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COVID-19 disease induced alteration of oxidative stress and clinical laboratory parameters in moderate and severe patients

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Abstract. – OBJECTIVE: The aim of our study was to present the clinical alterations of CRP, LDH, neutrophil to lymphocyte ratio, platelets to lymphocyte ratio, D-dimer, blood gas analyses, vitamin D, VEGF, IL-6, IFN-γ, CD4+, CD8+) and their correlation with oxidative stress index (OSI) in hospitalized COVID-19 patients.

PATIENTS AND METHODS: Oxidative stress index and clinical parameters were determined at admission and/or 7 days after hospitalization in 50 patients divided in moderate and severe group.

RESULTS: In the moderate group of patients, a good correlation (R^2 = 0.7400, p<0.05) was found between OSI and PLR, D-dimers and LDH at admission and after 7 days. The OSI correlated well with vitamin D, INF-γ, IL-6, CD4+, CD8+ and the absolute CD8 cell number on admission (R^2=0.7635, p<0.05). Vitamin D deficiency (15.37 ng/mL ± 2.81) was observed at admission in the severe group, accompanied by increased levels of IL-6 (295.3 pg/mL ± 40.06), INF-γ (1.603 pg/mL ± 0.134), VEGF (546.8 pg/mL ± 124.2) compared to non-infected individuals. All patients had low partial pressure of oxygen, although it did not show statistically significant difference between the two groups.

CONCLUSIONS: All investigated parameters were altered in both groups of patients and a good correlation between them was demonstrated.

Key Words:
COVID-19, Oxidative stress, Clinical parameters, Disease severity.

Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was first identified as a novel human pathogen in December 2019 and since then it has caused a worldwide pandemic[1]. Course of infection goes through several stages: replication of SARS-CoV-2 where patients manifest non-specific symptoms, dysregulated immune response, multiple organ damage and recovery[2]. Patients are categorized in the range of asymptomatic or mild to moderate, severe or critical[1]. Fatal outcomes due to the respiratory system damage and multi-organ failure in severe patients are associated with excessive inflammatory response, i.e., cytokine storm, evident by high blood levels of cytokines, chemokines and C-reactive protein (CRP). Several abnormal parameters correlated with poor outcome are commonly reported in patients with COVID-19, such as lymphopenia, neutrophilia, elevation in D-dimers and CRP levels[3]. In addition, high levels of IL-6, IL-10, IL-2R and TNF-α have been reported in patients with severe COVID-19, although some authors[4-6] suggest that other cytokines, such as IL-1β, IL-1RA, IL-8, IL-18 are included in the COVID-19 pathogenesis.

Oxidative stress (increased reactive oxygen species and reactive nitrogen species) presents an important pathway that contributes to numerous inflammatory pathological processes, including COVID-19 disease. The oxidative damage imposed on host tissues *via* polymorphonuclear cells and macrophage activation may lead to tissue damage and organ dysfunction[2,7,8]. In addition, to the present inflammation in COVID-19 patients, processes like inhibition of ACE-2 activity, endothelial dysfunction, disseminated intravascular coagulation and hemoglobin denaturation resulting in disturbances of iron metabolism by releasing toxic free iron are closely related to increased oxidative

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stress level. These patients also have lower levels of antioxidant substances (vitamin C, E, A and D) due to their increased utilization in counterbalancing the negative effects of the free radicals[9-11].

Lymphocyte subsets present important role in the performance of the adaptive immune system. Often, patients with severe form of the disease have lymphopenia, accompanied with lower number of CD4+ and CD8+ T cells[12]. Circulating CD4+ and CD8+ cells show greater reduction observed in critically ill patients which on the other hand contribute to the cytokine storm. This has been discussed in detail in a recent thorough review by Lagadinou et al[13].

In addition to the extensive research that has been performed in the past year, we have contributed to this field by investigation of the changes in the oxidative stress parameters. Herein, in details we present the alterations in the oxidative stress index (OSI) and other clinical parameters (CRP, LDH, neutrophil to lymphocyte ratio NLR, platelets to lymphocyte ratio PLR, D-dimer, blood gas analyses, vitamin D, VEGF, IL-6, IFN-γ, CD4+, CD8+) in 50 SARS-CoV-2 infected patients. Moreover, we investigated the correlation between all above mentioned clinical parameters in these patients that were divided in two groups: moderate and severe.

Patients and Methods

Study Patients

A total of 50 patients with COVID-19 hospitalized at the University Clinic for Infectious Diseases and Febrile Conditions, Skopje, Republic of North Macedonia, previously confirmed to have SARS-CoV-2 infection by Real-Time Reverse Transcriptase-Polymerase Chain reaction assay (RT-PCR) from nasal and pharyngeal swab specimen, were included in this study. Interim Guidance for Clinical Management of COVID-19 issued by the WHO was used for classification of patients in moderate and severe group.

The study was approved by Ethics Committee of the Faculty of Medicine, University of Ss Cyril and Methodius, Skopje, Republic of North Macedonia.

Clinical Characteristics, Laboratory Assessment and Methods

Medical records from all patients who have signed informed consent were used in order to obtain demographic characteristics, medical history, clinical symptoms and signs, concomitant medication, outcome data, as well as laboratory analyzes. Clinical improvement on physical examination, oxygenation, body temperature back to normal at least for three days and an improvement of lung infiltration (confirmed by chest X-ray examination) was used as discharge criteria.

Laboratory assessment consisted of complete blood cell count, blood biochemistry, coagulation profile, oxidative stress parameters (d-ROM, PAT) and inflammation markers at admission and after 7 days. Arterial blood gas parameters (pH, partial pressure of carbon dioxide (pCO_2), partial pressure of oxygen (pO_2), bicarbonate (HCO_3), base excess (BE), base excess of extracellular fluid (BEecf) analyses were performed at admission and additionally when individual patients showed relevant clinical worsening of their symptoms or oxygen insufflation was required.

Serum vitamin D concentration, cytokine profile and lymphocyte subsets were measured only at hospital admission.

Oxidative stress index (OSI) was calculated using values obtained for d-ROMs fast test and the PAT test measured by the spectrophotometer FRAS5 with normal reference values less than 40 given by the manufacturer (H&D srl, 43124 Parma, Italy).

The High Sensitivity Evidence Investigator™ Biochip Array technology (Randox Laboratories, Crumlin, UK) was used to perform simultaneous quantitative detection of IL-6, VEGF and IFN-γ. Additionally, by flow cytometry, CD4+, CD8+ and the absolute number of CD8 cells were quantified using BD FACS Canto™ II analyzer (BD-Biosciences, San Jose, CA, USA) on lysed whole blood samples. All samples were collected and analyzed immediately after hospital admission.

Statistical Analysis

Data were described as number and/or percentage or mean with standard errors of mean (SEM), where appropriate. Differences between groups were explored using the *t*-test followed by one-way ANOVA where appropriate. A *p*-value less than 0.05 was considered significant. All analyses were made using the statistical program GraphPad Prism 9 (LaJolla, CA, USA).

Results

The aim of this study was to present the alterations of the clinical and biochemical parameters frequently used as prediction factors for disease

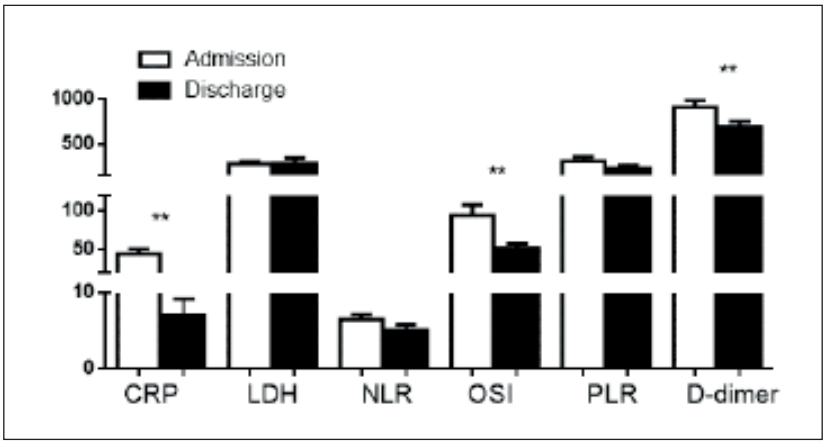

Figure 1. Graphical presentation of clinical laboratory parameters in patients (n=20) with moderate Covid-19 at admission and after 7 days of hospitalization (discharge). $^{**}p<0.05$.

severity in hospitalized patients infected with SARS-CoV-2. In our previously published studies, we have demonstrated that oxidative stress markers have good correlation with CRP, LDH and NLR in patients with COVID-19 which can be used for early identification of high-risk individuals and prevent their sudden deterioration[11,14,15]. This is in line with the alteration of redox balance that can contribute to a wide range of pathologies (cancer, neurodegeneration, atherosclerosis, inflammation etc.). Moreover, we share results in regard to the correlation between all investigated clinical laboratory parameters that are commonly used in the evaluation of COVID-19 patients with the oxidative stress index. Additionally, we present overview of the blood gas analyses in the COVID-19 patients on hospital admission.

Demographics and Clinical Characteristics of Patients

Among 50 hospitalized patients, 30 of them were classified as severe and 20 of them were classified as moderate cases with mean age of 58.0±9.94 years and 52.05±12.84 years, respectively. The average time from onset of symptoms to hospital admission was 10.52±2.33 days. More than 50% of all patients had high body temperature, dyspnea, malaise and cough on admission and had reported comorbidities, such as hypertension, diabetes and chronic cardiac disease more than 60 % and 77 % in the moderate and severe group, respectively.

Moderate group

Patients with moderate form of the disease were adults with clinical signs of pneumonia, but no signs of severe pneumonia, including $SpO_2 \geq 90\%$ on room air. In this group (n=20) on admission, abnormal values for all investigated parameters were observed. The mean values ± SEM for CRP, LDH, NLR, OSI, PLR and D-dimers are shown in Figure 1 at admission and on 7th day of hospitalization (discharge). Namely, statistically significant difference was observed for CRP (p=0.0001), OSI (p=0.008) and D-dimers (p=0.003) between hospital admission and on 7th day of hospitalization. A good correlation (R^2 = 0.7400, $p<0.05$) was found between OSI and PLR, D-dimers and LDH at admission and after 7 days of hospitalization. Additionally, ANOVA testing shows that OSI has a good correlation (R^2=0.7635, $p<0.05$) with vitamin D, INF-γ, IL-6, CD4+, CD8+ and the absolute CD8 cell number at admission. The mean values of gas analyses are presented in Table I.

Severe group

Severe cases additionally met at least one of the following conditions: $SpO_2 < 90\%$ on room air, respiratory rate >30 breaths/minute or presence of severe respiratory distress.

In the severe group (n=30) of patients' abnormal values for CRP, LDH, NLR, OSI, PLR, D-dimer, vitamin D, IL-6 and INF-γ were observed. In comparison with the moderate group, the mean values of the investigated parameters in the severe group were higher upon admission and on discharge. In this group, only six patients recovered, 24 had deterioration of their condition and died (second blood sample for nine of them was obtained and for 15 patients second blood sample could not be taken).

Table I. Clinical laboratory parameters on hospital admission expressed as Mean ± SEM.

Parameter	Moderate (n=20)	Severe (n=30)	Reference values	*p* (*t*-test)
pH	7.486 ± 0.0085	7.465 ± 0.0075	7.35-7.45	0.0745
pCO_2	38.67 ± 0.85	37.18 ± 1.287	35-40	0.3922
pO_2	57.37 ± 2.47	56.30 ± 2.406	75-100	0.7659
HCO_3	28.04 ± 0.731	28.67 ± 1.097	20-26	0.6708
BE	6.984 ± 0.9694	3.668 ± 0.7599	-3.0-3.0	**0.0092**
BE_{Ecf}	7.422 ± 1.103	2.956 ± 0.876	-3.0-3.0	**0.0025**
$SapO_2$ (%)	93.33 ± 0.5216	86.26 ± 1.114	90-100	**0.0001**
Na+	137.6 ± 0.8492	138 ± 1.038	135-145	0.7839
K+	4.058 ± 0.1159	4.187 ± 1.099	3.5-5.5	0.9245
Ca^{2+}	1.155 ± 0.009	1.120 ± 0.022	1.0-1.32	0.2175
Lactate	1.984 ± 0.1825	2.254 ± 0.1734	0.5-2.2	0.3035
CD4+	0.6765 ± 0.0653	0.1711 ± 0.0184		**0.0001**
CD8+	0.3157 ± 0.033	0.134 ± 0.011		**0.0001**
CD8+ Abs number	317.4 ± 32.42	99.63 ± 10.78	200-800	**0.0001**
IL-6 (pg/mL)	40.48 ± 3.147	295.3 ± 40.06	2.135 ± 0.453*	**0.0001**
INF-g (pg/mL)	0.789 ± 0.096	1.603 ± 0.134	0.389 ± 0.082*	**0.0001**
VEGF (pg/mL)	74.68 ± 3.956	546.8 ± 124.2	27.04 ± 4.708*	**0.0033**
Vit D (ng/mL)	29.51 ± 2.86	15.37 ± 2.81	30 - 100	**0.0014**

*Values from non-infected individuals from our previous research.

The mean values ± SEM for CRP, LDH, NLR, OSI, PLR and D-dimer are shown in Figure 2 where a statistically significant differences were observed between all investigated parameters at hospital admission in patients that survived ($p<0.05$). On the other hand, for the deceased patients, a statistically significant differences were obtained in case of NLR ($p=0.0008$) and D-dimers ($p=0.0001$).

A good correlation ($R^2 = 0.9722$, $p<0.05$) was found between OSI and D-dimers and LDH at admission and after 7 days of hospitalization. The severe group of patients at admission was vitamin D deficient (15.37 ng/mL ± 2.81) and had increased levels of IL-6 (295.3 pg/mL ±40.06), INF-γ (1.603 pg/mL ±0.134**)** and VEGF (546.8 pg/mL ±124.2) compared to non-infected individuals. Moreover, the absolute CD8 cell number was depleted in comparison to the reference values (Table I).

Discussion

Generally, infection with SARS-CoV-2 is related with oxidative stress, proinflammatory state, cytokine production and cell death. Namely, oxidative stress is characterized by an imbalance between ROS production and inadequate neutralization of free radicals. It has been already discussed that respiratory viruses, including coronaviruses, cause changes in redox balance in infected cells, inducing inflammation followed by tissue damage[16].

Our study shows clinical evidence that oxidative stress is increased in COVID-19 patients and its alteration could have impact on the progression of the disease. Namely, patients in both moderate and severe group on admission had significantly higher values for OSI, but those with continuous increasing of OSI upon hospital admission developed severe form of the disease that resulted in death (OSI value 84.03 *vs.* 107.8). This increase in the oxidative stress index was mainly due to the enhanced ROS production that presumably overwhelmed the cellular antioxidant buffering capacity. The concentrations of free radicals on admission were above 400 U.CARR in both study groups which is above the upper limit of normal reference values (250-300 U.CARR) according to the manufacturer. This is in line with the finding that the present neutrophilia in vulnerable patients generates an excess of ROS that aggravates the host immunopathological response developing severe form of the disease. In contrary, as it can be seen from the results for OSI in the moderate group of patients, this index is decreasing (OSI: 94.2 *vs.* 52.25) and patients have a better clinical outcome which is supported with decreased levels of the free radicals (d-ROM: 431.25 *vs*. 334.3 U.CARR) after 7 days of admission. Moreover, d-ROM continued to increase in the subgroup of critically ill patients who did not survive in comparison to the severe patients who recovered (d-ROM: 462.4 *vs.* 325.3 U.CARR).

The cytokine storm occurs when large numbers of leukocytes are activated and release a high concentration of proinflammatory cytokines.

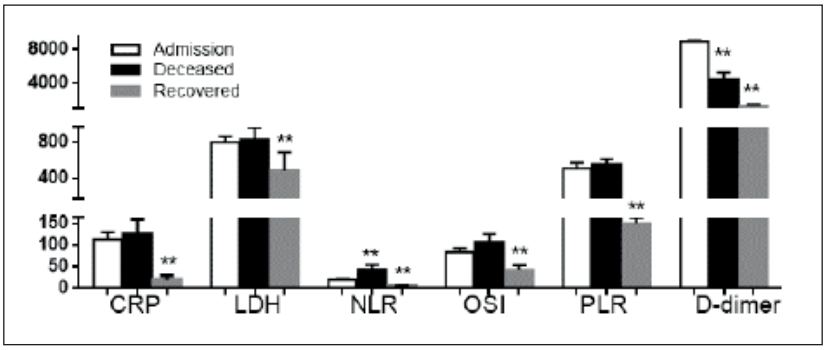

Figure 2. Graphical presentation of clinical laboratory parameters in patients (n=30) with severe COVID-19 at admission and after 7 days of hospitalization. $^{**}p<0.05$

Our results support the cytokine storm hypothesis where we have observed statistically significant difference between the severe and the moderate group of patients at admission for the tested cytokines IL-6 (p=0.0001), VEGF (p=0.0001) and IFN-γ (p=0.0033). Our study revealed that the investigated cytokines and biomarkers were significantly increased in the severe group of patients in comparison with the moderate group, which was accompanied with coagulopathy as determined by deterioration of the platelet related parameters (PLR, D-dimers, IL-6, VEGF). Since it is well established that COVID-19 is a multifactorial disease, the progression of the disease must be evaluated through analysis of several interconnected clinical and laboratory parameters.

Namely, the markers of inflammation (CRP, LDH, NLR, PLR and D-dimers) were significantly higher as it can be seen in Figure 1. The hematological disorders in regard to the changes in the blood cell count were more pronounced among the severe COVID-19 patients compared to the moderate group. The statistical significance was observed on patients' hospital admission for lymphocytes, white blood cells, neutrophils, red blood cells and hematocrit when compared severe *vs.* moderate group (p<0.05). However, after 7 days, red blood cells, hemoglobin, hematocrit and lymphocytes were significantly altered in the subgroup of patients who died as compared to the values on admission (p<0.05). In addition, the patients in severe group had higher CRP levels on admission which is considered to be a reliable marker of acute inflammation followed by higher NLR, a very sensitive indicator of infection and sepsis. On admission, patients with moderate form of the disease, had PLR and D-dimers values (318.2 and 866 ng/ml, respectively) lower in comparison to the severe group (512.36 and 8852 ng/ml, respectively). After 7 days, these parameters were decreased in the patients with the moderate form of the disease and in the subgroup of the severe patients who recovered. COVID-19 progression was evidenced by continuous deterioration of these parameters in the subgroup of patients who died (PLR 553.1 and D-dimers 4411 ng/ml). Hence, we can conclude that higher PLR and D-dimers were associated with poor clinical outcome compared to the patients in the moderate group.

Lower vitamin D levels were observed in the severe group of COVID-19 patients on admission when compared to the moderate group, but still in the lower range of the reference values for both groups. The supplemental therapy of vitamin D could contribute in a positive manner in COVID-19, mainly due to its antioxidant, immunomodulatory and anti-inflammatory effects[17].

Our results add value to the evidence that lymphopenia, especially low count of CD4+ and CD8+ T cells is commonly observed in COVID-19 patients. Namely, we have obtained decreased number for both parameters especially in the severe group of patients. Even though the mechanism for the COVID-19 lymphopenia is still not clear, we consider that the lymphocytes are important factor for the performance of the adaptive immune system and can be used in the identification of the critically ill patients. Moreover, data suggest that the release of IL-6 and the granulocyte colony-stimulating factor by T lymphocytes and monocytes can be suggested as a key factor to the cytokine storm[18,19].

All patients had low partial pressure of oxygen, although it did not show statistically significant difference between the two groups, but it has clinical importance indicating the need for supplemental oxygen therapy in both groups with moderate and with severe disease. In addition, measured oxygen saturation was lower in the severe group of patients (86.26%) and this parame-

ter has strong statistical significance, supporting the clinical indication for application of oxygen therapy. The analysis showed that in both groups the partial pressure of carbon dioxide and pH values were within the reference ranges, the levels of bicarbonates were slightly increased, but without a significant difference between the groups. The base excesses were increased in the group of patients with moderate disease, which was statistically significant, but no acid/base disturbances occurred within any of the group. Similar results were reported by Mumoli et al[20] at hospital admission of 88 COVID-19 patients, as well as by Doaei et al[21] who measured similar values of the blood gas parameters in critically ill patients with COVID-19 to those reported in our study. Additionally, our results are in agreement with one retrospective observational study that used decision tree machine learning model for prediction of prognosis of COVID-19 by blood gas parameters[22]. On the other hand, Deniz et al[23] detected a mild increase of pH and bicarbonate and relatively low pCO_2 in COVID-19 patients compared to non-COVID-19 individuals.

In addition, blood gas analysis showed normal values of electrolytes and lactates in all patients, and it did not have statistical difference between the groups. However, these results of electrolytes and lactates should be interpreted with caution, since the analysis were performed from capillary blood sample and the values might differ from those taken from venous or arterial blood sample, that are more commonly used.

Several limitations of our study need to be mentioned. First, due to the relatively small sample size of COVID-19 patients, the obtained results warrant further verification in prospective clinical studies with larger patient cohorts. Second, a more frequent blood sampling could provide additional information regarding the dynamic and complex pathophysiological processes that occur during disease progression.

Conclusions

In summary, all investigated clinical parameters including oxidative stress index were strongly altered in severe COVID-19 patients and could be used as supportive tools that help to distinguish patients at risk for developing severe/critical COVID-19 which are urgently needed in medical decision making in order to establish proper treatment strategies and hopefully reduce the disease mortality.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Informed Consent

Informed consent was obtained from all patients included in this study.

Funding

No funding was requested for this study.

Data Availability Statement

Data are available upon request to the corresponding author.

Authors' Statement

DZ, MP and EA contributed to study conception, design, and data interpretation. MP and DZ contributed to statistical data analysis, oxidative stress parameters analyses and preparing the draft manuscript. EA, KS and KG analyzed and interpreted the medical data record, and the blood gas analyses. KGj and KP contributed to data collection. All authors contributed to the final approval of the version to be published.

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