RESEARCH ARTICLE

# Correlation of lymphocyte subsets and inflammatory biomarkers with disease severity in COVID-19 patients

Khawar Abbas, Wajiha Musharraf, Mirza Naqi Zafar, Jawahar Lal, Sunil Dodani, Syed Adibul Hasan Rizvi

## Abstract

**Objective:** To determine the correlation of lymphocyte subsets and soluble serum inflammatory biomarkers with disease severity in coronavirus disease-2019 infection.

**Method:** The retrospective study was conducted at the Department of Immunology, Sindh Institute of Urology and Transplantation (SIUI), Karachi, Pakistan from September 1 to November 30, 2021, and comprised data of patients admitted from June to July 2021 who tested positive for coronavirus disease-2019 on the basis of reverse transcription-polymerase chain reaction of nasopharyngeal swab specimens. The patients were categorised into severe group A and non-severe group B. Initial investigations included complete blood count, neutrophil-to-lymphocytes ratio, C-reactive protein, D-Dimers and serum ferritin levels. Lymphocyte subsets included cluster of differentiation-3+, cluster of differentiation-4+/ cluster of differentiation-3+, cluster of differentiation-8+ T lymphocytes, cluster of differentiation-19+B lymphocytes, cluster of differentiation-16+ cluster of differentiation-56+ Natural Killer cells and serum cytokine levels of interleukin-2, interleukin-4, interleukin-6, interleukin-10, tumour necrosis factor-alpha and interferon gamma. They were correlated with disease severity. Data was analysed using SPSS 20.

**Results:** Of the 54 patients, 33(61.1%) were males and 21(38.9%) were females. There were 29(53.70%) patients in group A with median age 52 years (interquartile range: 43.5-65 years), and 25(46.29%) in group B with median age 50 years (interquartile range: 36.5-59 years) ($p$=0.241). Disease was significantly more severe in male patients compared to female ($p$=0.002). In group A, cluster of differentiation-3+ T cells were reduced in 21(72.4%) patients, cluster of differentiation-8+ T cells in 16(55.2%), cluster of differentiation-4+ T cells in 23(79.3%) and cluster of differentiation-19+ B cells in 8(27.6%). In group B, cluster of differentiation-3+ T cells were reduced in 10(40%) subjects, cluster of differentiation-8+ T cells in 7(28%), cluster of differentiation-4+ T cells in 12(48%) and cluster of differentiation-19+ B cells in 4(16%) patients. Serum cytokine levels were not significantly different between the groups ($p$>0.05). In group A, 7(24.13%) patients died, and in such cases, the neutrophil-to-lymphocytes ratio was significantly higher ($p$=0.037).

**Conclusion:** Pro-inflammatory markers and cytokine levels increased, while lymphocyte subsets decreased with increasing severity of the disease.

**Keywords:** COVID-19, C-reactive protein, Ferritin, Lymphocyte subsets, Interleukin-6. (JPMA 74: 78; 2023)

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## Introduction

The novel coronavirus disease-2019 (COVID-19) pandemic spread throughout the entire globe with considerable adverse effect on global public health.[1] Its clinical manifestations, severity and outcome differed from one individual to another. In the vast preponderance of cases (80%), it was mild. However, in 10-15% of cases, the disease was more severe, especially in elderly patients with pre-existing conditions like hypertension (HTN) and diabetes mellitus (DM).[2-4]

It has been reported that the coronaviruses, particularly Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) viruses, have a profound effect on immune system dysregulation.[5-7] As a result, T cell and B cell alterations may foretell the disease severity of coronavirus infections as well as the clinical outcomes. T lymphocytes play important role not only in cell mediated, but also in humoral immune response. T lymphocyte subsets include cluster of differentiation-CD3+T cells, CD4+ helper T cells and CD8+ cytotoxic T cells. B lymphocytes participate in the humoral arm of the acquired immune system, while Natural Killer (NK) cells are part the innate immune system, but they also participate in the process of antibody-dependent cell-mediated cytotoxicity (ADCC).[8]

A large number of biomarkers have been studied for an early diagnosis, prognostication and prediction of COVID-19 disease severity. A number of recent studies have reported that T lymphocyte subsets correlate with the severity of disease in COVID-19 patients. Similar alterations in lymphocyte subsets were found in patients with SARS and MERS disease outbreaks.[9] In addition, several soluble

[1-4]Sindh Institute of Urology, and Transplantation (SIUT), Karachi, Pakistan.
**Correspondence:** Khawar Abbas. e-mail: drkhawar_imuno@yahoo.com
ORCID ID. 0000-0003-4349-8083

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serum inflammatory biomarkers have also been investigated for their diagnostic, prognostic and predictive value. Inflammatory markers, like C-reactive protein (CRP), procalcitonin (PCT), serum ferritin, neutrophil-to-lymphocyte ratio (NLR) and interleukin-6 (IL-6), have been found to be significantly correlated with increased risk of the development of severe COVID-19 disease.[5-8] On the other hand, many other researchers did not find any significant correlations of these inflammatory markers with disease severity.[10,11]

The current study was planned to investigate changes in the lymphocyte count, lymphocyte subsets, as well as soluble serum inflammatory markers, and to correlate these parameters with disease severity in COVID-19 patients.

## Materials and Methods

The retrospective study was conducted at the Department of Immunology, Sindh Institute of Urology and Transplantation (SIUT), Karachi, Pakistan from September 1 to November 30, 2021, and comprised data of patients admitted to COVID-19 ward and intensive care unit (ICU) from June to July 2021 who tested positive for COVID-2019. Approval was obtained from the institutional ethics review committee (SIUT-ERC-2022/A-302 dated 31 October, 2022), but because of the retrospective nature of the study, informed patient consent and sample size calculation were not considered. SARS-coronavirus 2 (SARS-CoV-2) infection was established by positive reverse transcription-polymerase chain reaction (RT-PCR) of nasopharyngeal swab specimens using relevant kits (Maccura Biotechnology Company Ltd., China) in accordance with the manufacturer's instructions. Children, pregnant women and patients with end-stage renal disease (ESRD) on haemodialysis and kidney transplant recipients were excluded. The data of those included was categorised into severe group A and non-severe group B.

On admission, non-severe disease was defined as patients presenting with cough and fever, but no sign of pneumonia radiologically. Severe disease was defined as patients presenting with fever and cough along with radiological features of pneumonia.

All patients had undergone routine laboratory tests, complete blood count (CBC) with NLR, CRP, D-Dimers and serum ferritin. NLR was calculated by dividing absolute neutrophil count by lymphocyte count (normal range: 1-3). Lymphocyte subset analysis was done using a fluorescence-activated cell sorting (FACS) flow cytometer (FACSCanto, BD Biosciences, United States). Briefly, CD3+CD4+/ CD3+CD8+ T cells, CD19+ B cells, and CD16+CD56+ NK cells were measured as per the manufacturer's instructions. CRP was measured by particle-enhanced immunoturbidimetric assay (normal: <0.5mg/dl). D-Dimers were also measured by particle-enhanced immunoturbidimetric assay (normal: <0.5mg/L). Serum ferritin was measured by paramagnetic particle, chemiluminescent immunoassay technique (normal; males 21.8-274 ng/ml for males and 4.63-204 ng/ml for females). Serum levels cytokines IL-2, IL-4, IL-6, IL-10, tumour necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) were measured with a Cytometric Bead Array (CBA) kit on FACS flow cytometer (FACSCanto, BD Biosciences, US) as per the manufacturer's instructions.

Data was analysed using SPSS 20. Frequencies and percentages were calculated for qualitative variables, while mean±standard deviation and median with interquartile range (IQR), were used, as appropriate, for quantitative variables. Kolmogorov-Smirnov test was used for assessing data normality. Continuous variables were compared between the groups by using student's t-test or Mann-Whitney U test. Bivariate analysis for categorical variables was performed using chi-square test. P<0.05 was taken as statistically significant.

## Results

Of the 54 patients., 33(61.1%) were males and 21(38.9%) were females. There were 29(53.70%) patients in group A with median age 52 years (IQR: 43.5-65 years), and 25(46.29%) in group B with median age 50 years (IQR: 36.5-59 years) ($p$=0.241). Disease was significantly more severe in male patients compared to female ($p$=0.002).

On the day of admission, which was on average 5.3±1.5 days after the onset of disease, all 29(100%) patients in group A had fever compared to 12(48%) in group B ($p$<0.001). In group A, all 29(100%) patients presented with chest imaging abnormalities with multiple bilateral patchy opacities compared to none in group B. DM was present in 8(27.6%) patients in group A compared to 6(24%) in group B ($p$=0.9). HTN was found in 14(48.3%) patients in group A versus 9(36%) in group B ($p$=0.4). Chronic obstructive pulmonary disease (COPD) was present in 2(6.9%) patients in group A compared to 2(8%) in group B ($p$=0.9). Most patients presented with cough (79.3% in group A vs. 60% in group B), sore throat (25.15% in group A vs. 15% in group B), diarrhoea (3.4% in group A vs. 16% in group B) and myalgia (41.4% in group A vs. 24% in group B) in both groups.

With respect to haematological parameters and inflammatory markers, IL2, IL4, IL6, IL10, TNF-α and IFN-γ levels were not significantly different between the groups ($p$>0.05). NLR and lymphocyte counts showed significant differences ($p$<0.05). Total leukocyte count (TLC) was

slightly lower in group A in comparison with group B ($p$>0.05). However, lymphocyte count showed comparatively greater reduction in group A compared to group B ($p$<0.001). This resulted in a rise in NLR in group B in comparison with group B ($p$<0.001) (Table 1).

Lymphocyte subsets were analysed in all 54 patients at the time of presentation (Table 2). In group A, CD3+ T cells were reduced in 21(72.4%) patients, CD8+ T cells in 16(55.2%), CD4+ T cells in 23(79.3%) and CD19+ B cells in 8(27.6%). In group B, CD3+ T cells were reduced in 10(40%) subjects, CD8+ T cells in 7(28%), CD4+ T cells in 12(48%) and CD19+ B cells in 4(16%) patients. CD16+CD56+NK cells were reduced in 6(20%) in group A and in 10(40%) in group B ($p$<0.05).

In group A, Overall, there were 7(24.13%) deaths in group A and none in group B. The mortality was significantly more in male patients ($p$=0.001), and NLR was significantly increased in such patients ($p$=0.037).

**Table-1:** Comparison of blood counts, inflammatory markers in non-severe and severe group of COVID-19 patients.

| Baseline variables | Normal ranges | All patients (n=54) | Non-severe group of COVID-19 patients (n=25) | Severe group of COVID-19 patients (n=29) | p-value |
|---|---|---|---|---|---|
| Leukocytes x $10^9$/L) | 4-11 | 5.58 (5.16- 12.47) | 8.83 (5.16-12.77) | 7.23 (4.93-11.49) | 0.686 |
| Neutrophil x$10^9$/L) | 2.0-7.0 | 6.06 (3.28-9.58) | 6.48 (2.51- 9.51) | 5.11 (4.01-10.13) | 0.300 |
| Lymphocytes x$10^9$/L) | 1.0-3.0 | 11.75 (5.38-17.87) | 17.43 (13.46- 22.29) | 7.20 (4.55-12.05) | <0.001 |
| NLR | 1-3 | 6 (3.30- 10.65) | 3.85 (1.33-6.38) | 8.10 (3.80-16.90) | <0.001 |
| C-Reactive Protein (mg/dl) | <0.5 | 4.55 (1.42- 10.30) | 2.35 (0.65-8.63) | 4.70 (2.08-10.90) | 0.198 |
| D-Dimer (mg/l) | <0.5 | 0.72 (0.38- 2.36) | 0.66 (0.36-2.83) | 0.77 (0.44-1.27) | 0.960 |
| S. Ferritin (ng/dl) | 4.63-274 | 737.20 (454.92- 157.57) | 641.06 (409.05-1180.15) | 813.75 (454.92-1776.00) | 0.499 |
| **CYTOKINES** | | | | | |
| IL-2 (pg/mL) | 0-4.1 | 1.91 (1.50- 2.10) | 2.01 (1.58-2.76) | 1.56 (1.42- 1.93) | 0.082 |
| IL-4 (pg/mL) | 0-3.2 | 2.43 (2.01- 3.31) | 2.86 (2.09-4.72) | 2.32 (1.83- 3.21) | 0.113 |
| IL-6 (pg/mL) | 0-2.9 | 14.87 (5.62- 51.75) 90.7% | 10.83 (5.21-26.61) | 21.35 (5.59-62.14) | 0.271 |
| IL-10 (pg/mL) | 0-5.0 | 3.47 (2.29- 5.27) | 3.28 (2.26-5.33) | 3.60 (2.58-5.03) | 0.617 |
| TNF-α (pg/mL) | 0-23 | 2.31 (1.48- 2.84) | 2.42 (1.87-3.45) | 1.77 (1.17- 2.73) | 0.077 |
| IFN-y (pg/mL) | 0-18 | 2.81 (1.84- 3.42) | 2.77 (1.77-3.37) | 2.85 (1.94- 3.77) | 0.910 |

COVID-19: Coronavirus disease-2019, IL: Interleukin, TNF-α: Tumour necrosis factor-alpha, IFN-y: Interferon gamma. All values are in median±interquartile range.

**Table-2:** Lymphocyte subset comparison between severe and non-severe groups of COVID-19 patients.

| Lymphocyte subsets | Normal ranges | All patients (n=54) | Non-severe group of COVID-19 patients (n=25) | Severe group of COVID-19 patient (n=29) | *p*-value |
|---|---|---|---|---|---|
| CD3+ T Cells/ul | 690-2540 | 548 (311.0-1217.75) | 933.0 (546-1748.50) | 341 (204.0- 707.0) | <0.001 |
| CD3+ T Cells % | 55-84 | 57.4 | 40 | 72.4 | <0.011 |
| CD#+CD4+ T Cells/ul | 410-1590 | 350.50 (141.50- 547.25) | 420 (332.50-989.0) | 175 (119-391.50) | <0.001 |
| CD4+ T Cells % | 31-60 | 64.8 | 48 | 79.3 | <0.023 |
| CD3+CD8+ T Cells/ul | 190-1140 | 302.50 (120.50- 529.25) | 507.0 (177.50-639.50) | 174 (73.50-323.50) | <0.001 |
| CD3+CD8+ T Cells % | 13-41 | 42.6 | 28 | 55.2 | <0.013 |
| CD19+ B Cells/ul | 90-660 | 241 (100.75- 352.75) | 340.0 (178.0- 498.50) | 187 (89 -292) | <0.001 |
| CD19+ B Cells% | 6-25 | 22.2 | 16 | 27.6 | 0.195 |
| CD16+56+(NK Cells/ul) | 90-590 | 126.50 (60.50- 221) | 157 (102.0- 308.50) | 116 (39- 189) | <0.030 |
| CD16+56+(NK Cells) % | 5-27 | 31.5 | 20 | 41.4 | 0.028 |

COVID-19: Coronavirus disease-2019, CD: Cluster of differentiation. All values are in median±interquartile range.

## Discussion

The current study investigated the clinical characteristics and immunological parameters in systemic circulation in patients with COVID-19. The median age in the study was 50 years and severe disease was seen in the slightly elderly age group having median age 52 years. However, the age difference was not significantly different. Similar age pattern was observed in other studies.[12,13] The age-associated variation in the severity of COVID-19 disease may partly be explained by diminished cellular and humoral immune functions.[14]

Mean duration of symptoms was 5.3 days, which was in line with Rizvi et al.[4] The epidemiological investigations reported across different global regions showed greater morbidity and mortality in males compared to females.[15-18] In the current study, the frequency of COVID-19 disease was also higher among male patients (61.1%) compared to females (38.9%). The lower susceptibility of females to COVID-19 could be explained by the protection from the X chromosome, which plays a vital role in both innate and acquired immune responses.[19] The severity of disease was also more in males than females in the current study ($p$<0.002). Also, about 75% patients had at least one comorbid condition. Higher prevalence of comorbidities was found in those with severe disease compared to the non-severe group. These findings are consistent with previous studies[17,20] which established that COVID-19 was more likely to affect those with underlying diseases that cause weak immunity.

COVID-19 patients suffer from a wide range of symptoms that may range from mild to severe.[21] Fever is one of the most frequent

COVID-19 symptoms, but the patients do not always exhibit fever, and some patients do not develop any symptom. In the current study, fever was found in all patients in the severe group, while it was found in 48% of the non-severe group ($p$<.001). A meta-analysis found that fever was the most frequent symptom (85.6%) in COVID-19 cases.[22] In another meta-analysis, the frequency of fever in critical or severe COVID-19 patients was 91.69%.[23]

Regarding inflammatory markers, serum ferritin, CRP and D-dimer concentrations were higher in the severe group. However, this was not statistically significant. As in the current study, Chen et al. did not find any significant difference in severe and non-severe groups with respect to inflammatory markers.[24] However, other investigators did find high CRP in the severe group that correlated with the severity of COVID-19.[25-28]

NLR in the current study was significantly higher in patients with severe disease ($p$<0.001), which is similar to the findings of Wang et al.[29] Chan et al.[30] established that NLR was a prognostic marker to differentiate severe from non-severe group. Liu et al.[31] reported that NLR was the most useful prognostic parameter predicting the outcome for severe COVID-19.

All the soluble cytokines showed a variable pattern and no significant differences were found between the groups. However, IL-6 concentrations were greater in the severe group compared. Gupta et al. explored type 1 T helper (Th1)/Th2/Th17 cytokine pattern during different stages of COVID-19 infection and found dynamic expression of the markers. Only IL-6 showed a significant difference.[10] In particular, increased severity of the disease process was not associated with increased cytokine concentrations except for cytokine IL-6. Several other studies showed the association of raised IL-6 with disease severity and poor outcomes.[32-37]

Lymphocyte subtypes play a critical role in the body's humoral and cellular immune-responses against foreign agents. Each cell type controls and regulates the other cell type. The present study demonstrated pronounced lymphopenia as well as reduction of lymphocyte subsets, particularly in the severe disease group. The study showed that CD3+ T cells were reduced in 40% of the non-severe patients compared to 72.4% severe ones ($p$=0.011). CD8+T cells showed reduction rate of 28% compared to 55.2% respectively ($p$=0.013). CD4+ T cells also demonstrated reduction rate of 48% in non-severe patients compared to 79.3% in the severe ones ($p$=0.023). Wang et al.[32] found decrease of CD4+ T cells in 52.9% in non-severe group compared to 95.24% in the severe group. Similarly, decrease of CD8+ T cells was reported in 28.4% in the non-severe group and 61.9% in the severe group. Similar pattern of lymphocyte reduction was also found by He et al. in SARS infection, suggesting that the immune system reacts in a similar manner when countering homologous coronavirus infections.[33] In the study on SARS[34] the frequency of lymphopenia was seen in 84% patients, CD4+ T cells declined in 100% patients, CD8+ T cells declined in 87%, B cells decreased in 76% and NK cells decreased in 55% patients.

The mortality of SARS COVID has been reported as more than 10% and MERS COVID as >35%.[35] The reported mortality rate from COVID-19 has ranged from 1.4% to 28%. 4 In the current study, 12.96% patients expired and all these patients belonged to the severe group and predominantly males ($p$<0.001). Several epidemiological investigations found differences in the rate of progression and disease outcome among different genders. In comparison to women, men were more likely to suffer from higher mortality.[19,36-38]

The current study has limitations. The retrospective design of the study may limit the strength and reliability of the findings. Besides, the cross-sectional study was done at a single centre with inevitable selection bias. All parameters were tested only once at the time of initial presentation and no serial estimations were performed. Large-scale longitudinal studies are needed to validate the current findings.

## Conclusion

Pro-inflammatory markers CRP, NLR and cytokine levels, particularly that of 1L-6, increased with increasing severity of the disease. Conversely, all lymphocyte subsets decreased in severe disease.

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**Conflict of Interest:** None.

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**Author Contribution:**

KA: Concept, design, acquisition, analysis and interpretation of data, primary drafting, final approval.
WM: Concept, design, acquisition of data, primary drafting, final approval.
MNZ: Concept, design, critical review, final drafting, final approval.
JL: Acquisition, critical review, final approval.
SD: Acquisition, critical review, final approval.
AUHR: Final approval