from making a healthy and accurate interpretation. Further studies with larger sample sizes of severely ill patients with COVID-19 are needed.

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Authors' contribution: Fadime ERSOY DURSUN: Acquisition of clinical and biological data, data analysis and interpretation, and statistical analysis. Yasemin ÇAĞ: Data analysis and interpretation. Ender İĞNECİ and Burcu IŞIK GÖREN: Data analysis and interpretation. Ferhat ARSLAN: Acquisition of clinical and therapeutic data. Ferruh Kemal İŞMAN: Acquisition of clinical and therapeutic data. Tülin AKARSU AYAZOĞLU: Manuscript correction and linguistic review. Mustafa Haluk VAHABOĞLU: Study concept and design, data analysis and interpretation, manuscript correction, and linguistic review.

All authors read and approved the final version of the manuscript.

Conflict of interest: The authors declare no conflicts of interest.

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