Presentation of cytokine profile in relation to oxidative stress parameters in patients with severe COVID-19: an observational pilot study [version 1; peer review: 1 approved with reservations]

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Abstract
Introduction: COVID-19 can be worsened by hyper-production of cytokines accompanied by increased level of oxidative stress. The aim of this study was to investigate the correlation between a set of cytokines and the markers of the oxidative stress.

Methods: The levels of cytokines IL-2, IL-4, IL-6, IL8, IL-10, VEGF, IFN-γ, TNF-α, IL-1α, MCP-1 and EGF were determined by using High Sensitivity Evidence Investigator™ Biochip Array technology. The oxidative stress parameters (d-ROM, PAT, OS index) were measured in serum on FRAS5 analytical photometric system.

Results: IL-6, IL-8, IL-10, VEGF, MCP-1 and EGF were significantly higher (p<0.05) in the patients with severe COVID-19 with increased levels of IL-2, IFN-γ, TNF-α and IL-1α. The d-ROM, OS index, and PAT were significantly higher (p<0.05) in severe COVID-19 patients. IL-6 demonstrated the strongest correlation with all of the markers of the oxidative stress, d-ROM (r=0.9725, p=0.0001), PAT (r=0.5000, p=0.0001) and OS index (r=0.9593, p=0.012). Similar behavior was evidenced between IFN-γ and d-ROM (r=0.4006, p=0.0001), PAT (r=0.6030, p=0.0001) and OS index (r=0.4298, p=0.012).

Conclusion: The oxidative stress markers show good correlation with the tested cytokines which can be measured at the beginning of the disease in a primary care setting to predict the course of COVID-19.

Keywords
oxidative stress, COVID-19, cytokines
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Author roles: **Petrushevska M**: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Resources, Software, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Zendelovska D**: Conceptualization, Formal Analysis, Investigation, Methodology; **Atanasovska E**: Conceptualization, Methodology, Writing – Review & Editing; **Eftimov A**: Investigation; **Spasovska K**: Conceptualization, Investigation, Methodology

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1. Introduction
Cytokine storm syndrome has been widely discussed and proposed as one of the underlying aetiologies of respiratory failure in patients infected with SARS-CoV-2. Pro-inflammatory cytokines play a key role in large number of respiratory viral infections by activation of the adaptive immune response and, when this response is not controlled, it can lead to involvement of the lung tissue in the course of ARDS or can result in severe damages of multiple organs. For example, following influenza viral infection, an excessive amount of reactive oxygen species (ROS) is produced in several tissues including alveolar epithelium and endothelium for which induced expression of cytokines through activation of Toll-like receptors (TLR3, TLR7 and TLR8, retinoic acid inducible gene I and members of NOD-like receptor family) stand in the background of the pathogenesis. Oxidative stress is typical for infection of human respiratory syncytial virus, rhinoviruses, and many other viruses. This has been discussed in previously published reviews and as well, several experimental studies suggest that cytokine storm correlated with direct tissue injury and lead to unfavourable prognosis of severe form of the COVID-19 disease. Briefly, particularly high levels of IL-6, IL-10, IL-2R and TNF-α have been reported in patients with severe form of the disease, although other authors suggest that more cytokines, such IL-1β, IL-1RA, IL-8, IL-18 are included in the COVID-19 pathogenesis.

Authors have suggested that the innate immune response follows same pathway for SARS-CoV-2 infection. Namely, ROS is a strong ligand and a direct mediator in the NLPR3 (inflammasome) trigger. Moreover, NF-xB, which is activated by ROS, triggers transcriptional levels of NLPR3 are enhanced by TLR and NLR ligands. This means that the inflammasome is increased by ROS either directly or indirectly. To the addition of ROS, H2O2 activates NF-xB to produce inflammatory cytokines. Hyperproduction of IL-6, TNF-α, IL-1β, IP-10, GCSF, MCP-1, MIP1-α/CCL3 and elevated blood ferritin are also observed in patients infected with SARS-CoV-2.

For this purpose, and in the light to share more experimental data as evidence to the suggested pathogenesis of COVID-19 with the scientific community, we have utilized a highly standardized cytokine assay to measure plasma levels of 11 inflammatory cytokines potentially associated as key factors with the cytokine storm syndrome. Afterwards, we have investigated which of these cytokines involved in the cytokine storm of COVID-19 show good association/correlation with the oxidative stress markers determined with fast and inexpensive photometric analytical method. Moreover, the relation between the cytokines, oxidative stress markers and the most commonly used inflammation-related biomarkers (CRP, D-dimers, PLR, NLR and LDH) in severe form of the disease was investigated.

2. Methods
2.1 Study design, patients profile and data collection
52 patients with COVID-19 were hospitalized at the University Clinic for Infectious Diseases and Febrile Conditions, Skopje, Republic of North Macedonia at the beginning of the pandemic within a period of 1 month. 14 patients classified with severe COVID-19 (nine males and five females) with a mean age of 58.36 years (range from 36 to 71 years) were included in this study. The diagnosis and classification of COVID-19 were based on the Interim Guidance for Clinical Management of COVID-19 issued by WHO. Severe cases in addition to severe pneumonia met at least one of the following conditions: SpO2 <90% on room air, respiratory rate >30 breaths/minute or presence of severe respiratory distress. All patients were confirmed to have SARS-CoV-2 infection by real-time reverse transcriptase-polymerase chain reaction assay (RT-PCR). Severe form of COVID-19 as primary exposure variable, demographic characteristics, medical history, clinical symptoms and signs, concomitant medication, outcome data, as well as laboratory analyzes were obtained from the patients’ medical records were other predictor variables. The study flow chart is shown in Figure 1.

The study was approved by the local ethics committee (Ethics Committee of the Faculty of Medicine, University of Ss Cyril and Methodius, Skopje, Republic of North Macedonia, No #03-366/7) and complies with the STrengthening the Reporting of Observational studies in Epidemiology (STROBE) statements for reporting of observational trials.

2.2 Method for determination of d-ROMs, PAT and oxidative stress index
PAT (total antioxidant power, iron reducing) and d-ROMs (plasma peroxides) were measured on a FRAS5 analytical photometric system (H&D, Italy). Samples were collected and analyzed immediately after hospital admission. The instructions of the manufacturer were followed for the both tests. The d-ROM and PAT are reported in equivalents of H2O2 and ascorbic acid, respectively. Oxidative stress index (OSI) presents information obtained from d-ROMs Fast test and the PAT test that is automatically calculated by the manufacturer’s software (OB manager, FRAS5, H&D, Italy) with normal reference values less than 40.

2.3 Cytokines profile assay
The High Sensitivity Evidence Investigator™ Biochip Array technology (Randox Laboratories, GB) was used to perform simultaneous quantitative detection of multiple analytes from a single patient sample (14 SARS-CoV-2 infected and 20 non-infected individuals).
100 μL of plasma was used in biochip carriers, following by incubation on thermo-shaker for 1 hour at 37°C and 370 rpm and 16–20 hours incubation at 4°C. Afterwards, carry out of two wash cycles and 300 μL conjugate was added into each well followed by another incubation of 1 hour at 37°C and 370 rpm. At the final step after twice washing the carriers, fluorescent dye was added to carriers according to protocol and carriers were captured by Evidence Investigator Array. Results were processed automatically using EvInvest software and levels of cytokines IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN-γ, TNF-α, IL-1α, MCP-1 and EGF were calculated as pg/mL.

2.4 Statistical analysis

Exposure variables were summarized using descriptive statistics. Data were described as number and/or percentage, or median and range or mean and standard deviation (SD) or standard error of mean (SEM), where appropriate. Differences between groups were explored using the t-test followed by Mann–Whitney where appropriate. A p-value less than 0.05 was considered significant. For purpose of control and comparison between groups, we have analyzed samples of 20 healthy individuals with negative RT-PCR test for SARS-CoV-2 (12 males and eight females, mean age 54). Spearman r coefficient of correlation was performed. All analyses were made using the statistical program GraphPad Prism 9 (USA) (RRID:SCR_000306); an open-access alternative is JASP (RRID:SCR_015823).

3. Results

3.1 Demographics and laboratory findings

All 14 patients with a mean age of 58.36 years had severe form of the disease. The average time from onset of symptoms to hospital admission was 10.52 ± 2.33 days (range 7–16 days). All of them had underlying medical conditions at admission. The most frequently reported comorbidities were hypertension, diabetes and chronic cardiac disease. The most prominent and disturbing symptoms reported by the patients on admission were high body temperature (80%), dyspnea (64%), malaise (62%) and cough (56%). The mean value of all clinical laboratory parameters upon hospitalization are presented in Table 1. Abnormal values for CRP, LDH, PLR, D-dimer and NLR were observed. The mean ± SEM value for CRP was 144.7 ± 21.37 mg/L, LDH was 823.4 ± 80.02 IU/L, PLR was 538.2 ± 85.09, NLR was 17.08 ± 2.058, and D-dimer was 2688 ± 499.1 ng/mL. All 14 patients had increased values for ALT, AST and WBC in comparison to the individuals not infected with SARS-CoV-2. The observed statistically difference between the two groups was significant in all cases (p < 0.05).

3.2 Cytokine profile, oxidative stress parameters and commonly used biomarkers

As presented in Table 1, 11 cytokines (including chemokines and growth factors) were analyzed in 14 patients infected with SARS-CoV-2 with severe form of the disease and these values were compared with individuals without SARS-CoV-2 infection. In this comparison, statistically significant increase (p < 0.05, t-test) was observed for IL-6, IL-8, IL-10, VEGF, MCP-1 and EGF in the SARS-CoV-2 patients, while IL-2, IFN-γ, TNF-α and IL-1α were increased but this difference was not significant when compared to the individuals without SARS-CoV-2 infection (p < 0.05, t-test). Important finding of this pilot study is that the parameters of the oxidative stress, d-ROM (448.8 ± 30.37 U.Carr), OSI index (107.7 ± 14.38) and PAT (3048 ± 100.1 U.Carr) were significantly higher (p < 0.05, t-test) in severe COVID-19 patients when compared to the not infected individuals (Table 1). Moreover, we have investigated the correlation among the investigated cytokines, the oxidative stress parameters and CRP, LDH, PLR, D-dimer and NLR. The Spearman r coefficient of correlation between all these parameters is presented as a heat-map on Figure 2. The heat-map confirmed a positive and significant correlation between all cytokines and the parameters of the oxidative stress (d-ROM, PAT and OSI), except a negative correlation between IL-10 and the total antioxidant capacity, PAT. The correlation was not considered to be significant between OS index and the IL-8 (r = 0.3762, p = 0.8552) and between d-ROM and VEGF.
Table 1. Laboratory findings in severe COVID-19 patients and non-infected individuals expressed as mean ± SEM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Severe COVID-19 patients mean ± SEM (n = 14)</th>
<th>Not infected individuals mean ± SEM (n = 20)</th>
<th>p (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 (pg/mL)</td>
<td>250.1 ± 39.07</td>
<td>2.135 ± 0.453</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL-2 (pg/mL)</td>
<td>4.426 ± 2.177</td>
<td>2.005 ± 0.402</td>
<td>0.2818</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>1.936 ± 0.268</td>
<td>1.956 ± 0.137</td>
<td>0.3150</td>
</tr>
<tr>
<td>IL-8 (pg/mL)</td>
<td>108 ± 19.79</td>
<td>7.159 ± 1.298</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>11.14 ± 4.551</td>
<td>0.916 ± 0.219</td>
<td>0.0001</td>
</tr>
<tr>
<td>VEGF (pg/mL)</td>
<td>530.7 ± 147.1</td>
<td>27.04 ± 4.708</td>
<td>0.0001</td>
</tr>
<tr>
<td>IFN-g (pg/mL)</td>
<td>1.487 ± 0.745</td>
<td>0.389 ± 0.082</td>
<td>0.3889</td>
</tr>
<tr>
<td>TNF-a (pg/mL)</td>
<td>5.223 ± 0.751</td>
<td>3.646 ± 0.757</td>
<td>0.090</td>
</tr>
<tr>
<td>IL-1a (pg/mL)</td>
<td>0.4614 ± 0.263</td>
<td>0.2153 ± 0.0422</td>
<td>0.7210</td>
</tr>
<tr>
<td>MCP-1 (pg/mL)</td>
<td>891 ± 92.35</td>
<td>89.61 ± 12.18</td>
<td>0.0001</td>
</tr>
<tr>
<td>EGF (pg/mL)</td>
<td>65.37 ± 17.46</td>
<td>24.28 ± 5.367</td>
<td>0.0318</td>
</tr>
<tr>
<td>d-ROM (U.Carr)</td>
<td>448.8 ± 30.37</td>
<td>271 ± 5.590</td>
<td>0.0001</td>
</tr>
<tr>
<td>PAT (U.Carr)</td>
<td>3048 ± 100.1</td>
<td>2406 ± 71.55</td>
<td>0.0001</td>
</tr>
<tr>
<td>OSI</td>
<td>107.7 ± 14.38</td>
<td>21 ± 2.527</td>
<td>0.0001</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>144.7 ± 21.38</td>
<td>2.1 ± 0.05</td>
<td>0.0001</td>
</tr>
<tr>
<td>LDH (IU/L)</td>
<td>823.4 ± 80.02</td>
<td>156 ± 20.31</td>
<td>0.0001</td>
</tr>
<tr>
<td>NLR</td>
<td>17.08 ± 2.058</td>
<td>1.5 ± 0.02</td>
<td>0.0001</td>
</tr>
<tr>
<td>PLR</td>
<td>538.2 ± 85.09</td>
<td>113 ± 10.35</td>
<td>0.0001</td>
</tr>
<tr>
<td>D-dimer (ng/mL)</td>
<td>2688 ± 499.1</td>
<td>225 ± 22.75</td>
<td>0.0001</td>
</tr>
<tr>
<td>WBC (×10^3/L)</td>
<td>14 ± 2.004</td>
<td>6.1 ± 1.365</td>
<td>0.0019</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>51.93 ± 7.171</td>
<td>28.96 ± 2.658</td>
<td>0.0018</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>61.210 ± 7.283</td>
<td>30.56 ± 3.487</td>
<td>0.0002</td>
</tr>
</tbody>
</table>

Figure 2. Spearman r presented as a heatmap between the investigated cytokines, oxidative stress parameters, and commonly used biomarkers in COVID-19.
interleukins (IFNs, IL-1, IL-6, IL-10, IL-17 and TNF-α) were reported.7,13,18,19

Our study revealed that several cytokines and biomarkers were significantly increased in infected SARS-CoV-2 patients with severe form of the disease in comparison to those who were not, which was accompanied with coagulopathy as determined by deterioration of the platelet related parameters (PLR, D-dimer, IL-6) and MCP-1 as thrombosis related indicator. Huang et al. (2020) reported that MCP-1 levels were much higher in critical ICU patients and additionally that the platelet count was lower in those patients that do not survive.19 Patients from our study were all with severe form of COVID-19 and all of them had died during hospitalization. Moreover, in our patients several of the cytokines had been increased more than 10-fold above the levels of the non-infected that we considered as a baseline. It is worth noting, the statistically significant increase of the VEGF levels more than 10-fold that can be related to the essential role of VEGF in endothelial cell activation by binding to cell surface VEGF receptors. VEGF up-regulation was observed in several viral infections and it has been investigated as a target for potential therapy development.20 In addition, Huang et al., report higher levels of VEGF in hospitalized COVID-19 patients.19

The strong correlation between the investigated cytokines (including chemokines and growth factors), the oxidative stress parameters and some of the commonly used biomarkers (CRP, D-dimers, NLR, PLR) are in line with the proposed cytokine storm as underlying mechanism of the infection. The cytokine storm syndrome occurs when large numbers of leukocytes are activated and release a high concentration of proinflammatory cytokines, with IL-6, IL-10, IFN-γ, MPC-1, IL-1, IL-2 and IL-8 being the foremost. Generally, SARS-CoV-2 infection is associated with oxidative stress, the proinflammatory state, cytokine production, and cell death demonstrated by increase in ROS levels and an alteration of antioxidant defense during the infection.11,21

Even though limited published data are available, we believe that SARS-CoV-2 in line with other RNA viruses triggers oxidative stress by disturbing the pro-antioxidant-antioxidant balance.3,22,23 We have demonstrated the significantly higher level of the d-ROM and OS index values in the infected patients with SARS-CoV-2 when compared with those who were not infected, supporting the hypothesis that viral infection will increase the oxidative stress and complicate the course of the disease. Whilst we consider that the OS index value presents an important parameter that we can have an impact on against COVID-19, by supplementation with antioxidants especially when there is applicable knowledge for several nutraceuticals/vitamins (vitamin C, vitamin D, curcumin, selenium, quercetin and other polyphenols) with proven anti-inflammatory, antioxidant and antiviral capacity.24,25

There are several limitations of the study besides being a single-center experience and a pilot study with only severe and critically ill patients. The herein presented patients were hospitalized at the beginning of the global pandemic when no specific and official guidelines were issued and available to assist the need for hospitalization. They had symptoms developed several days prior being hospitalized, however we believe that these symptoms were not life threatening and the hyper-inflammatory phase was at its beginning stage which is deemed by the obtained levels of the cytokines and the oxidative stress index. Nevertheless, further studies concerning COVID-19 patients with high levels of d-ROMs and OS index are warranted to determine whether supporting antioxidant therapy can reduce the possibility for the fatal outcome of the critically ill COVID-19 patients.
5. Conclusion
This observational pilot study demonstrates a good correlation between the panel of tested cytokines and the parameters of the oxidative stress measured by a fast photometric method that could be used at the beginning of the disease to predict whether COVID-19 will develop in severe form. The presented results will contribute to support the evidences that the cytokine storm syndrome leads as an immunopathogenesis during SARS-CoV-2 infection and by using the oxidative stress parameters (d-ROM, PAT, OS index) physicians can provide timely and early interventions in COVID-19 patients.

Author contributions
MP, DZ, EA contributed to the conception and design of the study. MP and DZ contributed to the oxidative stress parameters analyses, collated the data for the study, and completed all statistical analysis of data. MP wrote the first draft of the manuscript. AE performed the cytokine assay. KS and EA contributed to the clinical evaluation and medical data collection from the COVID-19 patients. All authors read and approved the final version of the manuscript.

Data availability
Underlying data

Data are available under the terms of the Creative Commons Zero “No rights reserved” data waiver (CC0 1.0 Public domain dedication).

References

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I have read the manuscript with great interest. The authors have made a comprehensive study on an important topic. I have some minor recommendations for the authors:

1. The inflammatory background of COVID-19 should be discussed.

2. Treatment options and targeted therapies should be discussed in more detail.

3. Severe COVID-19 complications should be discussed in more detail.

4. The clinical implication of findings obtained from the present study should be underlined.

5. The following literature by Tanacan et al. 2021 may be useful and in my opinion, the mechanisms behind the possible immunologic processes behind COVID-19 may help the authors to improve their manuscript.

References


Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**
Yes

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Perinatology, maternal-fetal medicine, prenatal diagnosis

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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