Original Article

ISSN: 1735-0344 *Tanaffos 2022; 21(3): 293-301*

TANAFFOS

Evaluation of Lymphocyte Subtypes in COVID-19 Patients

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Received: 19 January 2022
Accepted: 23 June 2022

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Background: Although many aspects of the COVID-19 disease have not yet been clarified, dysregulation of the immune system may play a crucial role in the progression of the disease. In this study, the lymphocyte subsets were evaluated in patients with different severities of COVID-19.

Materials and Methods: In this prospective study, the frequencies of peripheral lymphocyte subsets ($CD3^+$, $CD4^+$, and $CD8^+$ T cells; $CD19^+$ and $CD20^+$ B cells; $CD16^+/CD56^+$ NK cells, and $CD4^+/CD25^+/FOXP3^+$ regulatory T cells) were evaluated in 67 patients with confirmed COVID-19 on the first day of their admission.

Results: The mean age of patients was 51.3 ± 14.8 years. Thirty-two patients (47.8%) were classified as severe cases, and 11 (16.4%) were categorized as critical. The frequencies of blood lymphocytes, $CD3^+$ cells, $CD25^+FOXP3^+$ T cells, and absolute count of $CD3^+$ T cells, $CD25^+FOXP3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, and $CD16^+56^+$ lymphocytes were lower in more severe cases compared to the milder patients. The percentages of lymphocytes, T cells, and NK cells were significantly lower in the deceased patients. ($p= 0.002$ and $p= 0.042$, $p=0.006$, respectively).

Conclusion: Findings of this cohort study demonstrated that the frequencies of $CD4^+$, $CD8^+$, $CD25^+FOXP3^+$ T cells, and NK cells differed in the severe cases of COVID-19. Moreover, lower frequency of T cells and NK cells could be predictors of mortality in these patients.

Key words: COVID-19; Immune system; Lymphocytes

INTRODUCTION

Coronavirus disease 19 (COVID-19) was first reported in Wuhan, China, in December 2019. The etiologic agent was an emerging coronavirus named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In a short period, COVID-19 spread to other countries and led to a pandemic (1). By 17 May 2021, 163 M confirmed cases of COVID-19, including 3.3M deaths, were reported to the World Health Organization (2). The first case from Iran was noted on the 19th of February 2020, and by 25 October 2020, 574,856 confirmed cases and 32,953 deaths (3). Although about 80 percent of affected patients have mild symptoms, the disease can be severe enough to need admission among 20% of patients, and 5% lead to respiratory or other organ failure and even death (4).

Despite global efforts to determine the pathophysiology of COVID-19, many aspects of the disease have not been recognized. Dysregulation of the immune system seems vital in the progression of COVID-19 (5, 6). Lymphocyte subsets, particularly cytotoxic T lymphocytes, dendritic cells, and natural killer (NK) cells, play an essential role in the pathogenesis and elimination of viral infections (7, 8). Lymphopenia has been recognized as a predictor of severe outcomes among COVID-19 patients (9). Alternations in the phenotypes and numbers of lymphocyte subsets have been reported in COVID-19 patients (10-14). Studies have demonstrated a decrease in the frequencies of regulatory T (Tregs) cells ($CD4^+CD25^+FOXP3^+$) and $CD45RA^+$Treg cells in patients with severe COVID-19 (15, 16). The importance of the alterations of these parameters, especially T_{reg} cells, $CD27^+$, and $CD38^+$ lymphocytes during the disease, has not been well studied. In a previous study, we observed an increasing trend in total T cells, T helpers, cytotoxic T cells, activated lymphocytes, and natural killer cells among recovering patients with COVID-19 who responded to treatment (17).

Therefore, in the current study, we did detailed immunophenotyping of blood lymphocytes on admission time and aimed to assess their correlations with clinical outcomes and the severity of the disease.

MATERIALS AND METHODS

Patients

In the study period of 23 to 30 May 2020, 67 confirmed COVID-19 patients were enrolled in the study at Masih Daneshvari Hospital, Tehran, Iran. The selected patients were symptomatic, and the SARS-CoV-2 RNA in the samples was confirmed by a WHO-confirmed reverse transcriptase-polymerase chain reaction (RT-PCR) assay. The disease's severity was evaluated by measuring resting oxygen saturation (O_2sat) levels and respiratory rate. Patients with pulmonary infiltration confirmed by chest X-ray (CXR) or computerized tomography (CT) and O_2 sat >93% with ambient air were classified as moderate (24 cases) and those with O_2 sat ≤93% or a respiratory rate higher than 30 breaths/min were categorized as severe (32 cases). Patients who were admitted to the intensive care unit (ICU), those who needed noninvasive or mechanical ventilation, and cases with acute respiratory syndrome distress (ARDS) or shock were classified as critical (11 cases) (18, 19). The study protocol was approved by the ethics committee of the National Research Institute of Tuberculosis and Lung Diseases (approval number: IR.SBMU.NRITLD.REC.1399.037). Written informed consent was obtained from all participants or their legal guardians. All experiments were performed in accordance with relevant guidelines and regulations.

Therapeutic approach

All patients were under supportive care consisting of intravenous fluids and supplemental oxygen. Based on the recommendations of the Iranian national guideline for COVID-19 management at the time of the study (details mentioned elsewhere (20)), all patients had received lopinavir/ritonavir 400/100 mg for seven days.

Blood sampling

On the first day of admission, before the initiation of treatments, peripheral blood (2 mL) was collected in EDTA anticoagulated tubes. All blood samples were tested within six hours after sampling. Total blood counts (CBC) and $CD3^+$, $CD4^+$, $CD8^+$ T cells; $CD19^+$ and $CD20^+$ B lymphocytes; $CD16^+$ $CD56^+$ (NK cells), and regulatory T cells (CD4/CD25 and FOXP3+) frequency and absolutes counts (cells/µl) were measured.

Briefly, the samples were centrifuged, and then red blood cells were removed by adding lysis buffer. Subsequently, white blood cells were harvested and washed with cold PBS. Then, the cell-surface Fc receptors were blocked with 2.4 G2 (PharMingen, San Diego, CA,

USA) before staining. Antibodies used for flow cytometry were phycoerythrin (PE)-conjugated anti-human CD4, CD19, CD16/56 antibodies (PharMingen, USA) for staining CD4 T cells, CD19 for B cells, CD16/56 for NK cells. CD8 cells were stained by anti-human CD8 conjugated with an allophycocyanin (APC) antibody. For CD3 T cells, fluorescein isothiocyanate (FITC)-conjugated anti-human antibody (PharMingen, USA) was used. All antibodies were added to the cells and incubated at 4°C for 30 min in a dark place. For immunophenotyping of the Treg cells, surface staining of CD4 and CD25 markers was performed by mouse anti-human CD25-FITC (Biolegend, San Diego, CA, USA) and CD4-PE (Immunostep, Salamanca, Spain) for 30 min at 4 °C. Then the cells were washed and incubated in fixation and permeabilization solution buffer (BD Biosciences, San Diego, CA, USA) at 4°C for 15 min. Following this stage, the cells were washed with cold PBS, and intracellular staining was performed with FOXP3-APC Abs (eBioscience, California, USA) at 4°C for 30 min. Isotype-matched antibodies were used for all the samples. Then, the cells were washed, and 10000 events were analyzed by FACS (FACSCalibour, USA). The data were analyzed using FlowJo software version 8.

Statistical analysis

Categorical variables were presented as numbers and frequencies, and continuous variables as medians and interquartile ranges. Categorical data were compared using the chi-square test or Fisher's Exact test. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to test the normality of the data. For comparison of medians between different groups of severity, the Kruskal-Wallis test was used, and the differences between the two groups were analyzed using the Mann–Whitney test. SPSS Statistics version 21.0 software was used for statistical analyses. All reported P values were two-sided, and a value of less than 0.05 was considered statistically significant. The diagram was drawn using the R software.

RESULTS

Baseline characteristics

During the study period, 67 patients with COVID-19 were enrolled. The mean age of the cases was 51.3 ± 14.8 years, 31 (46.3%) were male, and 52.2% had at least one comorbid condition. Twenty-four (35.8%) patients were classified as moderate, 32 (47.8%) as severe, and 11 (16.4%) were categorized as critical cases. Demographics, basic characteristics, and the outcomes of the treatment are summarized in Table 1. Patients with O_2Sat less than 93 were significantly older than others (54±15 versus 46±14 years, p=0.040). Also, the rate of underlying diseases (40.3% versus 11.9%, p=0.021), particularly among diabetics (22.4% versus 4.5%, p=0.047), was significantly higher among cases with hypoxia. In terms of the outcomes, mortality was more common among patients with hypertension (6%) in comparison to the others (4.5%) p=0.040 (Table 1). The median age, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) were not significantly different among patients with varying severities of the disease. In patients in critical conditions, the median of lactate dehydrogenase (LDH) was higher than the severe and moderate cases (p-value of 0.005 and 0.001, respectively).

Table 1. Demographic and characteristics of 67 confirmed COVID-19 cases

Parameter		N (%)
Age (mean ± SD)		51.35 ± 14.83 years
Gender	**Male**	31 (46.3)
	Female	36 (53.7)
Underlying disease		35 (52.2)
DM		18 (26.9)
HTN		15(22.4)
Chronic heart diseases		8 (11.9)
Chronic lung diseases		6 (9.0)
BMI≥40		2 (3.0)
Other comorbidities		4 (5.5)
The days from symptom onset (mean ± SD)		10.2 ± 5.1 days
Severity	**Moderate**	24 (35.8)
	Severe	32 (47.8)
	Critical	11 (16.4)
O_2 saturation	**>93**	24 (35.8)
	≤93	43 (64.2)
Outcome	**cure**	60 (89.6)
	death	7 (10.4)

SD: standard deviation; BMI: body mass index; DM: Diabetes mellitus; HTN: Hypertension

Immunophenotyping and severity of the disease

Leukocyte counts, percentages of neutrophils and lymphocytes, and the percentages and absolute counts of lymphocyte subsets were compared among patients with different disease severities. Cases with more severe diseases had a higher rate of blood neutrophils. Conversely, the percentage of blood lymphocytes, $CD3^+$ cells, $CD25^+FOXP3^+$ T cells; and absolute count of $CD3^+$ T cells, $CD25^+FOXP3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD16^+56^+$ lymphocytes were lower in more severe cases in comparison with the milder cases (Figure 1). No significant differences were found in the $CD4^+/CD8^+$ as well as other parameters.

Cellular subsets in moderate, severe, and critical COVID-19 patients

The frequency of polymorphonuclears in the patients in critical was significantly higher than those in the severe (p=0.0038) and moderate (p = 0.00094) COVID-19 patients (p=0.00051). Moreover, the percentage of PMNs in severe patients was higher than that in moderate patients (p=0.027) (Figure 1A). The frequency of lymphocytes in critical patients was significantly lower than those in severe (p=0.0049) and moderate (p=0.0011) COVID-19 patients (p=0.00026), and the percentage in severe patients was lower than in moderate COVID-19 cases (p=0.0065) (Figure 1B).

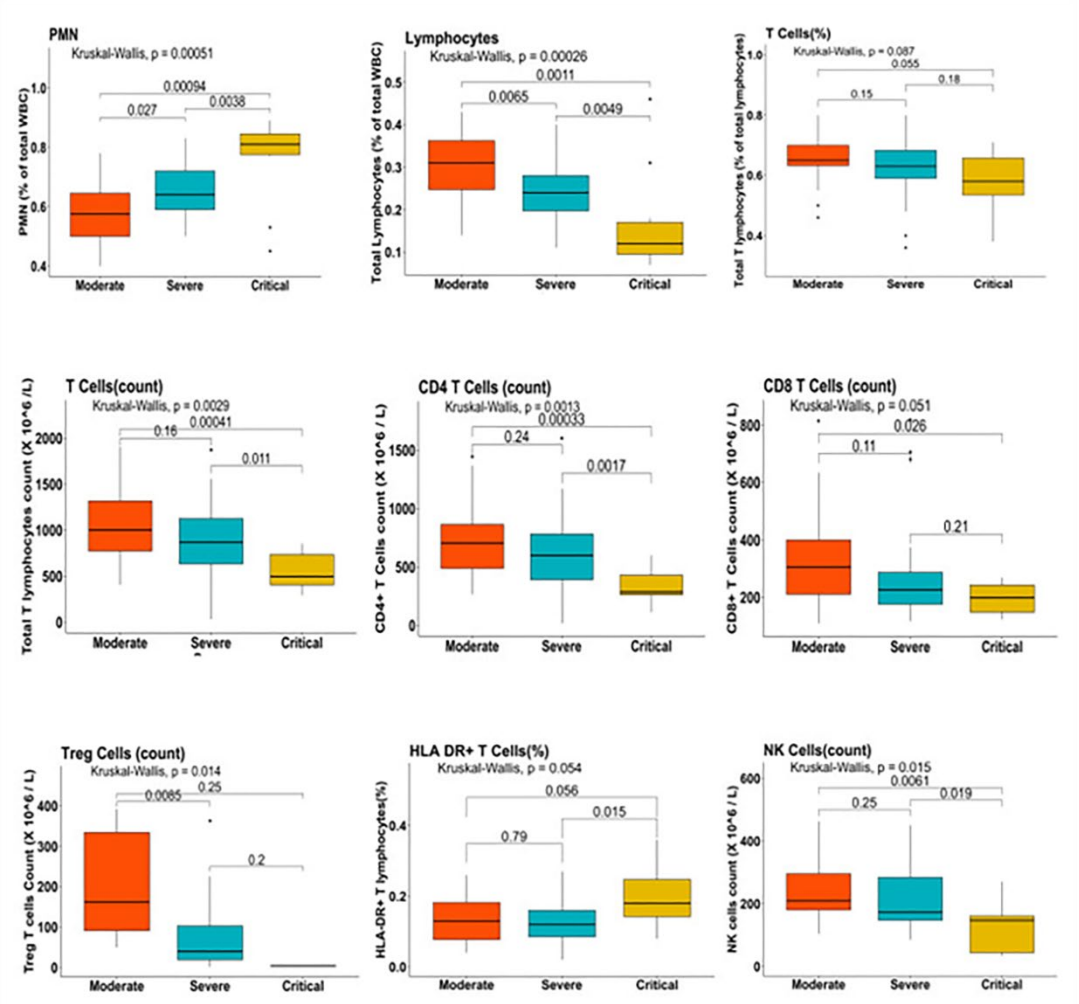

Figure 1. Comparison of on admission cellular subsets in COVID-19 patients with different disease severity
Abbreviations: T_{reg}: regulatory T lymphocyte (CD4+CD25+FOXP3+), NK: natural killer

The frequency of total T lymphocytes in critical patients was not significantly different from severe (p=0.18) and moderate (p=0.055) patients (p=0.087) (Figure 1C). The total lymphocyte count was significantly lower in critical patients than in severe (p=0.011) and moderate (p=0.00041) patients (p=0.0029). However, there were no significant differences in total lymphocyte count between severe and moderate patients (P=0.16) (Figure 1D). There was a significant difference in CD4+Tcell count between critical COVID-19 patients and severe (p=0.0017) and moderate group patients (p=0.00033) (Figure 1E). On the contrary, there was no significant difference in CD8+Tcell count between critical and severe COVID-19 patients (p=0.21). On the contrary, CD8+Tcell count in critical patients was significantly higher than in moderate COVID-19 patients (p=0.026) (Figure 1F).

The T_{reg} cell count in severe COVID-19 patients was significantly lower than in moderate patients (p=0.014). However, the T_{reg} count in critical patients was similar to severe (p=0.2) and moderate (0.25) patients (Figure 1G).

The frequency of HLA-DR+T lymphocytes in critical patients was significantly higher than in severe (p= 0.015) patients. No significant differences was found in the frequency of HLA-DR+T lymphocytes in critical and moderate COVID-19 patients (p=0.056) (Figure 1H). There was significant difference in NK cell frequency between critical patients and severe (p=0.019) and moderate (p=0.0061) cases (p=0.015) (Figure 1I). Deceased patients had a total leukocytes [median; 11380, interquartile range (IQR), 6370-15350 (cell/µl)] and neutrophils percentage in peripheral blood 78% [IQR; 73-88%] which was significantly higher than [5775, IQR; 4140-7800 (cell/µl) and 62%, IQR; 53-72%, respectively] recovered patients (p=0.023 and p=0.001 respectively) (Table 2).

Table 2. Distribution of cellular subsets among COVID-19 patients concerning outcome

Parameters	Outcome				p-value
	Cure		Death		
	Median	IQR	Median	IQR	
White blood cells	5775	4140-7800	11380	6370-15350	**0.023***
Neutrophils (%)	62	53-72	78	73-88	**0.001***
Lymphocytes (%)	25	19-34	12	8-20	**0.002***
Total lymphocyte (c)	1397	1115-1860	1342	733-1776	0.499
CD3$^+$ cells (%)	64	59-69	60	38-62	**0.042***
CD3$^+$ cells (c)	877	623-1215	598	495-806	0.146
CD4$^+$ cells (%)	41	36-47	36	24-43	0.056
CD4$^+$ cells (c)	593	385-803	364	248-497	0.065
CD8$^+$ cells (%)	16	14-23	15	12-23	0.573
CD8$^+$ cells (c)	242	161-346	201	116-267	0.305
CD19$^+$ cells (%)	11	8-15	13	11-24	0.058
CD19$^+$ cells (c)	142	94-250	183	60-399	0.354
CD20$^+$ cells (%)	11	7-15	13	11-24	0.072
CD20$^+$ cells (c)	135	87-241	179	58-399	0.376
CD27$^+$ cells (%)	16	10-26	22	13-29	0.492
CD27$^+$ cells (c)	278	126-510	269	134-437	0.827
CD25$^+$FOXP3$^+$ cells (%)#	3	1-8	1	0-11	0.350
CD25$^+$FOXP3$^+$ cells (c)#	48	9-144	19	0-311	0.467
CD16$^+$56$^+$ cells (%)	16	11-18	6	5-13	**0.006***
CD16$^+$56$^+$ cells (c)	204	149-300	76	43-185	**0.040***
CD4$^+$/CD8$^+$ ratio	2.6	1.7-3.3	2.4	1.2-3.4	0.637
CD3$^+$ HLA-DRL$^+$ cells (%)	4	0-7	5	0-10	0.410
CD3$^+$ HLA-DRL$^+$ cells (c)	54	0-123	35	0-88	0.536
CD38 cells (%)	11	8-16	14	9-17	0.359
CD38 cells (c)	167	121-227	114	100-403	0.595

* Statistically significant

FOXP3 marker was checked for 35 patients. (c): count, IQR: interquartile range

Moreover, the percentage of lymphocytes in the patients who passed away [12%, IQR; 8-20%] was significantly lower than the surviving ones [25%, IQR; 19-34%, p=0.002] (Table 2). The frequency of T cells (CD3+) [60%, IQR; 38-62%] in the deceased group was lower than in those who were cured. [64%, IQR; 59-69%, p=0.042)]. The NK Cell percentages [6%, IQR; 5-13%] and counts [76 (cell/µl), IQR; 43-185 (cell/µl)] in the non-surviving group were significantly lower than in the cured group [16%, IQR; 11-18% and 204 (cell/µl), IQR; 149-300 (cell/µl), respectively (p=0.006 and p=0.04) (Table 2).

DISCUSSION

In this study, we found a significant difference in the total lymphocyte counts and immunophenotypes ($CD4^+$ cells, $CD8^+$ cells, $CD25^+FOXP3^+$ regulatory T cells, NK cells) in patients with severe COVID-19. The role of immune dysregulation caused by SARS-CoV-2 has been demonstrated in the pathogenesis of COVID-19 disease (21, 22). The virus may cause immune system exhaustion (5, 8), cytokine storm, and hyper inflammation (23-25). Different subsets of lymphocytes have prominent roles in the immune system's competence against viral infections (7), and any disturbance in their population induces immune system dysregulation (10, 13, 26). Lymphopenia and decreased numbers of $CD4^+$ and $CD8^+$ subsets of T cells have been reported in the COVID-19 patients (27). Several studies have demonstrated a significant decrease in the total number of lymphocytes among patients with COVID-19 (21, 28, 29).

Moreover, emerging studies in patients with COVID-19 have reported a correlation between cellular subsets and disease severity. Still, the available information regarding the outcome of the disease is not well understood (10, 13, 15, 21, 30, 31). Several studies reported a strong association between low levels of total lymphocytes, $CD4^+$ T cells, and $CD8^+$ T cells and the severity of the disease in patients with COVID-19 (8, 13-15, 21, 26).

In this regard, $CD4^+$ T cells play an essential role in regulating the immune response against viral infections. This is achieved through activating the network between immune cells via the secretion of pro- and anti-inflammatory mediators. Besides this function, some subsets of T cells can lyse cancerous and infected cells. In this line, $CD8^+$ cytotoxic T cells can destroy virus-infected host cells (32). The precise mechanism of lymphopenia and reduction of the counts of T cells in COVID-19 disease is not yet elucidated. Only a limited number of studies have suggested that SARS-CoV-2 can directly destroy lymphocytes, particularly T cells (15). Chu et al. demonstrated that MERS-CoV (another Betacoronavirus) could infect and induce apoptosis in T cells. However, this has not been reported in the SARS virus infection (33), although it is speculated that they might play a role in the pathogenesis of COVID-19. A recent study has demonstrated infiltration and sequestration of lymphocytes(mainly $CD8^+$ T cells) in the affected organs, especially the pulmonary interstitium (29). In patients infected with SARS-CoV-2, it is demonstrated that approximately 80% of the infiltrated inflammatory cells in the lungs are $CD8^+$ T cells (34). The shift of the immune cells from blood to the tissue, as shown in some autopsies (29, 35), may explain the lower count of circulating T cells, especially $CD8^+$ cells in patients with severe COVID-19.

Weiskopf et al. showed an increase in the CD4:CD8 ratio in ten moderate-to-severe COVID-19 patients (36). Moreover, Diao et al. compared this ratio between patients under ICU care and other hospitalized cases and found a greater ratio among the former(21). Nonetheless, in the current study, we did not observe any significant difference in this ratio between severe and non-severe COVID-19 patients (14, 15).

This could be due to a parallel reduction in both T cell subsets in severe cases. Regulatory T cells (Treg) are vital to immunological homeostasis and reduce excessive inflammatory responses against viral infections. (37). Qin et al. observed lower blood levels of Treg cells among COVID-19 patients, particularly in severe cases (15). In the current study, a correlation between Tregs and the severity of the disease was detected. However, in a study by Chen

et al., despite the reduction of Tregs in both severe and moderate cases, the total Treg cells were similar in the two groups (26). In severe cases, they observed a significantly lower proportion of naive Tregs and a slightly higher proportion of memory Tregs. As Treg lymphocytes can inhibit the overstimulation of the immune response against pathogens, their reduction may justify the tissue damage in severe cases of COVID-19 patients (38).

In the current study, the total number and frequency of B cells in COVID-19 patients was controversial. In this line, Chen et al. reported that the count of B lymphocytes was not significantly different in severe disease cases (26). However, they found a higher proportion of B cells in severe cases and observed a more significant decrease in T cells in this group of patients. Conversely, in a meta-analysis study, Wang et al. observed a significant reduction in B cells among more severe cases due to an obscure mechanism (10, 14).

Most studies have found no association between the NK cell counts and the severity of COVID-19 disease (14, 26). However, the current study's findings align with a meta-analysis by Huang (10), which noted lower NK cell levels in patients with severe COVID-19 (39).

In our study, a higher number of polymorphonuclear cells and a lower proportion of lymphocytes, T cells, and NK cells were observed, making them suitable candidates as predictors of mortality in COVID-19 patients.

In support of this hypothesis, the findings of the present study demonstrated that in patients with normal T cells, a lower mortality rate was observed compared to those with lower T cell counts. Recently, in a correspondence, two studies have shown a correlation between $CD4^+$ and $CD8^+$ T cell counts and the rate of death in patients with COVID-19 (14, 21).

The current study had limitations due to the variability and diverse future of COVID-19, making it difficult to generalize the outcomes. Due to the immune system's complexity, prospective multicenter studies with large samples of patients need to be designed and conducted. Moreover, a detailed analysis of cellular subsets with multi-colored staining of the subsets of T cells such as Th9, Th17, and Th22 T cells is needed to assess the time-based changes in immune response after infection with SARS-Co-V-2 and their prognostic value should be investigated.

In conclusion, our results suggest that total lymphocyte counts and the difference of cellular immunophenotyping (including $CD4^+$, $CD8^+$, $CD25^+FOXP3^+$ T cells, and NK cells) may be correlated with the severity of COVID-19. A reduction in the levels of these cells may suggest their diagnostic suitability as predictors of the severity of the disease; however, this needs to be investigated in detail in the future.

Acknowledgement

The authors acknowledge from all participants and clinicians and nurses for helping and reminding the memory all patients whom died from COVID-19 during this study.

Statement of Ethics

All protocol has been approved by ethic committee of Maish Daneshvari Hospital.

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