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RESEARCH ARTICLE

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Immunophenotyping of lymphocytes and monocytes and the status of cytokines in the clinical course of Covid-19 patients

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Abstract

Lymphopenia, T cell subgroup changes, and cytokine level differences are important in the early diagnosis and treatment of Covid-19 cases and similar pandemics. We aimed to investigate the T cell, monocyte subgroups, and cytokine differences according to disease severity. A total of 46 volunteers were included in the study. According to disease status, there were three groups (control, mild, and severe). The age, gender, smoking status, temperature, heart rate and oxygen saturation, complete blood count, C-reactive protein (CRP) was noted, and flow cytometric analyses were performed for T cell and monocyte subgroups, and cytokine levels. Temperature, heart rate, SPO_2, white blood cell (WBC), lympocyte count, trombocyte count, neutrophil/lymphocyte ratio, D-dimer and CRP levels, lymphocyte %, lymphocyte/monocyte rate, monocyte subtypes (%), $CD3^+$, $CD4^+$, $CD8^+$ cell counts, interleukin (IL)-1β, TNF-alpha, monocyte chemoattractant protein (MCP)-1, IL-6, IL-8, IL-10, IL-18, IL-23 were significantly different between groups. CRP, IL-8, neutrophil/lymphocyte ratio, NK cells (%) have positive correlation and negative correlation was observed at lymphocyte (count), lymphocyte (%), lymphocyte/monocyte, classical monocyte (%), lymphocyte (count), $CD3^+$ (count), $CD4^+$ (count). As conclusion, lymphocyte (%), Lymphocyte (count), CRP levels, $CD3^+$ and $CD4^+$ cell counts strongly correlate with disease severity are valuable parameters for determining the prognoses of Covid-19.

KEYWORDS

cell-mediated immunity, cytokines, lymphocyte, monocyte

1 | INTRODUCTION

The Covid-19 disease develops due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus and has caused pandemic for the last 2 years.[1]

Severe Covid-19 patients have alterations in some of the blood parameters, such as increasing procalcitonin, D-dimer, C reactive protein (CRP), and a number of the pro-inflammatory cytokines in the literature.[2] SARS or middle east respiratory syndrome coronavirus (MERS) have significant lymphopenia like as Covid-19.[3,4] Lymphopenia is a typical profile in Covid-19 cases for >60 years old, and it is very important for predicting severity, and mortality. It is also known that Covid-19 patients have lower monocyte counts.[4,5] Higher white blood cell (WBC) and neutrophil counts have been reported in non-survivors than survivors, and the neutrophil/lymphocyte ratio is higher in severe Covid-19 cases.[6]

It has been reported that the evaluation of T lymphocyte subgroups together with other clinical findings may be beneficial for the prognosis of Covid-19 disease in many studies. The evaluation of T lymphocyte subgroups, when considered together with other

clinical findings could be useful for the prognosis of Covid-19 disease.[7] T lymphocyte numbers decreased significantly during Covid-19 cases.[4,5]

Cytokine storm has been reported for many diseases (malignancy, rheumatological pathologies, sepsis, etc.), including Covid-19.[8] In severe Covid-19 patients, systemic hyper inflammation and cytokine storm develop which causes rapid clinical deterioration.[9] Cytokine storm which leads to multiple organ failure and even death due to systemic hyper inflammation is caused by excessive cytokine release.[10] It has been shown that some of the anti-inflammatory drugs, such as corticosteroids, could be useful for Covid-19.[11]

In our study, we investigated the role of T lymphocyte, monocyte subtypes, and cytokine values in the evaluation of the severity of Covid-19 disease.

2 | MATERIALS AND METHODS

This study was designed as a prospective cohort study and initiated following the approval of the local clinical research ethics committee. A total of 83 patients who applied to the emergency between October 01, 2020 and December 01, 2020 with a history of fever, cough, shortness of breath, and having contact history with a Covid-19 case constitute the population of the study, and 31 of these patients whose Covid-19 polymerase chain reaction (PCR) results were positive were included in the study. The Covid-19 cases were divided into two groups due to their oxygen requirement, and hospitalization status. Control volunteers were selected from patients who did not have any known chronic disease, had a negative PCR test for Covid-19, and were hospitalized due to elective inguinal hernia operation. The control cases were equal in age, and gender with Covid-19 cases. A total of 15 control volunteers (Group 1) were included. A written informed consent form was obtained from all of the volunteers and registered to a public trials system (NCT04531345).

The age, gender, and smoking status of Covid-19 patients were questioned and their temperature, heart rate, and oxygen saturation were measured concurrently with patients' admission to the emergency. At the same period, complete blood count, D-dimer, ferritin, fibrinogen, CRP, and lung tomography were evaluated. In the control group, age, gender, and smoking status were questioned, fever, pulse, and oxygen saturation were measured, as well as a complete blood count, and CRP values were noted. From all the volunteers participating in the study, two separate tubes of blood were taken into 5 cc hemogram tubes, and flow cytometric analysis was performed within 24 h (FACSLyric™, BD Company, cat no: 659180). There was no antibody in the first tube. The blood with antibody separated into two tubes. In the first tube with antibody, V500-C labeled CD45 (BD Company, cat no: BD 655873), APC/Cyanine7 labeled CD3 (Biolegend, cat no: 344818), PerCP/Cyanine5.5 labeled CD4 Biolegend, cat no: 357413), PE/Cyanine7 labeled CD8 (Biolegend, cat no: 344711) was added according to the amount of recommended by the manufacturer. V500-C labeled CD45 (BD Company, cat no: BD 655873), APC/Cyanine7 labeled CD3 (Biolegend, cat no: 344818), APC labeled CD14 (BD Company, USA, cat no: BD 345787), FITC labeled CD16 (BD Company, cat no: BD 560996), Percp-Cy5.5 labeled CD56 (BD Company, cat no: BD 560842) antibodies were added according to the amount of recommended by the manufacturer. Ten thousand events/sample were acquired. The ratios of total lymphocytes, and monocytes, $CD3^+$ lymphocytes, $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, natural killer (NK) cells, $CD14^{++}$, $CD16^-$ classical monocytes, $CD14^+$ and $CD16^+$, intermediate monocytes, and $CD14^{lo}$ and $CD16^+$ Nonclassical monocytes were evaluated with the flow cytometry (FACSLyric™, BD Company, cat no: 659180). At the same time, blood was taken from all volunteers in a 10 cc gelly biochemistry tube, centrifuged at 1800 rpm for 5min, serum was separated, and stored in 2 ml eppendorf in −80°C freezer after necessary labeling for cytokine measurements. After collecting the serum of all volunteers included in the study, the serum was removed from the −80 degrees freezer and dissolved. Interleukin (IL)-1β, IFN-α2, IFN-γ, TNF-α, monocyte hemoattractant protein (MCP)-1, IL-6, IL -8, IL-10, IL-12p (p70), IL-17A, IL-18, IL-23, and IL-33 were measured using flow cytometry (Cube 8™, Sysmex, Japan, cat no: CY-S-3068R_V3) and cytokine measurement kit (LEGENDplex™ Human Inflammation Panel 1, Biolegend, cat no: 740808).

2.1 | Statistical analysis

The analysis of the data was performed with SPSS version 22.0 computer program. The distribution of variables was measured with the Kolmogorov–Smirnov test. Homogeneity of variance was evaluated by applying Levene analysis of variance to the groups for the independent, and normally distributed variables. One-way analysis of variance (ANOVA) test was performed for between independent, normally distributed, and homogeneous parameters, Brown–Forsythe ANOVA test was performed for independent, normally distributed, and homogeneous parameters, and Kruskal–Wallis test were used for independent, and nonnormally distributed variables. In case of a significant difference in the analysis for normally distributed parameters, the Tukey test was used for homogeneous variance, Tamhanes's T2 test was used for inhomogeneity variance as post hoc analysis. The data which not distributed normally the Dunn Z-test for the post hoc tests after the Kruskal–Wallis test. D-Dimer was compared with Mann–Whitney *U*-test, ferritin, and fibrinogen was compared with Student *t*-test between mild, and severe Covid-19 cases. Chi-square test, Fisher's Exact Test were performed for the qualitative variables. Spearman correlation coefficients were used for nonnormally distributed parameters, Pearson correlation coefficients were used for normally distributed parameters in the correlation according to disease severity. The interpretations of the correlation analysis were performed according to "0.00–0.19: very weak, 0.20–0.39: weak, 0.40–0.59: moderate, 0.60–0.79: strong, 0.80–1.0: very strong."

3 | RESULTS

The Covid-19 cases ($n = 31$) were determined based on the order of admission to the hospital. The mean age of all volunteers was 60.57 ± 12.82 years, and the F/M ratio was 9/14. The distribution of Covid-19 cases by groups was determined as 15 patients for the outpatient mild group (Group 2) and 16 patients for the hospitalized severe group (Group 3), and 15 healthy volunteers (Group 1) for the control group. Demographic and vital data analysis of the volunteers are given in Table 1, results of hemogram and biochemical parameters in Table 2, flow cytometric immunophenotyping results in Table 3 and sample of images of the groups Figure 1, serum cytokine results in Table 4, flow cytometric cell counts in Figure 2.

TABLE 1 Evaluation of the demographic and vital signs of the volunteers

		Control ($n = 15$)	Mild ($n = 15$)	Severe ($n = 16$)	p-value
Age (year) (mean ± SD)		60.20 ± 8.71	58.13 ± 14.91	63.19 ± 14.19	0.554[a]
Gender	Female	6	5	7	0.757[b]
	Male	9	10	9	
Smoking status	Yes	4 (26.7%)	8 (53.3%)	5 (31.3%)	0.268[c]
	No	11 (73.3%)	7 (46.7%)	11 (68.8%)	
Temperature (°C) (mean ± SD)		36.78 ± 0.25	36.96 ± 0.68	37.34 ± 0.55	0.018[d]
Heart rate/min (mean ± SD)		75.67 ± 9.57	82.00 ± 8.87	90.56 ± 12.95	0.001[a]
SPO_2 (%) (median, [min-max])		98.00 (95.00–99.00)	96.00 (86.00–98.00)	93.00 (68.00–98.00)	0.003[e]

[a]One-way analysis of variance.
[b]Chi-square test.
[c]Fisher's exact test.
[d]Brown-Forsythe.
[e]Kruskal–Wallis test.

TABLE 2 Comparison of hemogram and biochemistry values of the groups

	Control ($n = 15$)	Mild ($n = 15$)	Severe ($n = 16$)	p-value	Corelation (r, p)
WBC (10^3/μl) (mean ± SD)	8.46 ± 1.80	5.57 ± 2.51	7.39 ± 4.09	0.049[a]	$r = -0.135$ $p = 0.371$
Neutrophil (10^3/μl) (mean ± SD)	5.28 ± 1.57	3.992.19	5.33 ± 3.41	0.182[a]	$r = 0.062$ $p = 0.681$
Lymphocyte (10^3/μl) (mean ± SD)	2.39 ± 0.91	1.31 ± 0.49	1.21 ± 1.16	0.000[b]	$r = -0.614$ $p = 0.000$
Platelets (10^3/μl) (mean ± SD)	310.60 ± 68.39	210.93 ± 58.62	216.88 ± 114.93	0.003[b]	$r = -0.404$ $p = 0.005$
Neutrophil/Lymphocyte (median, [min–max])	2.34 (0.98–9.29)	2.71 (1.04–6.44)	4.46 (1.46–45.87)	0.021[c]	$r = 0.413$ $p = 0.004$
D-dimer (μg/ml) (median, [min–max])	-	0.66 ± 0.84	1.34 ± 1.25	0.031[d]	NA
Ferritin (ng/ml) (mean ± SD)	-	100.15 ± 92.16	185.90 ± 156.39	0.120[e]	NA
Fibrinogen (mg/dl) (mean ± SD)	-	407.98 ± 112.17	483.86 ± 83.08	0.150[e]	NA
C-reactive protein (mg/dl) (median, [min–max])	6.00 (2.00–16.00)	41.95 (3.47–268.90)	87.23 (3.02–268.90)	0.000[c]	$r = 0.644$ $p = 0.000$

Abbreviation: NA, not applicable.
[a]Brown-Forsythe.
[b]One way analysis of variance.
[c]Kruskal–Wallis test.
[d]Mann–Whitney U-test.
[e]Student t-test.

TABLE 3 Evaluation of immunophenotyping results with flow cytometry of groups

	Control (n = 15)	Mild (n = 15)	Severe (n = 16)	p-value	Corelation (r, p)
Monocyte (%) (mean ± SD)	5.50 ± 1.13	5.68 ± 1.85	4.92 ± 2.40	0.505[a]	r = −0.132 p = 0.382
Lymphocyte (%) (mean ± SD)	24.77 ± 5.50	19.02 ± 5.32	12.21 ± 6.55	0.000[a]	r = −0.674 p = 0.000
Lymphocyte/Monocyte (mean ± SD)	4.56 ± 0.96	3.70 ± 1.74	2.95 ± 1.87	0.026[a]	r = −0.395 p = 0.007
$CD3^+$ (%) (median, [min–max])	72.53 (65.58–75.37)	68.90 (45.10–81.01)	71.42 (11.88–80.40)	0.686[b]	r = −0.039 p = 0.798
$CD4^+$ (%) (mean ± SD)	42.96 ± 5.40	38.15 ± 10.73	33.76 ± 13.89	0.067[c]	r = −0.343 p = 0.020
$CD8^+$ (%) (mean ± SD)	22.84 ± 5.30	23.68 ± 8.73	27.30 ± 12.49	0.379[a]	r = 0.198 p = 0.187
CD4/CD8 (mean ± SD)	1.98 ± 0.55	2.00 ± 1.23	1.52 ± 1.22	0.365[a]	r = −0.183 p = 0.225
NK (%) (mean ± SD)	10.29 ± 5.03	14.37 ± 6.36	17.60 ± 12.05	0.067[a]	r = 0.343 p = 0.020
Classical monocyte (%) (mean ± SD)	86.72 ± 6.92	79.70 ± 8.22	79.45 ± 7.18	0.015[a]	r = −0.325 p = 0.011
Intermediate monocyte (%) (mean ± SD)	8.59 ± 6.92	13.65 ± 5.29	14.14 ± 7.69	0.050[a]	r = 0.325 p = 0.027
Nonclassical monocyte (%) (median, (min–max)}	1.40 (0.54–3.33)	4.52 (1.27–10.35)	3.31 (1.35–19.35)	0.000[b]	r = −0.521 p = 0.000
Nonclassical monocyte (%) (median, [min–max])	1.40 (0.54–3.33)	4.52 (1.27–10.35)	3.31 (1.35–19.35)	0.000[b]	r = −0.521 p = 0.000

[a]One-way analysis of variance.
[b]Kruskal–Wallis test.
[c]Brown-Forsythe.

The temperature, heart rate, SPO_2 parameters were significantly different between groups. Also, temperature, heart rate, and SPO_2 (p = 0.005, p = 0.001, p = 0.001, respectively) between control and severe groups, SPO_2 (p = 0.041) between control and mild groups had significant differences in subgroup analyses (Tables 1 and 5).

WBC, lymphocyte, platelets, Neutrophil/Lymphocyte ratio, D-dimer, CRP were significantly different between groups. In subgroup analyzes for hemogram, and biochemical parameters, there were significant differences at D-Dimer (p = 0.031) between mild and severe groups, at lymphocyte (count) (p = 0.000), CRP (p = 0.006), WBC (p = 0.006), platelet (p = 0.007) between control and mild groups, at CRP (p = 0.000), lymphocyte (count) (p = 0.000), platelet (p = 0.010), Neutrophil/Lymphocyte (p = 0.017) values between control and severe groups. CRP has a strong, the neutrophil/lymphocyte ratio has a moderate level of positive correlation according to the correlation analysis (Tables 2 and 5).

Lymphocyte (%), lymphocyte/monocyte, classical monocyte (%), nonclassical monocyte (%) were significantly different between groups. Also there were significant differences at lymphocyte (%) (p = 0.026) between mild and severe groups, at lymphocyte (%) (p = 0.026), classical monocyte (%) (p = 0.035), nonclassical monocyte (%) (p = 0.001) between control and mild groups, at lymphocyte (%) (p = 0.000), lymphocyte/monocyte (p = 0.019), classical monocyte (%) (p = 0.025), nonclassical monocyte (%) (p = 0.001) between control and severe groups, at lymphocyte (%) (p = 0.006) between mild and severe groups according to the subgroup analysis. NK cells (%) intermediate monocyte (%) has a weak level of positive correlation, lymphocyte (%) has a strong, lymphocyte/monocyte, $CD4^+$ (%), classical monocyte (%) have weak level of negative correlation according to the correlation analysis (Tables 3 and 5).

Significant differences were observed at IL-1β, TNF α, MCP-1, IL-6, IL-8, IL-10, IL-18, IL-23 between groups. Also, there were significant differences at IL-1 beta (p = 0.001), TNF-alpha (p = 0.025), IL-6 (p = 0.026), IL-8 (p = 0.048), IL-10 (p = 0.022), IL-18 (p = 0.033), IL-23 (p = 0.002) between mild and severe groups, at TNF-alpha (p = 0.012), IL-18 (p = 0.000) between control and mild groups, at IL-6 (p = 0.038), IL-8 (p = 0.006) between control and severe groups, at IL-1beta (p = 0.001), IL-10 (p = 0.044), IL-23 (p = 0.008) between mild and severe groups according to the subgroup analysis. Although MCP-1 was significantly different between groups, however, there were no significant differences at the subgroup analysis. IL-8 has

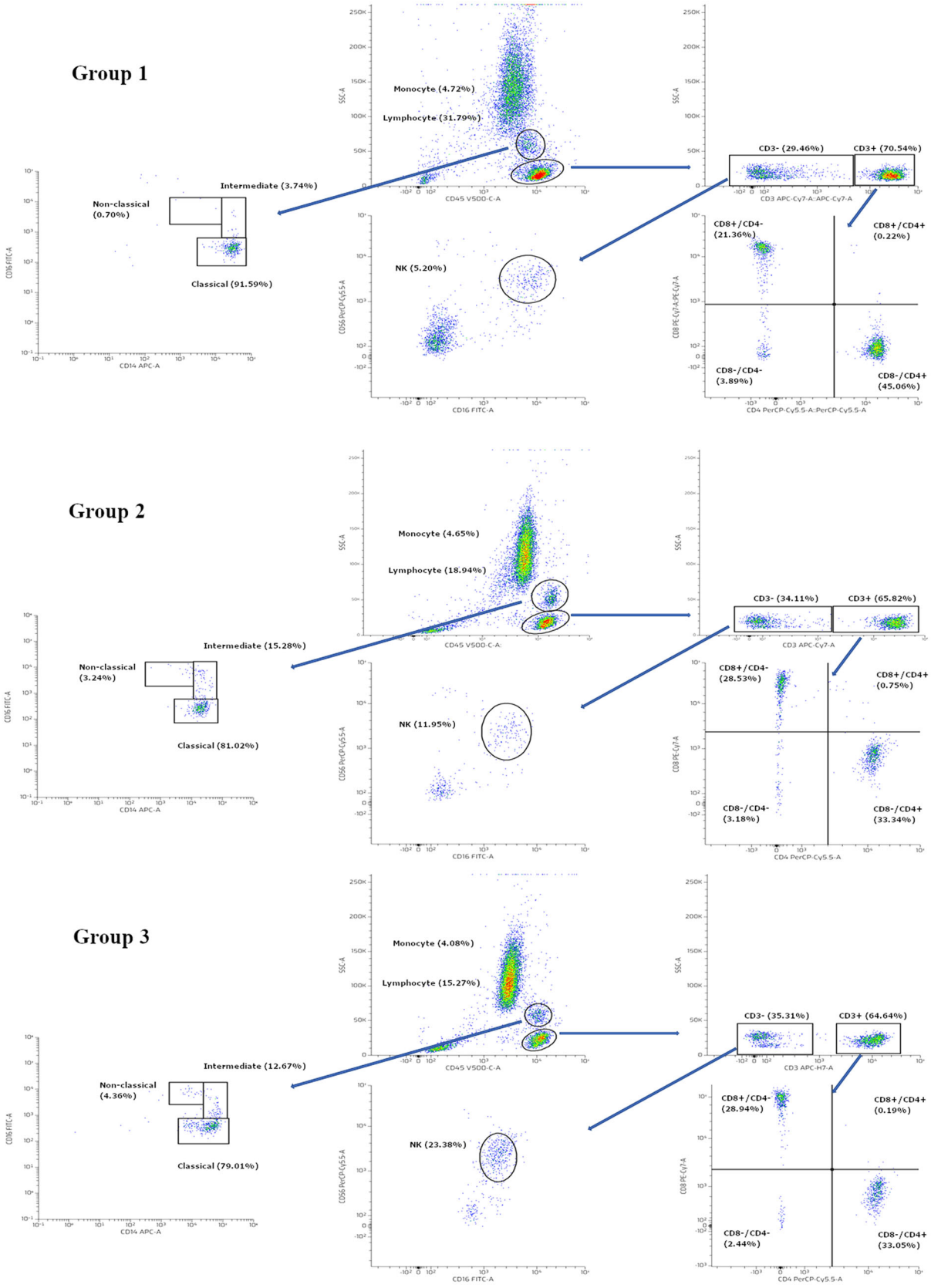

FIGURE 1 Gating strategies and flow cytometric analysis of lymphocytes and monocytes according to groups

TABLE 4 Comparison of serum cytokine levels of the groups

	Control (n = 15)	Mild (n = 15)	Severe (n = 16)	p-value	Corelation (r, p)
IL-1 beta (pg/ml) (median, [min–max])	4.61 (3.46–11.05)	3.46 (3.46–8.05)	7.45 (3.46–16.66)	0.002[a]	r = 0.201 p = 0.180
IFN-gama (pg/ml) (median, [min–max])	3.26 (3.26–7.41)	3.26 (3.26–5.73)	3.26 (3.26–12.51)	0.231[a]	r = 0.206 p = 0.169
IFN-alfa 2 (pg/ml) (median, [min–max])	2.91 (2.91–2.91)	2.91 (2.91–2.91)	2.91 (2.91–2.91)	1.000[a]	Not applicable
TNF-alfa (pg/ml) (median, [min–max])	6.72 (5.08–16.10)	5.08 (5.08–11.15)	5.35 (5.08–20.70)	0.012[a]	r = −0.104 p = 0.492
MCP-1 (pg/ml) (mean ± SD)	112.98 ± 58.25	64.22 ± 70.39	57.73 ± 61.40	0.041[b]	r = −0.340 p = 0.021
IL-6 (pg/ml) (median, [min–max])	8.34 (3.65–23.43)	5.81 (3.65–50.67)	18.13 (3.65–991.56)	0.018[a]	r = 0.377 p = 0.010
IL-8 (pg/ml) (median, [min–max])	10.99 (4.85–878.65)	16.21 (4.85–1114.51)	114.62 (13.81–1021.79)	0.008[a]	r = 0.461 p = 0.001
IL-10 (pg/ml) (median, [min–max])	4.11 (2.68–7.55)	3.48 (2.68–7.36)	6.15 (2.68–32.68)	0.046[a]	r = 0.239 p = 0.109
IL-12p70 (pg/ml) (median, [min–max])	3.99 (3.99–3.99)	3.99 (3.99–3.99)	3.99 (3.99–5.36)	0.147[a]	r = 0.255 p = 0.087
IL-17A (pg/ml) (median, [min–max])	4.15 (4.15–4.15)	4.15 (4.15–4.15)	4.15 (4.15–4.15)	1.000[a]	Not applicable
IL-18 (pg/ml) (median, [min–max])	96.00 (47.76–301.63)	37.17 (19.57–159.02)	72.61 (21.48–3805.52)	0.001[a]	r = −0.214 p = 0.153
IL-23 (pg/ml) (median, [min–max])	4.82 (4.82–11.70)	4.82 (4.82–9.34)	8.39 (4.82–24.06)	0.010[a]	r = 0.258 p = 0.083
IL-33 (pg/ml) (median, [min–max])	3.73 (3.73–3.73)	3.73 (3.73–3.73)	3.73 (3.73–6.63)	0.147[a]	r = 0.255 p = 0.087

[a]Kruskal–Wallis test.

[b]One-way analysis of variance.

moderate level of positive, MCP-1 has a weak level of negative correlation according to the correlation analysis (Tables 4 and 5).

There were significant differences at $CD3^+$ (count) (p = 0.000), $CD4^+$ (count) (p = 0.000), $CD8^+$ (count) (p = 0.004), between control and mild groups, at $CD3^+$ (count) (p = 0.000), $CD4^+$ (count) (p = 0.000), $CD8^+$ (count) (p = 0.005) between control and severe groups according to the subgroup analysis. Lymphocyte (count), $CD3^+$ (count), $CD4^+$ (count) has a strong level of negative correlation according to the correlation analysis (Figure 2, Table 5).

There was no mortality in Group 2 with mild disease, however, mortality was observed in 3 of 16 patients in Group 3, which had a more severe course.

4 | DISCUSSION

Covid-19 is a member of β-coronavirus like as MERS and SARS. Coronavirus infections could cause a cytokine storm due to sustained release of cytokines and chemokines, resulting in immune dysfunction and mortality.[12] Covid-19, similar to other cytopathic viruses, causes the release of certain molecular patterns that are recognized by extracellular toll-like receptors (TLRs) on alveolar macrophages and endothelial cells. These activate the proinflammatory cytokine transcription factors and interferon regulatory factors. In addition, intracellular proteins activate inflammasomes and convert pro-IL-1 to active IL-1.[13] Covid-19 cases are reported to present with tachypnea and tachycardia, fever, and a decrease in SPO_2 with increasing severity. It is also stressed that morbidity and mortality rates increase with age and smoking.[14] As reported in the literature, we also found tachycardia, low SPO_2, and high fever in Covid-19 patients, and although there was no significant difference, severe cases were older. Unlike most of the previous studies, we found higher smoking rates in cases of Covid-19, but were lower in severe patients compared to mild group.

Proinflammatory mediators such as IL-6, IL-10, MCP-1, IFN γ, TNF-α are significantly increased in the serum and correlated with disease severity and mortality in patients with Covid-19.[13,15] According to a study, researchers reported that IL-1β, IL-8, IL-10, IFN-ɣ, MCP-1, and TNF-α increased in Covid-19 cases according to a study.[15] Also, it was mentioned that especially IL-2 and IL-6 levels increased in the Covid-19 cases in another study, and there was a positive correlation with the severity of the disease.[16]

IL-6 is closely related to the severity of Covid-19, and it is stated in the literature that the threshold values of IL-6 are >55 pg/ml for severe disease and >80 pg/ml for mortality.[17] In addition to cytokine release, a decrease in lymphocyte and platelet counts and an increase in fibrinogen and acute phase reactants such as CRP and ferritin are also observed in Covid-19 patients.[18,19] Interleukin 10 which is an

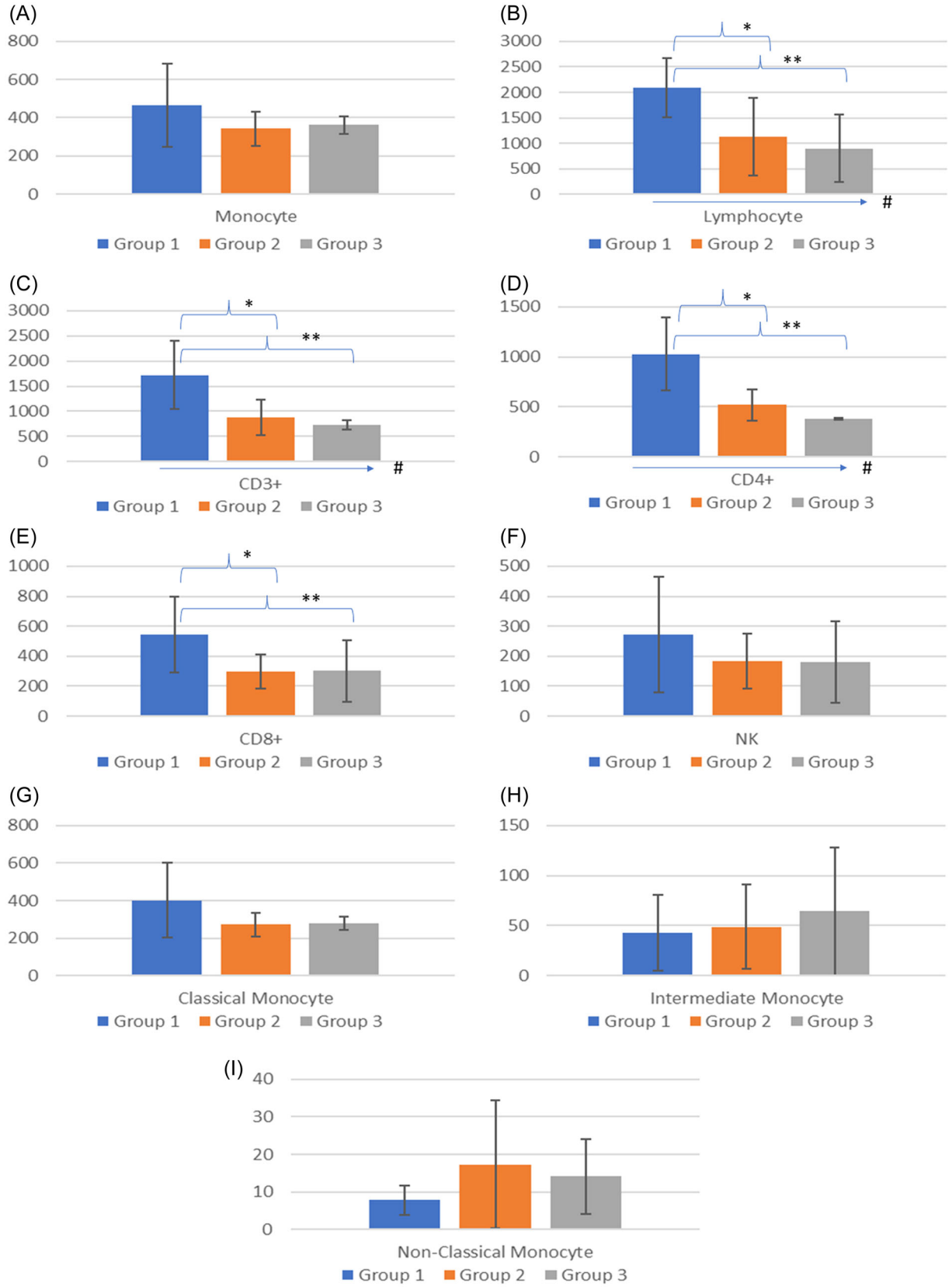

FIGURE 2 Cell counts of the lymphocyte and monocyte subtypes according to the groups. (A) Monocyte count (B) Lymphocyte count (C) $CD3^+$ lymphocyte count (D) $CD3^+CD4^+$ lymphocyte count (E) $CD3^+CD8^+$ lymphocyte count (F) NK cell count (G) Classical monocyte count (H) Intermediate monocyte count (I) Non-classical monocyte count. *$p < 0.05$ between control and mild groups. **$p < 0.05$ between control and severe groups (total lymphocytes have $r = -0.583$, $p = 0.000$, $CD3^+$ lymphocyte have $r = -0.625$, $p = 0.000$, $CD4^+$ cells have $r = -0.618$, $p = 0.000$ negative correlation results).

TABLE 5 The post hoc analysis results of the variables which are significant between groups

	Control versus mild groups (*p*-value)	Control versus severe groups (*p*-value)	Mild versus severe groups (*p*-value)
Temperature (°C)[a]	0.750	0.005	0.266
Heart rate/min[b]	0.246	0.001	0.077
SPO_2 (%)[c]	0.041	0.001	0.665
WBC[a]	0.006	0.729	0.440
Lymphocyte (count)[b]	0.000	0.000	0.654
Platelets[b]	0.007	0.010	0.979
Neutrophil/ Lymphocyte[c]	0.885	0.017	0.271
CRP[c]	0.006	0.000	0.820
Lymphocyte (%)[b]	0.026	0.000	0.006
Lymphocyte/ Monocyte[b]	0.312	0.019	0.392
Classical Monocyte (%)[b]	0.035	0.025	0.995
Nonclassical monocyte (%)[c]	0.001	0.001	1.000
IL-1 beta[c]	0.073	0.626	0.001
TNF-alfa[c]	0.012	1.000	0.098
MCP-1[b]	0.101	0.051	0.956
IL-6[c]	1.000	0.038	0.053
IL-8[c]	0.859	0.006	0.146
IL-10[c]	1.000	0.362	0.044
IL-18[c]	0.001	0.381	0.101
IL-23[c]	0.551	0.290	0.008
$CD3^+$ (count)[b]	0.000	0.000	0.694
$CD4^+$ (count)[b]	0.000	0.000	0.490
$CD8^+$ (count)[b]	0.004	0.005	0.996

[a]Tamhane.
[b]Tukey HSD.
[c]Dunn Z-test.

anti-inflammatory cytokine is also associated with the poor prognosis of Covid-19 patients.[20] This situation can be interpreted as a result of compensating for hyper inflammation, restoring homeostasis, and trying to reduce the tissue damage in severe Covid-19 cases. In a study evaluating Covid-19 disease severity and plasma cytokines in the literature, TNF-α, IL-1β, IL-6, IL-12, IL-23, IL-33 values were found to be statistically higher in individuals with severe disease. Also, it has been reported that IL-33 values are highly correlated with IL-23 and TNF-α in cases with severe Covid-19 disease.[21] In another study, no significant difference was observed in IL-1β, IL-1RA, IL-6, IL-8, IL-18, and TNF-α values between critically ill patients due to sepsis or ARDS and severe Covid-19 cases.[22]

In our study, we observed that CRP values increased significantly in Covid-19 cases and showed a positive correlation with the severity of the disease. Also, D-Dimer was significantly higher in the more severe group than mild Covid-19 patients but not ferritin and fibrinogen which is consistent with the literature. Interleukin 1β, TNF-α, IL-6, IL-8, IL-10, IL-18, and IL-23 values were found to be higher in severe Covid-19 patients according to our cytokine and chemokine values results. Also, severe group had higher IL-12 and IL-33 values but there was no differences between groups. When all of the groups are evaluated, it is observed that IL-6 and IL-8 increase with a positive correlation with the disease severity, while MCP-1 values show a negative correlation and decrease with the severity of Covid-19.

Lymphocytes and their subgroups are important in maintaining the functions of the immune system.[23] Cell surface markers such as CD3, CD4, CD8, CD19, CD16, and CD56 are used to distinguish cells in the immune system from each other. Therefore, the determination of lymphocyte subgroups in infectious diseases such as Covid-19 may help to provide important improvements in diagnosis and treatment by revealing the working mechanism of the immune system against these viruses.

Studies have reported that lymphopenia is associated with mortality, ARDS, and ICU need, and the lymphocyte counts significantly decreased in Covid-19 cases.[4] In addition, peripheral blood platelet counts are significantly reduced in Covid-19 patients. In the study of Lippi et al., it was reported that decreasing in thrombocytes is an associated poor prognosis.[19] We observed that lymphocyte counts decreased in both hemogram results and flow cytometric examination, and had a negative correlation with the severity of the Covid-19. Also, lower platelet levels were observed at Covid-19 patients.

It has been reported that the total lymphocyte count, $CD4^+$ T lymphocyte, $CD8^+$ T lymphocyte, and NK cell counts decreased significantly in Covid-19 patients and no difference was observed in the CD4/CD8 cell ratio according to a study about immunophenotyping of Covid-19. Compared to mild disease, a significant decrease was found in total lymphocyte, helper T lymphocyte, cytotoxic T lymphocyte counts in severe patients. There were not any significant differences in CD4/CD8 cell ratio and NK cells.[24]

Total lymphocyte, helper T lymphocyte, cytotoxic T lymphocyte counts decreased with negative correlation according to disease severity in our study. However, we did not observe any significant difference between groups in CD4/CD8 lymphocyte ratio, NK cell ratio, and NK cell counts, but NK cell ratios were increased and had a positive weak correlation with the severity of the disease. The decrease of NK cell counts in Covid-19 patients could be explained by the decrease of the total lymphocyte counts. The lymphocyte/monocyte ratios which negatively correlated with the Covid-19 severity also decreased in Covid-19 patients due to the decrease of the lymphocytes.

According to studies, there was a correlation between increased neutrophil/lymphocyte ratios and higher disease severity and worsened prognosis in Covid-19 cases.[2] Although there was a significant difference between the groups, the neutrophil/lymphocyte ratio increased in Covid-19 cases and was significantly correlated with disease severity.

There are very few studies in the literature examining the relationship between Covid-19 disease and monocyte subtypes. A study showed that the number of monocytes increased in mild diseases compared to healthy volunteers, and decreased with severity of disease. In addition, $CD16^-$ monocytes increased until severe Covid-19 disease, but decreased significantly in critical patients, and $CD16^+$ monocytes increased with the severity of the Covid-19.[25] However, we found no significant difference in the total monocyte ratios, which increased first in mild cases and decreased in cases of severe disease. Also, classical monocytes decreased and nonclassical and intermediate monocytes ratios increased in Covid-19 patients.

As the limitations of our study, the effects of the parameters could not be evaluated on mortality because the number of patients was low and the mortality rate was low in the patients. Also, the cytokines were measured with flow cytometry and the measuring range was limited to the minimum and maximum values.

In conclusion, lymphopenia which was negatively correlated with the severity of the disease was prominent in Covid-19 patients in our study and consistent with the literature. Neutrophil/Lymphocyte ratio, CRP, IL-8, NK (%), intermediate monocyte (%) which are positively correlated and lymphocyte (%), lymphocyte/monocyte ratio, classical monocyte (%), $CD4^+$ cell (%), lymphocyte (count), $CD3^+$ cell (count), $CD4^+$ cell (count) and MCP-1 levels which are negatively correlated with disease severity, seem to be valuable biomarkers for diagnosing and predicting Covid-19 severity. Among these results, CRP levels, lymphocyte (count), lymphocyte (%), $CD3^+$ and $CD4^+$ cell counts showed a strong correlation with disease severity. These results appear similar to those obtained in MERS-Cov and SARS-Cov infections, suggesting they might be useful for other similar viral pandemics in the future.

AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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