Interplay between lymphocyte subpopulation, inflammatory cytokines and their correlation with oxidative stress parameters in COVID-19

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ABSTRACT

Our objective was to investigate the inflammatory and oxidative stress markers in patients with moderate and severe form of coronavirus disease 2019 (COVID-19). In addition, we show the correlation between changes in lymphocyte subsets and markers of oxidative stress as a tool for patient classification. Interleukin-6 (IL-6) and VEGF were analyzed by utilizing a High Sensitivity Evidence Investigator™ Biochip Array technology. The total antioxidant capacity (PAT) and the free radical concentrations (d-ROM) were measured in serum utilizing analytical photometric system FRAS5. Peripheral blood was used to determine CD45 + mononuclear, B, T, and NK cells using a multi-parameter flow cytometric immunophenotypic test. Statistically significant differences in IL-6 and VEGF levels were observed between the two patient groups. Decreased values of the absolute number of lymphocytes and their CD4 + and CD8 + positive T cells, NK cells, and CD8 were obtained. In the moderate group, good correlations were found between IL-6 and VEGF and NK cells (r=0.6973, P<0.05; for IL-6 and r=0.6498, P<0, for VEGF. 05). Cytokines were correlated with CD45+ (r=0.5610, P<0.05; for IL-6 and r=0.5462, P<0.05 for VEGF). The oxidative stress index can be used as a cheaper alternative and as a triage tool between severe and moderate illnesses, after showing good correlation with more expensive patient classification analysis.

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Introduction

The etiology of coronavirus disease 2019 (COVID-19) still remains unknown, but in the last two years, the mechanisms involved in this disease are generally dependent on multiple modalities. The ultimate goal of the virus is to learn how to evade the host's immune system.[1,2] Namely, the pathogenicity of the virus, the comorbidities of infected individuals, and the ability of the host immune system to respond to induced cytopathic effects have a profound effect on the course and outcome of the disease. To date, the SARS-CoV-2 virus has affected more than 400 million people worldwide and killed more than 5 million people. According to the official data from the World Health Organization, most deaths, about 73%, occur in the population over the age of 65.

The humoral and cellular immunity uses T cells, B cells, natural killer (NK) cells in fighting viral infections and an effective immune response depends

on the activation of cytotoxic CD8+ T cells through killing the infected cells.[3] Results reported since the onset of pandemic showed that more than half of the patients had decreased lymphocyte counts, and pathological findings showed that patients who died of this disease had higher levels of pro-inflammatory CCR6 + Th17 + CD4 + T cells.[4] This infers that the overactivation of T-cells united with the high CD8 T cells cytotoxicity is partially responsible for the observed severe immune injuries in COVID-19 patients. Also, the decreased number of the CD8+ T cells and the high values for neutrophil-to-lymphocyte ratio (NLR) are related with augmented risk for disease severity and hence mortality in patients with severe form of the disease.[5-7] The high NLR in COVID-19 patients leads to redox disbalance as a result of the increased reactive oxygen species concentration. In addition, the activation of macrophages and the polymorphonuclear cells also has effect on the oxidative damage to the tissues and they can lead to organ failure.

The above-mentioned hypothesis, has been proposed as a tool for early identification of patients who need urgent hospitalization and treatment, which could have a potential impact on the disease severity and outcome.[8-14] The link between all proposed mechanisms could be the increased oxidative stress, which has also been proposed as an important player in COVID-19.[15,16] Recently published data suggest that cytokine storm correlates with direct tissue damage, leading to the development and poor prognosis of severe form of COVID-19 disease.[17] Previously published results from our research group show elevated levels of several cytokines (IL-6, VEGF and INF-g) in patients with severe COVID-19.[16] In connection with this observation, that elevated levels of IL-6 and VEGF were observed we have observed coagulopathy in these patients with severe COVID-19.

Even though large effort has been given in R&D of therapeutic agents or vaccines to fight this virus, still, lots of different diagnostic markers are used for making clinical decisions in the follow up of the disease. Some of them are quite expensive and this contributes to overburden of the public health institutions, especially in low income and developing countries. Our aim was to analyze several commonly used disease markers (inflammatory, clinical laboratory parameters and oxidative stress markers) and to provide comprehensive view of them for their future implementation in routine clinical practice. Additionally, our findings presented in this study could enhance the knowledge of the altered lymphocyte subsets and their correlation with the oxidative stress markers as a tool with prognostic power to differentiate between moderate and severe COVID-19.

Materials and Methods

Data collection and patients

Thirty-five (35) patients which had positive RT-PCR for SARS-CoV-2 were hospitalized at the University Clinic for Infectious Diseases and Febrile Conditions in Skopje, Republic of North Macedonia. All patients were not vaccinated since the study was performed before the onset of the national vaccination program. Sixteen (16) patients were classified with severe form of COVID-19 and nineteen (19) of them had moderate form of the disease were included in this study. The average age of all hospitalized patients included in this study was 56.79±10.16 years. Patients that were classified as severe in addition to severe pneumonia had at least one of the following parameters: SpO_2 less than 90% on room air, respiratory rate more than 30 breaths per minute or presence of severe respiratory distress. Demographic data, previous medical conditions, clinical symptoms and signs, use of other medications, outcome data, clinical laboratory analyzes were obtained from the medical records of the patients. All patients received standard of care, and supportive treatment was applied when needed.

Our study was officially approved by the Ethics Committee, Medical faculty, University of Ss Cyril and Methodius, Skopje, Republic of North Macedonia and was performed in accordance with the ethical principles for medical research involving human subjects stated in the Declaration of Helsinki.[18]

Determination of oxidative stress parameters (d-ROMs, PAT and oxidative stress index)

Samples were collected and analyzed immediately after hospital admission for the total antioxidant capacity (PAT) and the plasma peroxide (d-ROMs) concentrations. The analysis was performed on the FRAS 5 analytical photometric system (H&D, Italy). Oxidative stress index (OSI) was calculated automatically by the available software on the instrument. The parameters d-ROM and PAT are represented as equivalents of H_2O_2 and ascorbic acid, respectively.

Analysis of lymphocytes subsets

The multi-parameter flow cytometry (MPF) was performed on full blood immediately after sample collection. Immunophenotyping was done by using BD FACS Canto™ II analyzer on lysed whole blood samples following the Institutional protocol for multiparameter immunophenotyping.[19] Briefly, the immunostaining of cell suspension in the sample of lysed blood was performed with monoclonal antibodies (McAb) which were labeled with fluorochromes (FACS Lysing solution BD Biosciences; San Jose, CA, USA) We used the following monoclonal anti-

bodies: CD45+APC, CD3, CD4, CD8, CD16, CD19, and CD56, all manufactured by BD-Biosciences (San Jose, CA, USA). 100 µL of peripheral blood was incubated with appropriate McAb (15 min, room temperature), followed by addition of the lysing solution repetition of the incubation procedure. The sample was then washed with PBSA (3 times), afterwards the cells were resuspended in isotonic solution and data were collected. A dissolved but unstained sample was used as a control. CD45 gating strategy was used for analyzes (BD FACS Diva™ software).

Measurement of IL-6 and VEGF

For the simultaneous quantitative detection of multiple analytes from a single patient sample for IL-6 and VEGF we have used the High Sensitivity Evidence Investigator™ Biochip Array technology (Randox Laboratories, GB). 100 µL plasma sample was used in biochip carriers that were incubated on thermo-shaker (1 hour, 37°C, 370 rpm) followed by additional incubation (4°C, 20 hours). Subsequently, two wash cycles were carried out and 300 µL conjugate was added into each well of the plate followed by another incubation (1 hour, 37°C, 370 rpm). Finally, after washing the carriers (2 times), we have added a fluorescent dye to the carriers according to the protocol and carriers were captured using Evidence Investigator Array. EvInvest software was used for automatic data processing.

Statistical analysis

The data was presented using descriptive statistics. Simply put, the data was described in terms of numbers or means and mean standard error (SEM). Differences between groups were analyzed using t-test and Mann-Whitney as needed. The P-value less than 0.05 was considered statistically significant. We investigated the correlation by calculating Spearman's r coefficient.

All results presented were analyzed by the statistical program GraphPad Prism 9 (USA).

Results

Demographics and laboratory findings

The average time from the start of the symptoms until hospital admission was 10.48±2.08 days. Among the most upsetting symptoms reported by the hospitalized patients were high body temperature (78%), dyspnea (46%), fatigue (69%), tachycardia (62%) and cough (56%). Among the co-existing medical conditions most frequently were reported the following: hypertension (34%), diabetes (26%) and coronary artery disease (12.6%). Clinical laboratory parameters on admission in the patients with moderate and severe form of COVID-19 are shown in Table 1. In contrast to the moderate group statistically significant differences

Table 1. Clinical laboratory findings in severe and moderate COVID-19 patients on hospital admission. Results are expressed as mean±SEM.

	Moderate group (n=19) Mean±SEM	Severe group (n=16) Mean±SEM	P (Mann-Whitney, t-test)
CRP (mg/L)	41.61±9.45	113.9±30.58	0.0159
LDH (IU/mL)	266.4±24.10	805.0±88.70	<0.0001
ALT (U/L)	47.31±13.76	89.34±26.32	0.1482
AST (U/L)	46.33±8.07	78.05±13.63	0.0457
CK (U/L)	89.89±17.78	250.3±75.44	0.0671
D-dimer (ng/mL)	860.4±83.49	8838±3032	<0.0001
Hemoglobin (g/L)	128.8±2.46	140.8±3.36	0.0053
Hematocrit	0.38±0.01	0.41±0.01	0.0103
WBC ($\times 10^3$µL)	7.17±0.89	13.74±1.52	0.0002
PLT ($\times 10^3$µL)	328.7±25.96	287.3±20.82	0.2327
Neutrophils (%)	0.65±0.03	0.89±0.01	0.0001
Lymphocytes (%)	0.24±0.03	0.063±0.01	0.0001
Glucose (mmol/L)	9.88±1.99	15.01±4.66	0.0118
pH	7.402±0.009	7.565±0.0075	>0.05
pCO_2	37.99±0.80	36.19±1.083	>0.05
pO_2	55.97±2.97	54.15±2.961	>0.05
HCO_3	28.94±0.705	27.99±1.009	>0.05

SEM , standard error mean; CRP, C-reactive protein; LDH, lactate dehydrogenate; ALT, alanine aminotransferase; AST aspartate aminotransferase; CK, creatinkinase; WBC, white blood cells count; PLT, platelets.

were observed for all investigated parameters except for PLT, ALT and CK in the patients with the severe form of the disease.

Alteration of oxidative stress markers, inflammatory cytokines and lymphocyte subsets

Patients with moderate form of the disease had lower values of the measured concentration of free radicals (d-ROMs) and hence lower oxidative stress index when compared to the patients classified with the severe form of the disease (P=0.0001).

In addition, the moderate group had an increased total antioxidant capacity (PAT) compared to the patients in the severe group, but this difference was not considered to be statistically significant (P>0.05). In our previous study, the oxidative stress markers (d-ROM and OSI) showed good correlation with IL-6 and VEGF out of eleven screened cytokines as predictors of disease worsening in patients with severe COVID-19. In this study we have used them as parameters to potentially predict the cytokine storm and herein we report a statistically significant difference of IL-6 and VEGF levels between the moderate and severe groups of patients (P=0.0001).

In terms of the alteration of the lymphocyte subsets, we have observed decreased levels of absolute leukocytes counts, CD45+ mononuclear and its subsets CD4+, CD8+, CD3+, NK cells (P<0.05). These results are shown in Table 2. Also, CD19+ and CD45+ were decreased in the severe group in comparison to the group of patients with moderate COVID-19, but this difference was not considered to be statistically significant (P>0.05), probably due to the small sample size.

Moreover, we have investigated the correlation by calculation of the Spearman r among all investigated parameters in both groups separately and these results are shown as a heat-map in Figure 1. In summary, the oxidative stress parameters, OSI and d-ROM demonstrated a good correlation with CD45+ and CD4+. The total antioxidant capacity (PAT) correlated with CD8+ lymphocyte subset in the patients with the moderate form of COVID-19 and the patients with the severe form of the disease. Additionally, only in the moderate group, we have obtained a good correlation amongst the investigated cytokines (IL-6 and VEGF) and NK cells (CD16+CD56+) was obtained. In this case, the Spearman r in the case of IL-6 was 0.6973 (P<0.05) as in terms of VEGF, the Spearman r was 0.6498 (P<0.05), whereas in the severe group IL-6 and VEGF correlated with CD45+ (for IL-6, r=0.5610, P<0.05; and for VEGF, r=0.5462, P<0.05).

Discussion and Conclusions

One of our study objectives was to investigate the alteration of the lymphocyte subsets together with the inflammatory cytokines and the oxidative stress markers in patients with severe and moderate COVID-19. The final goal was to identify the most suitable laboratory parameters which can be used as a predictor for early detection of development a severe form of the disease, as well as identification of patients who may benefit from different therapeutic strategies (*e.g.*, cor-

Table 2. Alteration in oxidative stress markers, inflammatory cytokines and lymphocytes subsets in severe and moderate COVID-19 patients on hospital admission. Results are expressed as mean±SEM.

Parameter	Severe COVID-19 patients mean±SEM (n=16)	Moderate COVID-19 patients mean±SEM (n=19)	P (t-test)
IL-6 (pg/mL)	300.6±38.20	43.52±2.8	0.0001
VEGF (pg/mL)	550.6±119.3	78.56±3.1	0.0001
d-ROM (U.Carr)	472±11	353±16	0.0001
PAT (U.Carr)	2480±86	2680±150	0.2794
OSI	110.2±7.9	64.2±5.13	0.0001
CD4+	0.1711±0.0184	0.6765±0.0653	0.0001
CD8+	0.1034±0.0191	0.3157±0.0332	0.0001
NK	0.06225±0.01656	0.1498±0.02519	0.0087
Absolute CD8	99.63±10.78	317.4±32.42	0.0001
CD45+	0.7382±0.1188	1.266±0.1428	0.7594
CD19+	0.1852±0.0495	0.2686±0.0861	0.4296
CD3+	0.4038±0.0629	0.9057±0.0114	0.0009
Leukocytes	12.28±1.055	7.637±0.8581	0.0016

SEM, standard error mean; IL-6, interleukin-6; VEGF, vascular-endothelial growth factor; d-ROM, derivatives-reactive oxygen metabolites; OSI, Oxidative stress index; NK, natural killers.

ticosteroids) in order to further slow-down the symptom's deterioration.

Lymphocytopenia is a sign of a patient with a severe form of a disease associated with poor clinical outcome.[6-11] Liu *et al*. have shown that the degree of lymphopenia is higher in patients with severe COVID-19 compared to the mild cases.[12] They concluded that the degree of lymphocyte depletion could be considered as an indicator of the degree of immune system damage induced by viral infection. Akbari *et al*. performed meta-analysis, showing a significant reduction in the total number of lymphocytes and their subsets in COVID-19 patients, suggesting that they are a reliable indicator of disease severity.[20] CD45+ mononuclear cells play an important role in viral infection, and studies have shown that the density on the surface of T cells is reduced.[9,10] We also showed that the CD45 + count decreased in both groups, but the difference was not significant due to the large SEM or small sample size. In addition, the good correlations obtained between CD45 + and OSI in the moderate and severe groups are primarily against oxidative damage due to their location on the plasma membrane with easy access to extracellular oxidants. It may be due to the relative susceptibility.[11] Overall, the two parameters CD45+ and OSI can be considered as an appropriate diagnostic criterion to distinguish between severe disease progression and complications.

Oxidative stress index can be used as potential marker with a diagnostic value since we have obtained very high values in patients with severe COVID-19 as well as OSI demonstrated a good correlation with IL-6, CD45+, CD4+ and absolute number of CD8 cells. In the severe group, we have observed an increased level of IL-6, a pro-inflammatory cytokine, which probably contributes to the T lymphocyte deficiency (CD3+, and the subsets CD4+ and CD8+).[21,22] Several authors have reported that during viral infections with the virus, CD4+ T lymphocytes are activated into T helper cells and then they secret pro-inflammatory cytokines as IL-6. The activated immune cells enter into pulmonary circulation and can lead to serious lung injury.[16,20,23] Therefore, blocking IL-6 secretion could block immunopathological injury. Our results contribute to this immunopathological hypothesis, since we have evidenced a significant decrease in T cells count in terms of CD4+ (severe vs moderate) which on the other hand correlated well with the IL-6 and the OSI for both groups (P=0.0001).

This could be improved by treatment with corticosteroid therapy (*e.g*., for accelerated recovery of CD8+). Additionally, several clinical trials and investigations with monoclonal antibodies like tocilizumab (IL-6 receptor antagonist), sarilumab (IL-6 antagonist) and anakinra (IL-1 receptor antagonist) against selected cytokines are in progress, with promising results for suppression of the so-referred cytokine storm.[24-27] Since there is evidence for compromising the lymphocyte activity by the increased oxidative stress and inflammation, we consider that amelioration of the oxidative stress can be added as a concomitant therapeutical strategy in these patients. By utilizing the measurement of the oxidative stress markers as a not expensive diagnostic tool, clinicians could act faster and can predict the clinical response.

In contrast to the patients with the moderate form of COVID-19, the patients within the severe group had two to four-fold decreased values of all examined lymphocyte subsets. Similar behavior was reported by Balzanelli *et al*. who had investigated the dynamic

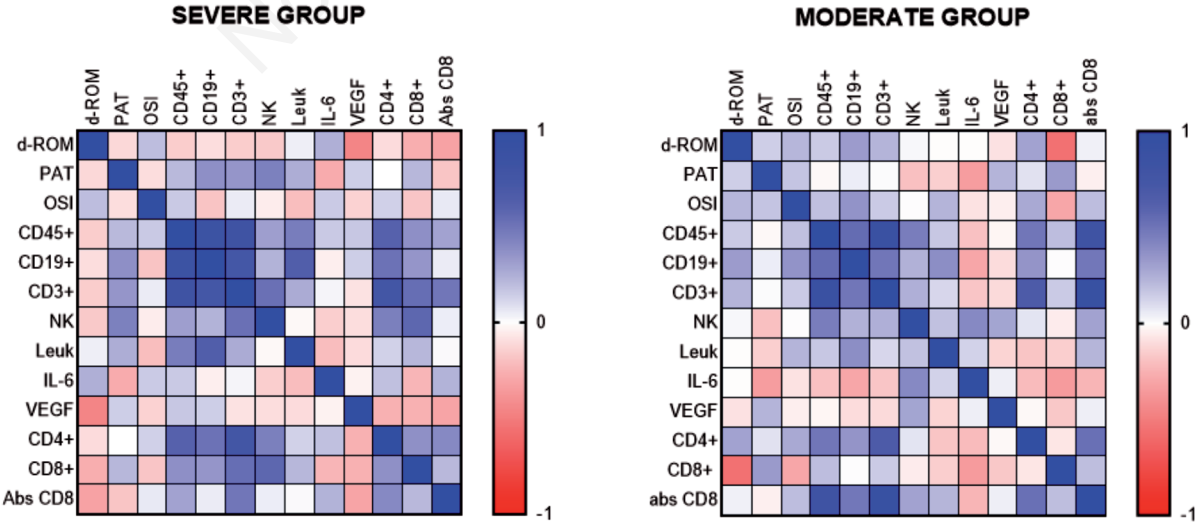

Figure 1. Heatmap presentation of Spearman r coefficient between the investigated markers of oxidative stress, inflammatory cytokines and lymphocyte subsets severe and moderate COVID-19 patients.

changes of lymphocyte subsets in COVID-19, COVID-like patients, general pulmonary patients, and healthy individuals. Moreover, we would also like to potentiate those patients with the moderate form of the disease who fully recovered, unlike patients from the severe group, where 8 of them had deterioration of the disease and died.[28] Moratto *et al*. reported enhancement in the CD3+, CD4+ and CD8+ cells count in moderate and severe patients who had improved their health status and survived in contrast to those who remained in critical phase and had very low counts of all subsets.[13] Based on our results the mean ratio between CD4+/CD8+ in the severe group was 1.65 and for the moderate 2.14. When a viral infection occurs, this ratio is disrupted as several researchers have reported during HIV infection which under normal health state is 2:1.[11] Upon our research and prior publishing our data, only limited research results were available in discussing the potential of CD4+/CD8+ as a predictor of disease progression.[7-9] This is mainly because of the small sample size, in combination big standard deviations which could be the reason for contradictory results obtained. Namely, Mahmoudi *et al*. concluded that the ratio between CD4+ and CD8+ T cell was lower in patients with severe form of COVID-19 compared to the patients with mild or moderate form of the disease.[9] Alternatively, Pallotto *et al*. reported higher CD4+/CD8+ in critically ill patients. Hence, until more studies are performed with larger population, we can speculate that this ratio can be reliably used in identification of critically and severe form of COVID-19.[7]

Our study has several drawbacks besides being a single-center experience with a small sample size, however the data reported were obtained at the beginning of the pandemic period when no specific and limited clinical experience was available and without any officially issued state guidelines to assist the need for hospitalization. It is worth noting, that measuring the dynamic changes in lymphocyte subsets would be of great importance in order to shade additional light in the pathogenesis of the disease. We believe that the obtaining a sample for analysis early at the beginning of the infection (before hospitalization) could shed more valuable insight in the development of the disease. Our results could contribute better understanding of the immune system response, including cell-mediated immunity as well as its link with the redox imbalance in the organism. It is worth asking, whether additional supplemental therapy which attenuates the oxidative stress might result in better immunological system response in these patients.[29] Alamdari *et al*. have shown that administration of several substances (vitamin C and N-acetyl cysteine) has beneficial effects in the treatment of critically ill patients in one single center phase 1 clinical trial.[30] This area of research has just begun to tackle the researchers for further investigation of the potential use of antioxidants.

In conclusion, we have found altered lymphocyte subsets, oxidative stress parameters and pro-inflammatory cytokines (IL-6, VEGF) in patients with COVID-19, for which we have demonstrated a significant difference between the moderate and severe group. In terms of clinical application, our data can be considered as evidence that the oxidative stress index could be used as a triage tool between the severe and the moderate form of the disease as a cheaper alternative after demonstrating a good correlation with more expensive analysis used for patients' classification.

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