

DNA damage and changes in oxidized biomolecules in COVID-19 patients treated in intensive care units: a single center experience

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Abstract. – OBJECTIVE: COVID-19 is a deadly disease. Investigations are being conducted on the underlying mechanisms to predict prognosis and reduce mortality rates. In this study, the extent of DNA damage and serum levels of oxidized biomolecules were investigated. We hypothesize that malondialdehyde (MDA) and protein carbonyl (PC) serum levels and DNA damage levels may be biomarkers that can be used in prognostic decision making and prediction of mortality in COVID-19 patients.

PATIENTS AND METHODS: Patients included in the study were divided into two groups according to their survival. These groups were compared in terms of serum MDA, PC levels, DNA damage degrees and mortality on the 1st, 3rd, and 5th days of ICU admission.

RESULTS: In patients who died, MDA levels increased over time ($p=0.023$), PC levels peaked on the third day of admission to the intensive care units (ICU), and then decreased, while DNA damage increased gradually ($p=0.013$). In surviving patients, MDA levels decreased over time ($p=0.018$); PC levels were at their peak on the first day of admission to the ICU and then decreased ($p=0.018$); DNA damage decreased initially, and then increased minimally compared to Day 1.

CONCLUSIONS: For COVID-19 ICU patients, serum levels of MDA and PC and degrees of DNA damage can strengthen prognostic decision-making and contribute to reducing mortality.

Key Words:

COVID-19, DNA damage, Oxidized biomolecules, Intensive care unit.

Introduction

The intensive care unit (ICU) is a special area where clinical stabilization is provided in critically ill patients, where a considerable number of

specialists personnel work, and where advanced monitoring and organ support can be offered. An effective ICU requires an integrated approach that extends beyond the limits of the ICU. It includes early warning and early response systems and a multidisciplinary approach to patient treatment before and during ICU stay, as well as comprehensive follow-up and quality palliative care¹. Although scoring systems and clinical findings provide a valuable framework for characterizing disease severity and informing ICU discharge, new strategies aimed at improving the quality of care provided to patients may in turn improve ICU performance.

Oxidative stress is a biochemical process characterized by an imbalance between the production of reactive oxygen species (ROS) and that of antioxidants, which induces oxidative damage of biomolecules and alters cellular physiology. The balance in this process has an important place in the protection of vital functions. Disruption of this balance causes damage to critical biomolecules and cells, potentially affecting all organismal mechanisms where reactive species are produced that cause oxidative stress². The hyperoxidative state, that occurs in sepsis and widespread inflammation, damages proteins, lipids, DNA, and RNA by the action of ROS^{3,4}. Lipids, proteins, and RNA can be transformed and replaced quite easily through resynthesis. However, damaged DNA cannot be repaired or replaced by repair mechanisms⁴. In general, oxidative stress is closely associated with the exacerbation of chronic inflammation, genomic instability, and cancer⁵.

Multidimensional scoring systems based on extensively measured clinical and physiological parameters improve the accuracy of the prognosis of patients admitted to ICU⁶. Consequently, there is a need to develop biomarkers with which

clinicians can further improve the accuracy of prognosis and risk estimation for patients in the ICU. This is crucial for early diagnosis and treatment. Advanced age, severe acute respiratory distress syndrome, sepsis (markers of inflammation), and certain underlying medical comorbidities are associated with high mortality rates^{7,8}. Death due to SARS-CoV-2 infection – causing COVID-19 – may be associated with oxidation of biomolecules such as lipids, proteins, and DNA. Measurements of the levels of these biomolecules can be used to improve the accuracy of risk estimation and prognosis for ICU patients, adding to multidimensional scoring systems. For COVID-19 ICU patients, serum levels of MDA and PC and degrees of DNA damage can strengthen prognostic decision-making and contribute to reducing mortality. Accordingly, in this study, malondialdehyde (MDA) and protein carbonyl (PC) serum levels and the extent of lymphocyte DNA damage were evaluated in blood samples of 25 COVID-19 patients (on the 1st, 3rd, and 5th days of ICU hospitalization). Lymphocyte DNA damage was detected using the comet assay technique. The correlations between these values and the prognosis and status of the study participant on discharge from the ICU were evaluated using measurements taken at different times during the patient's admission to a COVID-19 ICU.

Patients and Methods

Our study was performed in accordance with the principles of the Declaration of Helsinki and was approved by the Harran University Clinical Research Ethics Committee (Approval Number: HRÜ/22.16.13). From November 2021 to December 2021, heparinized venous blood samples were collected from 25 patients diagnosed with COVID-19 at the tertiary general ICU of Harran University Hospital. The patients were admitted to the ICU according to the necessity of ventilator support, coma scores, comorbidities, biochemical parameters, and aggressiveness of the clinical course. ICU patients with chronic diseases other than COVID-19 were excluded from the study. The patients were followed up for 1-4 weeks. Venous blood samples were drawn at the same time every day on the 1st, 3rd, and 5th day of admission to the ICU to determine MDA and PC serum levels and the extent of DNA damage.

Samples Preparation

First, plasma and mononuclear leukocytes were obtained from 5 mL of heparinized venous blood samples drawn from participants. For MDA and PC measurements, 3 mL of the heparinized venous blood sample was centrifuged at 1,800 g for 10 min. The plasma samples obtained after centrifugation were stored at -80°C until they were analyzed. The remaining 2 mL of the heparinized venous blood samples were layered onto Histopaque-1070 (Sigma, St. Louis, MO, USA) solution (1:1). The mixture of heparinized whole blood and Histopaque was centrifuged at +4°C and 500 g for 30 min to obtain peripheral mononuclear leukocytes. After centrifugation, the interface band containing leukocytes was aspirated with a pipette into a tube containing phosphate-buffered saline (PBS) in a 1:1 ratio, and then centrifuged at +4°C and 300 g for 10 min. Finally, the mononuclear leukocyte pellet was suspended in Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco Life Technologies, Grand Island, NY, USA), and cell counted. The mononuclear leukocytes were cryopreserved by diluting with 1.5×10^6 cells/mL to 2×10^6 cells/mL of freezing media [(RPMI 1640 medium containing 20% fetal calf serum (Sigma, St. Louis, MO, USA) and 5% DMSO (Sigma, St. Louis, MO, USA)]⁹. The cryopreserved leukocytes were stored at -80°C until comet assay analyses were performed.

Determination of MDA Levels

The plasma MDA levels were measured using the method utilized by Jain et al¹⁰. First, 800 µL of PBS buffer (pH 7.4) and 25 µL of butylated hydroxytoluene (Merck, Darmstadt, Germany) solution were added to 200 µL of plasma and the mixture was vortexed. Then, 500 µL of 30% trichloroacetic acid (Sigma, St. Louis, MO, USA) was added to the mixture and left on ice for 2 h. The resulting mixture was centrifuged (Hettich Universal 320R, Hettich Zentrifugen, Germany) at 2,000 g for 15 min at 25°C. Subsequently, 1 ml of supernatant was placed in a fresh tube. Then, 75 µL of 0.1 M ethylenediaminetetraacetic acid (EDTA; Sigma, St. Louis, MO, USA) prepared in 0.05 N NaOH (Merck, Darmstadt, Germany) and, 1% thiobarbituric acid (Sigma, St. Louis, MO, USA) were added to the supernatant and mixed. Finally, the mixture was placed in boiling water for 15 min, after which it was immediately cooled; its optical density at 532 nm was then measured. All measurements were performed

in duplicate on a multiplate reader (Varioskan™ LUX; ThermoFisher Scientific, Waltham, MA, USA). MDA levels were calculated using a molar extinction coefficient for MDA of $1.56 \times 10^5 \text{ cm}^{-1} \cdot \text{M}^{-1}$, and the results were expressed in nmol/mL.

Determination of PC Content

Plasma PC concentrations were measured using a 2,4-dinitrophenylhydrazine (DNPH) assay¹¹. The assay is based on the spectrophotometric detection of the reaction between DNPH and PC to form protein hydrazone. The total protein content should be measured to indicate the ratio of the PC level to the total amount of plasma protein. The plasma total protein content was measured using a μ Drop plate (Varioskan™ LUX; ThermoFisher Scientific, Waltham, MA, USA). Plasma PC levels were expressed as nmoL/mg protein.

Measurement of DNA Damage

A 37°C-water bath was used to thaw the cryofrozen peripheral blood mononuclear cells (PBMCs). After dissolution, 400 μ L of RPMI-1640 and 100 μ L of 10% dextrose were added to 500 μ L cell suspensions. The mixture was centrifuged at +4°C and 1,100 rpm for 10 min, and the cell pellets were then diluted in PBS to the desired cell density and kept on ice. DNA damage in the PBMCs was analyzed *via* a comet assay, per Singh et al¹² – with minor modifications. Ten microliters of PBMC suspension (approximately 20,000 cells) were mixed with 80 μ L of 0.7% low-melting-point agarose (Sigma, St. Louis, MO, USA) in PBS at 37°C. Subsequently, 80 μ L of this mixture was layered onto slides that had previously been coated with 1.0% hot (60°C) normal melting point agarose, and then covered with a coverslip at 4°C for at least 5 min to allow the agarose to solidify. The coverslips were then removed, and the slides submerged in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na; 10 mM Tris-HCl, pH 10-10.5; 1% Triton X-100; and 10% DMSO added just before use) for at least 1 h. Subsequently, the slides were immersed in freshly prepared alkaline electrophoresis buffer [0.3 mol/L NaOH (Sigma, St. Louis, MO, USA) and 1 mmol/l Na₂EDTA, pH >13] at 4°C for unwinding (40 min) and were then subjected to electrophoresis (25 V/300 mA, 25 min). After electrophoresis, the slides were stained with ethidium bromide (Sigma, St. Louis, MO, USA; 2 μ L/ml in distilled H₂O; 70 μ L/slide), covered with a coverslip, and analyzed under

a fluorescence microscope (Olympus, Tokyo, Japan) equipped with epifluorescence optics and a rhodamine filter (excitation wavelength: 546 nm, barrier filter: 580 nm). Images of 100 randomly selected nuclei from each participant (50 cells from each of the two replication slides) were visually analyzed¹³. Each image was classified based on the intensity of the fluorescence in the comet tail, and was attributed a value of either 0, 1, 2, 3, or 4 (from undamaged Class 0 to maximally damaged Class 4), so that the total score for each participant could fall between 0 and 400 arbitrary units (AU). Photomicrographs of representative samples are presented in Figure 1. All procedures were performed by the same biochemistry staff, and DNA damage was detected by a single observer who was unaware of the subject's diagnosis.

Statistical Analysis

The results are expressed as means \pm standard deviations (SD). Nonparametric continuous variables were compared using the Mann-Whitney U-test, and parametric variables were compared using the Student's *t*-test. Qualitative variables were assessed using the chi-square test, and correlation analyses were performed using Pearson's correlation test. Differences were considered to be statistically significant at $p < 0.05$. The data were analyzed using SPSS 25.0 program for Windows (IBM Corp., Armonk, NY, USA).

Results

A total of 25 patients with confirmed SARS-CoV-2 infection were enrolled in the study, comprising 10 (40%) females and 15 (60%) males. The participants were divided into groups based on their survival outcomes: 16 participants were discharged as *exitus* from the COVID-19 ICU, and the other nine participants had a healthy discharge status from the COVID-19 ICU.

MDA and PC levels and DNA damage results for three separate times for participants in the two study groups are presented in Table I and Figure 2. When the two groups were compared as two independent groups (i.e., Discharged as Exitus Group vs. Healthy Discharge Group), there was no statistical difference in the levels of MDA and PC and the extent of DNA damage between the Discharged as Exitus Group and the Healthy Discharge Group, except for the Day 5 MDA

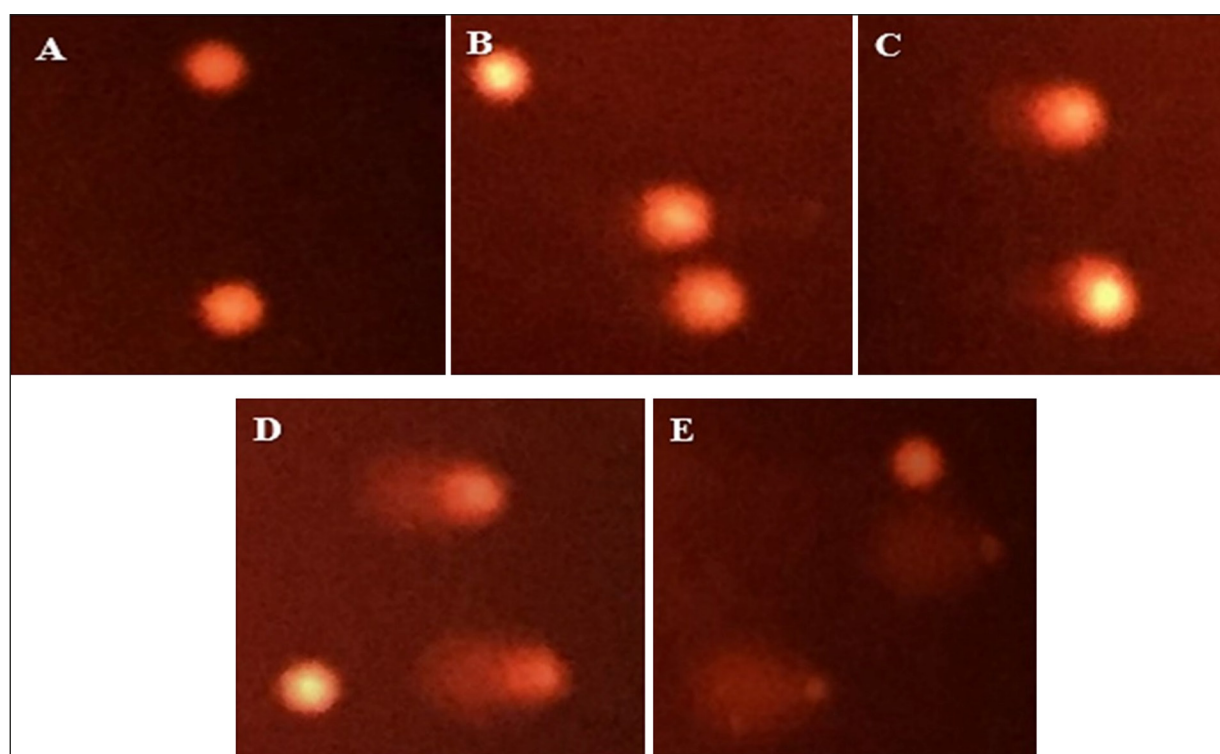


Figure 1. Photomicrographs were showed according to fluorescence intensity in the comet classes. **A**, Class 0-undamaged. **B**, Class 1. **C**, Class 2. **D**, Class 3. **E**, Class 4-maximally damaged. Slides were analyzed using a fluorescence microscope at a 20× magnification.

results ($p > 0.05$). The Day 5 MDA results for the Discharged as Exitus Group were higher than those of the Healthy Discharge Group for the same day, with a statistically significant difference ($p = 0.003$).

Table II presents the Freedman test analysis results for MDA and PC levels and DNA damage in the blood samples of participants who were discharged as exitus from the COVID-19

ICU. The MDA levels and the extent of DNA damage increased gradually with length of hospitalization at the COVID-19 ICU and were statistically significant ($p = 0.023$ and $p = 0.013$, respectively). However, PC levels, an indicator of protein oxidation, were found to have risen on Day 3 and Day 5 compared to Day 1. However, this difference was not statistically significant ($p = 0.472$).

Table I. Comparison of MDA, PC, and DNA damage levels in study groups according to days.

Days	Parameters	Exitus discharge (n = 16)	Healthy discharge (n = 9)	<i>p</i>
Day 1	MDA, nmol/mL	15.28 (6.86)	17.78 (9.98)	0.084
	PC, nmol/mg Protein	0.22 (0.11)	0.25 (0.03)	0.161
	DNA Damage, AU	99 (110.5)	120 (78)	0.713
Day 3	MDA, nmol/mL	16.69 (15.37)	14.5 (6.24)	0.275
	PC, nmol/mg Protein	0.24 (0.09)	0.2 (0.05)	0.682
	DNA Damage, AU	150 (91)	102 (74)	0.609
Day 5	MDA, nmol/mL	22.77 (8.59)	11.7 (3.28)	0.003
	PC, nmol/mg Protein	0.24 (0.08)	0.2 (0.02)	0.063
	DNA Damage, AU	173 (101)	128 (28)	0.31

Results are expressed as median (interquartile range). Bold values statistically significant at $p < 0.05$. *p*-value obtained Mann-Whitney U test. MDA – Malondialdehyde, PC – Protein Carbonyl, DNA – Deoxyribo nucleic acid

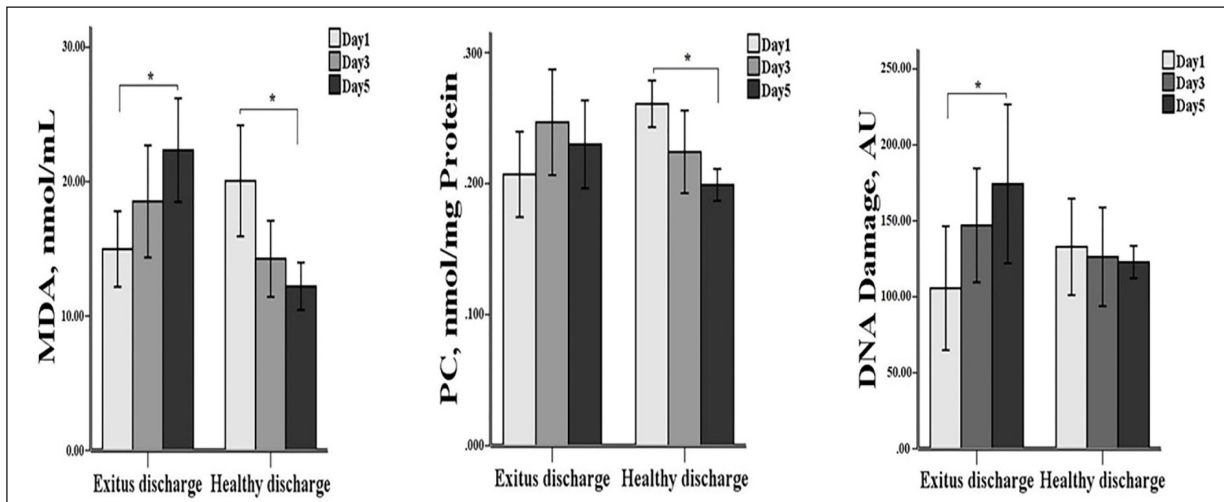


Figure 2. Oxidation of lipids (MDA), Oxidation of proteins (PC) and Oxidation of Genomic DNA (DNA Damage) during ICU. Results are expressed as mean \pm SE. Statistical differences were shown as. MDA – Malondialdehyde, PC – Protein Carbonyl, DNA – Deoxyribo nucleic acid, ICU – Intensive care unit * $p < 0.05$.

Table II. Change of MDA, PC, and DNA damage levels according to days in exitus discharge.

	Day 1	Day 3	Day 5	<i>p</i>
MDA, nmol/mL	15.28 (6.86)*	16.69 (15.37)	22.77 (8.59)	0.023
PC, nmol/mg Protein	0.22 (0.11)	0.24 (0.09)	0.24 (0.08)	0.472
DNA Damage, AU	99 (110.5)*	150 (91)	173 (101)	0.013

Results are expressed as median (interquartile range). *p*-value obtained Friedman test. Bold values statistically significant at $p < 0.05$. The statistical relationship between the two dependent groups was obtained using the Wilcoxon test. *Day 1 vs. Day 5. MDA – Malondialdehyde, PC – Protein Carbonyl, DNA – Deoxyribo nucleic acid.

Table III presents the Freedman test analysis results for MDA and PC levels and the extent of DNA damage in the blood samples of participants who had a healthy discharge status from the COVID-19 ICU. The MDA and PC levels decreased gradually with the length of hospitalization at the COVID-19 ICU, and this decrease was statistically significant. The *p*-value for both tests was 0.018. Although the extent of DNA damage differed from day to day, there was no statistically significant relationship between these values ($p > 0.05$).

Discussion

ROS oxidize various biological macromolecules, such as lipids, proteins, and nucleic acids, causing structural and functional changes in these molecules (i.e., oxidative stress)^{3,4}. It has been reported that many pathologies, including inflammation, sepsis, malignancy, cardiovascular diseases and stroke, and acute diseases that cause multiple organ failure (MOF), which are known to be the most common causes of death, are closely related to oxidative stress^{5,14-18}. A bas-

Table III. Change of MDA, PC, and DNA damage levels according to days in healthy discharge.

	Day 1	Day 3	Day 5	<i>p</i>
MDA, nmol/mL	17.78 (9.98)*	14.50 (6.24)	11.7 (3.28)	0.018
PC, nmol/mg Protein	0.25 (0.03)*	0.20 (0.05)	0.2 (0.02)	0.018
DNA Damage, AU	120 (78)	102 (74)	128 (28)	0.651

Results are expressed as median (interquartile range). *p*-value obtained Friedman test. Bold values statistically significant at $p < 0.05$. The statistical relationship between the two dependent groups was obtained using the Wilcoxon test. *Day 1 vs. Day 5. MDA – Malondialdehyde, PC – Protein Carbonyl, DNA – Deoxyribo nucleic acid.

al concentration of ROS is indispensable for the manifestation of cellular functions, but excessive levels of ROS damage cellular macromolecules, such as DNA, lipids, and proteins, eventually leading to necrosis and apoptotic cell death¹⁷. In this study, the serum levels of MDA and PC (which are the oxidized forms of these macromolecules) and the extent of DNA damage, were investigated in blood samples taken at different times from patients hospitalized at a COVID-19 ICU. At the end of a four-week follow-up, we found that MDA and PC serum levels and the extent of DNA damage increased over time in those discharged from the ICU dead (discharged as exitus) and decreased in those with a healthy discharge status. Because lipids and lipoproteins in biological membranes are major peroxidation targets, tests for lipid peroxidation are commonly used to determine the oxidative state¹⁹.

MDA is the product of lipid oxidation, and MDA levels have been used as a mortality biomarker in critically ill patients. MDA levels in individuals who die after traumatic brain injuries increase over time and are higher than in individuals who survive such injuries. This has been attributed to the loss of microvascular regulation, vasogenic edema formation, and cellular dysfunction due to high ROS levels²⁰. Similarly, patients with sepsis – who have the highest mortality rates in ICUs – had sustained high MDA serum levels during the first week of follow-up, which is associated with disease severity and 30-day mortality²¹. In our study, the MDA levels increased over time in patients discharged from the COVID-19 ICU dead and decreased over time in those with a healthy discharge status, and this was statistically significant ($p=0.023$ and $p=0.018$, respectively). These results support the findings of previous studies reporting an association between MDA levels and mortality and prognosis.

Another interesting oxidative stress biomarker is PC. PC concentration is a marker of oxidative damage to proteins and is not used as often as MDA in the evaluation of critically ill patients²². This is partly because oxidized proteins are broken down within hours or days, while oxidized lipids are detoxified within minutes. Another critical issue is that PC groups are formed early and are more stable than lipid peroxidation products. In this context, Regueira et al²³ found that lipoperoxidation and protein oxidative damage exhibited different kinetics in 21 patients with septic shock. Indeed, while lipoperoxidation increases with time, protein oxidative damage peaks at ear-

ly ICU admission and subsequently declines over time. Similarly, in our study results, PC levels peaked on the third day of ICU hospitalization among the ICU non-survivor group, and then decreased. Among the ICU survivor group, PC levels were at their highest levels on the first day of hospitalization, and then decreased. We believe that the PC level results we obtained for the ICU survivor and non-survivor groups contribute to validating the usability of this parameter as a clinical marker.

The comet assay is a versatile and easily performed DNA damage detection technique. The technique employs a genotoxicity test that does not require radioactive labeling, yields sensitive and fast results, and is also widely used because of its low cost¹³. It has been reported that DNA damage is high in many diseases associated with oxidative stress. Furthermore, it has been postulated that oxidative stress – which occurs in clinical conditions caused by microorganisms, e.g., pneumonia – may cause DNA damage and may be indirectly linked to disease severity and mortality. In a recent study²⁴ conducted in this direction, it was found that DNA damage was higher in patients with pneumonia (thus requiring ICU admission) than in the control group. In addition, it has been reported that the extent of DNA damage in patients with intubated pneumonia is lower than in patients with non-intubated pneumonia. It has been stated that the reason for the lower rate in intubated patients is better oxygenation and less respiratory workload due to oxygen support²⁴. MOF with high ICU requirement is the most serious complication of severe multiple trauma¹⁶. Experimental evidence indicates that MOF may be associated with stress-induced cell death *via* apoptosis in the trauma state. A study²⁵ has shown that DNA damage in severe trauma patients increases gradually from the initial point of hospitalization and then decreases in response to treatment. This increase is reported to be associated with apoptotic and necrotic DNA damage. In our study, DNA damage increased gradually in non-survivor patients and was statistically significant ($p=0.013$). In the ICU survivor group, the extent of DNA damage decreased on the third day in response to treatment. This decrease suggests that cellular homeostasis was maintained, and DNA repair mechanisms were activated; therefore, the decrease is an indicator of systemic recovery. We think that the minimal increase in DNA damage on the fifth day may be related to the length of stay at the ICU.

Limitations

There are some limitations to this study. Our study was conducted on a limited number of patients at a single center. Confirming our results with larger patient groups at multiple centers will make this study a significant contribution to the literature on this subject.

Conclusions

Our study reports that serum MDA and PC levels, and DNA damage increase in COVID-19 patients, which paves the way for morbidity and mortality. Serum MDA level is prominent in predicting both recovery and mortality. In addition, we think that the serum PC level and the degree of DNA damage are biomarkers that can be used to make prognostic decisions and predict mortality in patients in the ICU. In conclusion, for COVID-19 ICU patients, employing scoring systems and clinical findings in prognostic processes in combination with MDA and PC serum levels, and DNA damage degree will strengthen prognostic decision-making. These observations need to be confirmed using a large population sample.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethics Approval

This study was approved by the Harran University Faculty of Medicine Clinical Research Ethics Committee (Approval Number: HRU/22.16.13).

Informed Consent

Informed consent was obtained from the participants or their first-degree relatives for the study.

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Data Availability

The data supporting this article are available from the corresponding author on reasonable request.

Authors' Contribution

Erdoğan Duran designed the research, drafted the article, and conducted the research, project supervision, writing-reviewing and editing. Seyhan Taşkın, Başak Pehlivan, Hakim Çelik data collection, data checking, data entry and analysis. All authors read and approved the final version of the manuscript.

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