Original Article

Evaluation Of Lymphocyte Subsets In Hospitalized Covid-19 Patients In Pune

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Abstract

Background: The protective role of CD4+ and CD8+ T cells was hypothesized based on research on SARS and MERS patients, and recent data confirms their participation in SARS-CoV-2 infection. Lymphopenia, like other coronaviruses and viral infections, is a characteristic indication in people with advanced COVID-19 disease. The study was performed on hospitalized COVID-19 patients a tertiary care center Pune, (India), to analyze peripheral white blood cell changes in lymphocytes.

Methodology: The PBMCs were isolated from enrolled study participants and flow cytometry analysis was done to assess changes in peripheral white blood cells. The lymphocyte subsets including CD4+T cells, CD8+T cells, B cells, NK cytotoxic cells and NK regulatory cells were evaluated in COVID-19 patients with different disease category and compared to healthy controls.

Results: The immunophenotypic characterization of peripheral blood cell subset was done for 50 COVID-19 patients and 14 healthy controls. These data were evaluated based on the disease severity. A total of 50 COVID-19 patients were classified as mild (n = 18), moderate (n = 17), or severe (n = 15) cases. In patients with severe SARS-Cov-2 infection, lymphocyte counts, CD8+ T cell counts were significantly reduced while an increased ratio of CD4+/CD8+ T cells, cytotoxic NK cells and B cells was observed.

Conclusion: Our study showed that, SARS-CoV-2 infection was associated with changes in peripheral lymphocyte expression of certain subtypes. This observation can be used to predict better clinical outcomes for patients with aggressive treatment protocols for severe patients.

Keywords: COVID-19, CD4, T cells

INTRODUCTION

The protective role of CD4+ and CD8+ T cells was hypothesized based on research on SARS and MERS patients, and recent data confirms their participation in SARS-CoV-2 infection (1,2,3). Lymphocytes, particularly T cells,

play an important role in the cellular immune response by directly killing virus-infected cells via cytotoxic T lymphocyte CD8+ T cells and CD4+ T cell-mediated CD8+ T cell priming, which involves inducing B-cell differentiation in plasma cells to produce virus-specific antibodies (4). T and B cell responses to SARS-CoV-2 infection were detected in the blood around a week after symptoms began (5).

Several studies suggest that in the early stages of COVID-19, when individuals have no specific symptoms, white blood cell (WBC) counts and peripheral blood lymphocyte counts are either normal or slightly diminished. These symptoms, however, may vary as the condition progresses. Lymphopenia, like other coronaviruses and viral infections, is a characteristic indication in people with advanced COVID-19 disease.

In COVID-19 patients, the total number of lymphocytes, CD4+ T cells, CD8+ T cells, B cells, and natural killer cells (NK cells) decreases, with the loss in CD8+ T cells being the most significant. Lymphocytopenia is a reliable indicator of early SARS-CoV-2 infection and aids in contact tracing and disease progression during COVID-19 pneumonia. The majority of hospitalized patients have significant lymphopenia, which aids in the early detection of COVID-19 in critically ill patients.

These lymphocyte subtypes are required for the immune system to function properly. Similarly, viral infections, immunological disorders and different infectious diseases can cause dysregulation in lymphocyte subtype levels [7].

Unlike in SARS, Various studies, mainly from China, showed that CD8+ T cells were often an independent indicator of COVID-19 severity. CD8+T would be a strong indicator of clinical outcome in COVID-19 patients than CD4+T. on the other hand, both CD4+T and CD8+T cell counts may influence COVID-19 severity [8]. CD4+ & CD8+ T cell counts differed significantly among individuals with moderate & severe cases of COVID-19. Recent research has indicated that the total number of B cells and NK cells remains stable [9-11].

Therefore, the study was performed on COVID-19 patients admitted to a hospital in a tertiary care center Pune, (India), to analyze peripheral white blood cell changes in lymphocytes, which are important to examine the confounding variables, it is critical to elucidate the features of COVID-19 lymphocyte subsets, which may provide unique insights into the immunological process.

METHODOLOGY

This was a observational study carried out from July 2020 to December 2021. A total of 49 patients with confirmed SARS COV-2 infection admitted at Dr. D. Y Patil Medical College, Hospital and Research Centre, Pune was enrolled in the study. 14 healthy individuals were recruited as uninfected controls for immunophenotype comparison. All uninfected controls had no history of SARS-CoV-2 infection during the previous six months. All participants provided written informed consent at the time of enrolment in the study. The study was approved by the Institutional Ethics Committee of Dr D Y Patil Vidyapeeth (Ref No-)

Patients were classified as mild/moderate/severe according to based on National Clinical Management Protocol COVID-19, Revised version 3, dated June 13, 2020, by the Ministry of Health and Family Welfare, Government of India. Patients with SpO2 ≤ 90% on room air and/or respiratory rate ≥ 30/min. and supplementary oxygen were included in the severe disease group, patients with SpO2- 91 to 94% on room air and/or respiratory rate 24 to 29/min were included in the moderate disease group and patients with SpO2 > 94% on room air and/or respiratory rate up to 24/min were included in the mild disease group.

PBMC Isolation

EDTA anticoagulated peripheral blood (5 mL) was collected from each study participants. All samples were tested within 6 hours of blood collection. The peripheral blood mononuclear cells (PBMCs) and plasma were separated by density gradient centrifugation according to standard protocols and the plasma was at -80^{0}Cstored till further use.

Flow cytometry for Immunophenotyping

The PBMCs were incubated for 30 min at room temperature in Stain Buffer (BD Biosciences, San Jose, CA) with optimal concentrations of the fluorochrome conjugated antibodies. The monoclonal antibodies used for staining were as follows CD3 APCH7, CD4 BV480, CD8 FITC, CD45 BV421, CD56 BV480, CD19 APC and CD16 FITC (BD Biosciences, San Jose, CA).. The cells were further washed with stain buffer, fixed with 1%

Formaldehyde in PBS and acquired to obtain 100000 gated lymphocyte events on FACS Jazz (BD Biosciences, San Jose, CA). The data was analysed using FACS Flow Jo software V10.7 (BD Biosciences, San Jose, CA).
The predominant T-cell subsets identified were CD4+T cells and CD8+ T cells. Lymphocytes were identified on the basis of forward and side scatter and CD45+ cells were gated as it is a leukocytes marker. Further on CD45+ cells, CD3+ve cells were gated on basis of presence of the CD3 marker. Subsequently the CD3+CD4+T cells and the CD3+CD8+ T cells were identified.
For assessing the NK cells and B cell population, Lymphocytes were identified on the basis of forward and side scatter and CD3-ve cells were gated on basis of the absence of marker. After gating on CD3-ve cells, CD16 vs. CD56, NK cytotoxic cells (CD16+CD56+) cells and NK regulatory (CD16-CD56+) cells were identified. Additionally, on CD3-ve cells, CD19+ cells were gated as B cells

Statistical analysis

SPSS (Statistical Package for Social Sciences) version 26.0, IBM, USA, was used to analyze the data. Total counts (frequency), percentages, means, and standard deviations were generated as part of descriptive statistics for patient demographics. For continuous variables, an independent sample t-test/Mann Whitney U-test was employed, and for categorical connections, a Chi-squared or Fisher exact test was utilized. A p-value of less than 0.05 was considered statistically significant.

RESULTS

During the period from July 2020 and December 2021, 50 COVID-19 patients were included in the study with a mean (SD) age of 50.2(15.8) years and including 56% males while 44% were females. The demographic and Immunophenotyping characterization of enrolled COVID-19 participants were detailed in Table 1.
The PBMCs isolated from the COVID-19 infected individuals (Mild-18, Moderate-17, Severe-15) and healthy controls (n=14) were labelled with fluorescent labeled monoclonal antibodies. The frequencies of lymphocytes, T cell subsets and NK cells subsets were calculated.

Table 1 –Immunophenotypic characterization of T cell subtypes in COVID-19 patients.

	Normal (n=14)	**Mild (n=18)**	**Moderate (n=17)**	**Severe (n=15)**
Age (years)	42.4 +18.6	44.7 + 15.1	50.8+18.0	53.6+ 13.2
TLC cells/ul	6621+2546	8769+1951	4757+1038	5164+2036
% Lymphocytes	34.9+8.1	25.5+7.4	19.6+3.8	12.2+3.6
Absolute Lymphocytes	2224.8+885.6	2002.8+816.6	939.1+279.3	671.5+440.4
%CD4 (Mean ± SD)	35.4643 ± 7.0388	34.3735 ± 13.1644	34.8294 ± 10.5372	29.07 ± 13.8429
%CD8 (Mean ± SD)	29.9714 ± 10.2508	19.1435 ± 12.7449	19.7065 ± 9.7785	17.7307 ± 10.2889
CD4/CD8 (Mean ± SD)	1.3071 ± 0.4314	2.7148 ± 2.4108	2.2668 ± 1.3934	2.3284 ± 1.9411
% B Cells (Mean ± SD)	17.0979 ± 6.7076	41.6776 ± 27.8223	26.8419 ± 27.5243	32.7132 ± 29.3564

% NK Reg Cells (Mean ± SD)	14.4914 ± 9.5840	17.8694 ± 13.2583	19.8123 ± 22.5997	16.4133 ± 14.4506
% NK cytotoxic cells (Mean ± SD)	7.2736 ± 5.0338	13.0023 ± 9.094	14.2871 ± 17.5322	15.3153 ± 16.4344

The total number of white blood cells ($P < 0.05$), the frequency of lymphocytes ($P < 0.001$) and the absolute number of lymphocytes ($P < 0.001$) in patients with mild, moderate and severe covid-19 compared to healthy people decreased significantly (Figure 1).

Interestingly, comparable proportions of CD4+ T cells were observed in different categories (mild, moderate and severe) of COVID-19 patients compared to healthy subjects (Figure 2A). However, the frequency of CD8+ T cells in COVID-19 patients was significantly decreased in mild, moderate, and severe COVID-19 patients compared to healthy subjects, whereas frequencies of CD8+T cells among different disease categories of COVID-19 patients (Mild, Moderate and Severe) remain unchanged (Fig 2B). We also calculated the CD4/CD8 ratio and found that it was significantly higher in mild, moderate, and severely ill COVID-19 patients ($P<0.05$) when compared to healthy controls (Fig 2C).

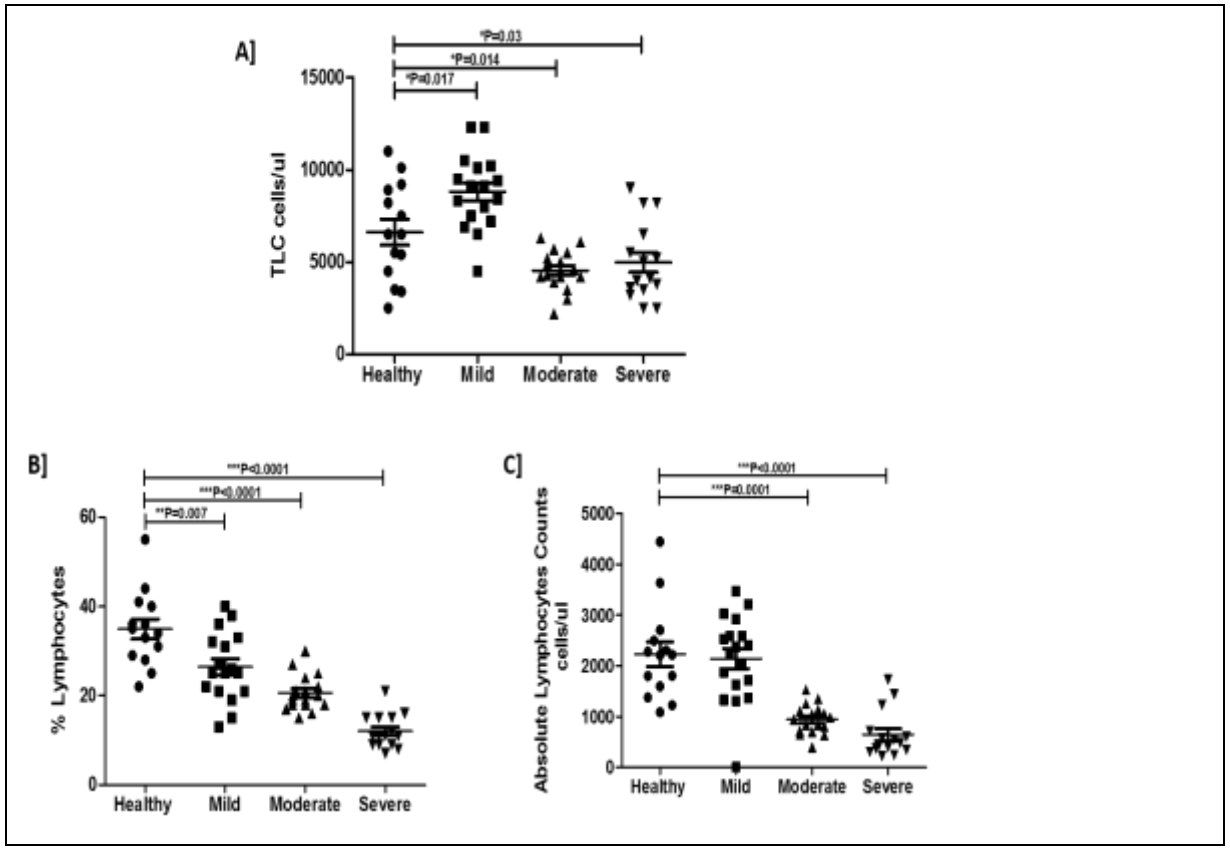

Figure 1[A] The scatter dot plot shows the total leukocyte count in healthy controls and COVID-19 patients with mild, moderate, severe disease. [B] The scatter dot plot shows the percentages of lymphocytes in healthy controls and COVID-19 patients with mild, moderate, severe disease. [C] The scatter dot plot shows the absolute lymphocyte count in healthy controls and COVID-19 patients with mild, moderate, severe disease

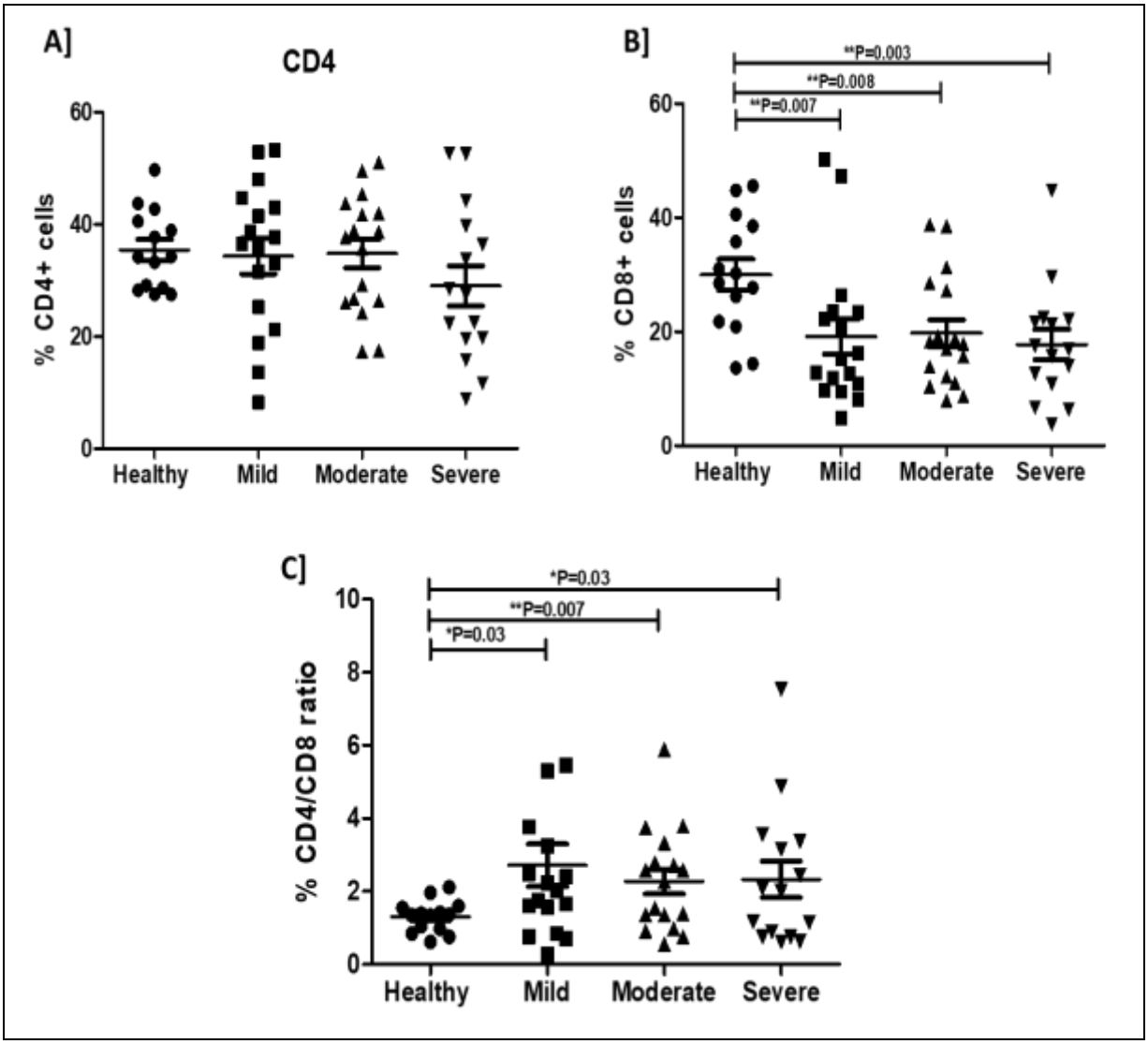

Figure 2 [A] The scatter dot plot shows the percentages of CD4+ T cells in healthy controls and COVID-19 patients with mild, moderate, severe disease. [B] The scatter dot plot shows the percentages of CD8+ T cells in healthy controls and COVID-19 patients with mild, moderate, severe disease. [C] The scatter dot plot shows the CD4/CD8 ration in healthy controls and COVID-19 patients with mild, moderate, severe disease

Further the B cells were identified as CD3-CD19+ cells. There is significant increase in frequencies of B cells in mild and severely ill COVID-19 patients ($P<0.05$) as compared to healthy controls. While moderately ill COVID-19 patients have comparable frequencies of B cells as compared to healthy controls (Fig 3A).

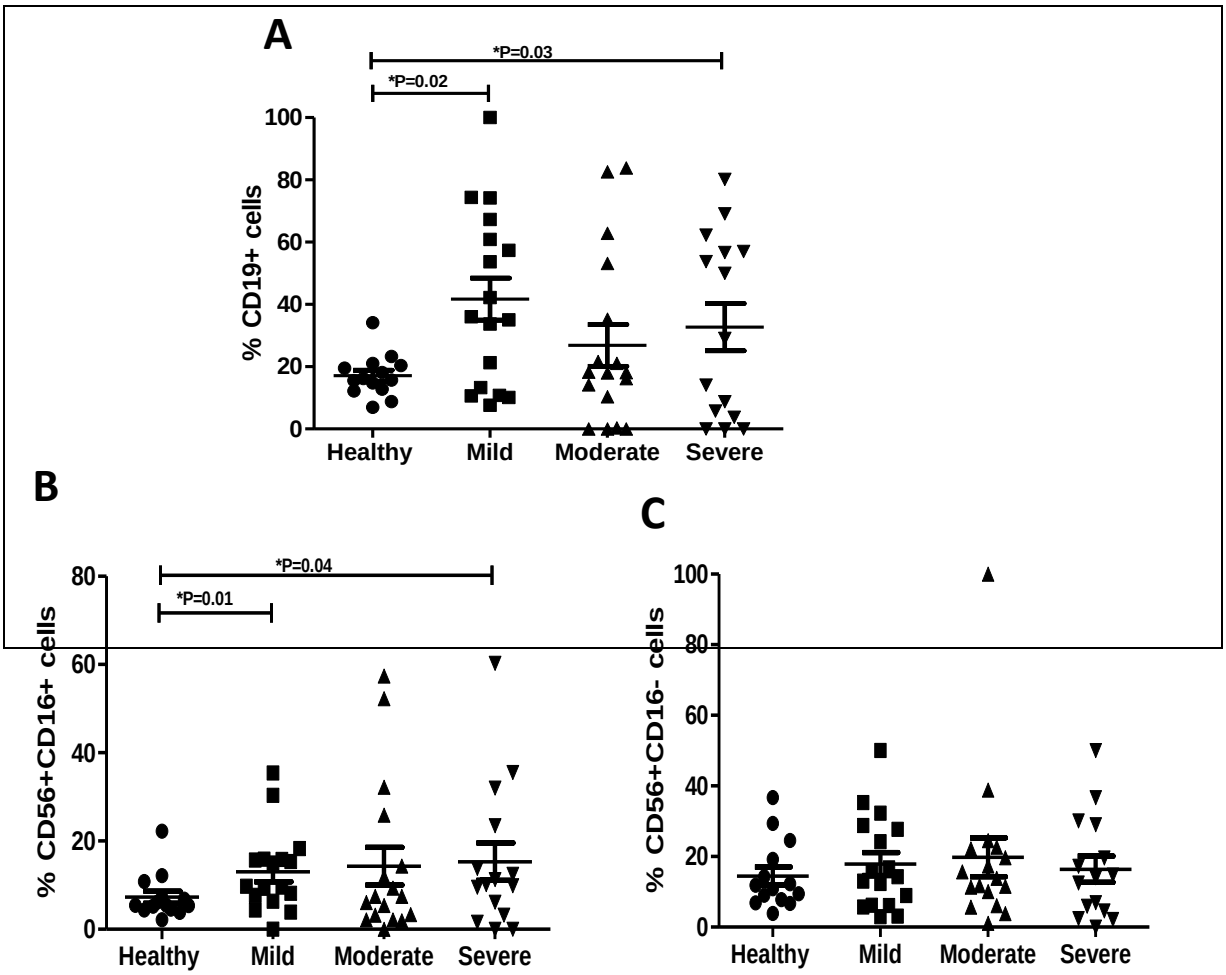

Figure 3 [A] The scatter dot plot shows the percentages of CD19+ B cells in healthy controls and COVID-19 patients with mild, moderate, severe disease. [B] The scatter dot plot shows the percentages of CD56+CD16+ NK Cytotoxic cells in healthy controls and COVID-19 patients with mild, moderate, severe disease. [C] The scatter dot plot shows the percentages of CD56+CD16- NK Regulatory cells in healthy controls and COVID-19 patients with mild, moderate, severe disease.

Additionally, we assessed the subsets of NK cells, which included NK cytotoxic (CD56+CD16+) and NK regulatory cells (CD56+CD16-) in the COVID-19 patients. We observed a significant increase in the frequencies of the NK cytotoxic cells in mild and severely ill COVID-19 patients as compared to healthy controls ($P<0.05$). Furthermore, the frequencies of NK cytotoxic cells in moderate COVID-19 patients remained unchanged. (Fig 3B). However, the frequencies of NK regulatory cells in healthy controls and COVID-19 patients were remain unchanged (Fig 3C).

DISCUSSION

This study examined the distribution of lymphocytes and their subsets in peripheral blood cells from patients with COVID-19 using flow cytometry analysis. Our results show that people with severe COVID-19 have reduced total WBC count, percentage lymphocytes, and absolute lymphocyte count, CD4+ T cells, CD8+ T cells, and CD4/CD8 ratio compared to healthy controls.

Variations in percentages of CD4+ T cells, percentages of CD8+ T cells, CD4+/CD8+ ratio, CD19+ B cells, and CD56+CD16 (regulatory) and CD56+ CD16+ (cytotoxic) NK cells in SARS- CoV-19 patients have been linked to disease severity.

The study shows a statistically significant reduction in lymphocyte count percentage with disease severity in the COVID-19 patient categories compared to controls. A significant cluster of cases was observed in moderate and severe patient categories within 1SD. This observation can be used to predict better clinical outcomes for patients with aggressive treatment protocols for severe patients. A recent study by Huang, Ian et al. and another study by Tan, L et al demonstrated Lymphopenia as a strong predictor of severity and hospitalization in COVID-19 patients [12,13].

In a study by Ziadi, Amra et al. lymphopenia was a predictor factor for the severity, occurrence of ARDS and a risk factor for mortality in the intensive care unit [14]. Additionally, it can be used to predict poor outcomes in patients with SARS-Cov-19 disease, consistent with our study.

CD4 + T cells show no significant differences between control and COVID-19 patients. However, the variable response of CD4+ T cells needs to be investigated with subgroup immunophenotyping studies and further investigated for confounding factors.

CD8+ T cells are significantly reduced in COVID-19 patients with disease severity and compared to controls. In addition, there is an increase in CD4+/CD8+ ratios in COVID-19 patients compared to controls. The finding can be used to predict patients with increasing disease severity. The increase in the ratio is due to the decrease in the number of CD8+ T cells rather than CD4+ T cells in all categories of COVID-19 patients.

According to previous studies, reduced CD4+ T cells and CD8+ T cells in COVID-19 infection have been associated with disease severity. Our results were consistent with the study conducted by De Zuani et al. and Liu, Jing et al., where they concluded that patients had significantly lower frequencies of CD8+ T cell subsets on admission, resulting in increased CD4 to CD8 T cell ratios [15,16].

The study found that there are no significant differences in CD19+ B cells between control and COVID-19 patients. However, the variable response of CD19+ B cells needs further investigation for confounding factors. Follow-up studies using flow cytometry after recovery for expression of B cell subsets also need to be investigated.

An increasing trend in the percentage of cytotoxic CD56+/CD16+ NK cells was demonstrated in SARS-Covid-19 patients compared to controls. There is a proportional increase in mean CD56+/CD16+ NK cell counts in all patient categories. These findings on the NK cell population in patients with COVID-19 disease were consistent with those reported by Laura B. et al [17].

CONCLUSION

SARS-CoV-2 infection was associated with changes in peripheral lymphocyte expression of certain subtypes. In patients with severe SARS-Cov-2 infection, lymphocyte counts, CD8+ T cell counts were significantly reduced while an increased ratio of CD4+/CD8+ T cells, cytotoxic NK cells and B cells was observed.

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