DNA damage in peripheral blood lymphocytes of severely ill COVID-19 patients in relation to inflammatory markers and parameters of hemostasis

Olgica Mihaljevic1,*, Snezana Zivancevic-Simonovic1, Vojislav Cupurdija3,4, Milos Marinkovic4, Jovana Tubic Vukajlovic6, Aleksandra Markovic5, Marijana Stanojevic-Pirkovic6, Olivera Milosevic-Djordjevic2,5

1Department of Pathophysiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
2Department of Genetics, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
3Department of Internal Medicine, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
4Department of Internal medicine, University Clinical Center Kragujevac, Kragujevac, Serbia
5Department of Biology, Faculty of Sciences, University of Kragujevac, Kragujevac, Serbia
6Department of Biochemistry, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

*Corresponding author. Department of Pathophysiology, University of Kragujevac, Faculty of Medical Sciences, Svetozara Markovica 69, 34000 Kragujevac, Serbia. E-mail: vrndic07@yahoo.com

Abstract

Bearing in mind that a variety of agents can contribute to genome instability, including viral infections, the aim of this study was to analyze DNA damage in hospitalized COVID-19 patients and its relationship with certain laboratory parameters. The potential impact of applied therapy and chest X-rays on DNA damage was also estimated. The study population included 24 severely COVID-19 patients and 15 healthy control subjects. The level of DNA damage was measured as genetic damage index (GDI) by comet assay. The standard laboratory methods and certified enzymatic reagents for the appropriate autoanalyzers were performed for the determination of the biochemical and hematological parameters. COVID-19 patients had significantly higher level of DNA damage compared with control subjects. The absolute number of neutrophil leukocytes was statistically higher, while the absolute number of lymphocytes was statistically lower in COVID-19 patients than in healthy controls. The analysis of the relationship between DNA damage and laboratory parameters indicated that GDI was positively correlated with interleukin 6 (IL-6) concentration and negatively with platelet count in COVID-19 patients. The level of DNA damage was slightly higher in female patients, in whom it was demonstrated a positive correlation of GDI with C-reactive protein (CRP) and procalcitonin. Likewise, there was a negative relationship of GDI with platelet count, and positive relationship of GDI and activated partial thromboplastin time (aPTT) in female population. The applied therapy (antibiotics, corticosteroid, anticoagulant, and antiviral therapy) as well as chest X rays has been shown to have genotoxic potential. The level of DNA damage significantly corresponds to the inflammatory markers and parameters of hemostasis in COVID-19 patients. In conclusion, inflammation, smoking habit, applied therapy, and chest X rays contribute to a higher level of DNA damage in COVID-19 patients.

Keywords: COVID-19; DNA damage; inflammation; hemostasis abnormalities

Introduction

Genome integrity is ensured by the constant monitoring of DNA repair and replication, and an uninterrupted cell-cycle development. The occurrence of genetic changes as a consequence of DNA damage and the lack of its repair due to the reduced DNA repair capacity [1] results in the genome instability. A variety of agents can contribute to DNA damage and genome instability, both endogenous and exogenous, including viral infections [2]. Viruses could induce the genotoxic lesions by acting on host DNA directly, or by stimulating the production of reactive oxygen species (ROS), and modulating signaling pathways involved in the cell cycle [3–5].

Severe acute respiratory syndrome coronavirus (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), has rapidly taken on pandemic proportions and become a global threat to human health around the world [6]. This RNA virus of high virulence and rapid transmissibility causes severe upper respiratory tract infection, which can be complicated by a massive inflammatory response and multiorgan dysfunction [7, 8]. Accordingly, there are abnormal laboratory results in COVID-19 patients as a consequence of some hematological, inflammatory, coagulation, hepatic, muscular, cardiac, and renal alterations [8, 9].

A storm of proinflammatory mediators induced by viral infection could play a role in DNA damage and the development of harmful somatic mutations, as well as in the disruption of cell cycle regulation [10, 11]. Specific proinflammatory mediators may induce the production of ROS in nonphagocytic
and phagocytic cells [12–14]. The resulting oxidative stress promotes genomic instability, by reducing gene expression for DNA mismatch repair. On the other hand, in vitro studies have shown that SARS-CoV-2 components could directly disrupt DNA repair by inhibiting the key DNA repair proteins [15] and DNA replication through the interaction with DNA polymerase enzymes [16]. The absence of adequate DNA repair, the weakening of cell cycle checkpoints, and an increased of cell proliferation results in the maintenance and accumulation of DNA damage which can be detected in peripheral blood lymphocytes.

Thus, the aim of our study was to analyze DNA damage in hospitalized COVID-19 patients as well as its association with certain laboratory parameters and the applied diagnostic and therapeutic agents. In addition, we evaluated the differences in the degree of DNA damage and complete blood count between COVID-19 patients and healthy controls. To the best of our knowledge, this is the first study about DNA damage in COVID-19 patients.

**Materials and methods**

**Study population**

The study enrolled 24 patients of COVID-19, hospitalized in the Clinic for Lung Diseases of University Clinical Center Kragujevac, who were sampled during August and September 2021. There were 16 (66.7%) males and 8 (33.3%) females of mean age of 55.83 ± 13.41 years. All patients fulfilled the criteria for COVID-19 set up by the World Health Organization [17]. Accordingly, the study included the subjects with SARS-CoV-2 infection, confirmed by real-time polymerase chain reaction, and with radiographically proven pneumonia (the presence of the consolidation of the lungs). They were not vaccinated prior to infection with COVID-19. At the time of sampling, each patient had antibiotic (antibiotic type depends on the severity of disease with the same regimen within the type, according to Europian Respiratory Society/American Thoracic Society guidelines for treatment of community-acquired pneumonia or hospital-acquired pneumonia), corticosteroid (methylprednisolone in a daily dose of 60–100 mg), and anticoagulant therapy (60 mg/24 h of enoxaparin sodium) as well as oxygen therapy. Also, all COVID-19 patients were treated with Favipiravir (6-Fluoro-3-oxo-3,4-dihydropyrazine-2-carboxamide), an antiviral agent, according to Treatment Guidelines for COVID-19. Before the enrollment in the study, none of the patients had received anti-interleukin 6 therapy. The blood samples were taken during the hospitalization, i.e. 2–3 days after chest X-ray and after the administration the therapies mentioned above. None of the patients had chronic inflammatory and autoimmune diseases, malignant diseases, and coagulation disorders. There were 11 smokers and 13 nonsmokers COVID-19 patients.

The control group comprised 15 healthy subjects, 9 (60.0%) males, and 6 (40.0%) females of mean age 33.80 ± 11.68 years. They were colleagues who were willing to engage in the study and who have not previously crossed COVID-19. Since four vaccines based on different techniques were used in our country, to avoid any influence of vaccine on the parameters measured, only nonvaccinated subjects were enrolled in control group. In all control subjects, the antigenic test for coronavirus was negative and they did not develop COVID-19 symptoms in the coming weeks. Besides, all hematological and biochemical parameters at the time of sampling were in the reference range. The control subjects had no acute or chronic infection, autoimmune diseases, malignant diseases, coagulation disorders, or other conditions that could affect the tested parameters. They had not been exposed to any known genotoxic agents. Three control subjects were smokers and 12 nonsmokers.

The study was planned according to the Declaration of Helsinki, and it was approved by the Ethical Committee of University Clinical Center Kragujevac (number 01/21-138). Written informed consent for participation in the study was obtained from all patients and control subjects.

**Sample size**

The target sample size was estimated by G*Power 3.1. Software, based on a study of similar design [18]. By using T test with α error = 0.05 and a power of 1–β = 0.95, we determined that the required sample size is 12 subjects. Based on this calculation, we included twice as many patients in our study.

**Measurement of DNA damage**

The alkaline version of the comet assay was performed according to the methodology of Singh et al. [19], with alterations suggested by Collins and Dušinská [20]. The comet assay was performed using two layers of agarose on microscope slides. Before the experiment, frosted microscope slides were cleaned with 96% ethanol and dried over a burner, and then clear slides were coated with a layer of 1% of normal-melting-point agarose and dried for 3 days.

Lymphocytes (the white ring) were isolated from whole heparinized peripheral blood, within 1 hr from blood sampling, at room temperature using Histopague-1077. Before processing, the blood was transported in containers at 4°C. Thereafter, the lymphocytes were washed twice in RPMI medium (by centrifugation at 1600 × g for 10 min) and resuspended in PBS. The cell viability, as assessed by the Trypan blue test, was >89%. Cell counting was performed using a light microscope with a hemocytometer filled with 10 µl homogenized cell suspension. Then, the lymphocytes were resuspended in PBS to obtain 1 × 10⁵ cells/ml.

One hundred microliters of cell suspension (10,000 cells) were mixed with 100 µl of 1% low melting-point agarose (dissolved in PBS, in a final concentration of 0.01 g/ml) and spread onto the slide per two drops of 90 µl and covered by a coverslip. The gels were left for about 2 min on ice in order to solidify agarose, and coverslips were removed, and then immersed to lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% dimethyl sulfoxide, pH 10) for 2 h, in the dark, at 4°C. After lysis of agarose-embedded cells, alkaline denaturation was performed in an electrophoresis buffer solution for 30 min (10 M NaOH, 200 mM EDTA, pH > 13) and slides were electrophoresed for 30 min at 25 V (0.7 V/cm) and 300 mA. Subsequently, the slides were washed in neutralizing Tris–HCl buffer three times for 5 min (0.4 M Tris, pH 7.5) and rinsed in distilled water. For visualization, slides were stained with 50 µl ethidium bromide (20 µg/ml). Experiments were performed in the dark to minimize the induction of additional DNA damage.

One hundred randomly selected cells (50 cells from each of two replicate gels) were scored, using a Nikon E50i fluorescent microscope at 400× magnification. The DNA damage
DNA damage in COVID-19

was quantified by visual classification of cells into five comet classes, according to the tail intensity and length, from 0 to 4: class 0—no damage; class 1—low damage; class 2—medium damage; class 3—high damage, and class 4—total destruction. Representative images of comets classified in five different classes are also seen in Figure 1. The Genetic Damage Index (GDI) was calculated for each sample following Pitarque et al. [21] using the following formula:

\[
GD1 = \frac{\text{Class 1} + 2 \times \text{Class 2} + 3 \times \text{Class 3} + 4 \times \text{Class 4}}{\text{Class 0} + \text{Class 1} + \text{Class 2} + \text{Class 3} + \text{Class 4}}
\]

Determination of biochemical and hematological parameters

The biochemical parameters were analyzed using standard accepted methods in Laboratory diagnostic service of the University Clinical Center Kragujevac. Serum concentrations of C-reactive protein (CRP), procalcitonin, interleukin 6 (IL-6), ferritin, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatine kinase (CK), pro-brain natriuretic peptide (pro-BNP), urea, and creatinine were measured by the reagents certified and validated for the use on Oly AU 680 (Beckman Coulter Inc. Brea, USA) for CRP, ferritin, LDH, AST, ALT, CK, urea and creatinine, and Cobas e 411 chemical analyzer (Roche diagnostics GmbH, Mannheim, Germany) for procalcitonin, IL-6 and pro-BNP. The reference ranges were as follows: CRP < 5 mg/l; procalcitonin < 0.5 ng/ml; IL-6 < 7 pg/ml; ferritin 20–300 μg/l; LDH 220–450 U/l; AST 0–40 IU/l; ALT 0–40 IU/l; CK < 171 U/l; pro-BNP < 125 pg/ml; urea 3–8 mmol/l; and creatinine 49–106 μmol/l.

The automated DxH 800 Hematology Analyzer (Beckman Coulter, Inc. Brea, USA) was used for the assessment of hematological parameters: hemoglobin level (range 138–175 g/l for males and 110–157 g/l for females), hematocrit (0.415–0.530 l/l for males and 0.356–0.470 l/l for females), blood count of erythrocytes (range 4.34–5.72 x 10¹²/l for males and 3.86–5.08 x 10¹²/l for females), leucocytes (3.70–10.0 x 10⁹/l), and platelets (135–450 x 10⁹/l). Based on the absolute counts of leucocytes’ subtypes (neutrophils, lymphocytes, monocytes) and platelets, the following indices were calculated: neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic inflammation response index (SIRI) (defined as multiplication of neutrophils and monocytes divided by lymphocytes count).

Blood coagulation parameters (prothrombin time [PT], activated partial thromboplastin time [aPTT], D-dimer, and fibrinogen) were determined by ACL TOP 350CTS (Beckman Coulter Inc. Brea, USA). The reference ranges of the coagulation parameters were as follows: PT 11.8–15.3s; aPTT 25–35s; D-dimer < 0.50 μg/ml; and fibrinogen 2–5 g/l.

Statistical analysis

The commercial SPSS version 20.0 for Windows was used for statistical analysis. All data were expressed as mean ± standard deviation. The differences in the analyzed parameters between two groups of subjects were evaluated using independent sample T-test (in the case of normal distribution of variables), respectively, Mann–Whitney test (in the case of non-normal distribution of variables). Bivariate correlation test (with determination of Pearson/Spearman coefficient) was performed to assess the relationship between the degree of DNA damage and laboratory parameters. Linear regression analysis was applied to identify predictors of DNA damage. P value less than 0.05 was considered as statistically significant.

Results

We studied the differences in the degree of DNA damage and complete blood count between COVID-19 patients and healthy controls as well as the potential association of GDI to certain laboratory parameters in COVID-19 patients.

Comet assay showed that DNA damage in peripheral blood lymphocytes of COVID-19 patients was significantly higher than in healthy subjects (1.86 ± 0.29 vs. 0.35 ± 0.06) (P < 0.001) (Table 1).

Our study also included analysis of the complete blood count in COVID-19 patients and healthy controls. We found that there were statistically significant differences in the number of neutrophil leukocytes and lymphocytes between COVID-19 patients and control subjects (Figure 2A). Accordingly, significant differences in NLR and SIRI were

Figure 1. The photographs of cells obtained by using a Nikon E50i fluorescent microscope at 400× magnification after performing the comet assay in severely ill COVID-19 patient (A) and healthy controls (B).
Table 1. Degree of DNA damage in peripheral blood lymphocytes of COVID-19 patients (a) and control subjects (b) measured by comet assay

| Patients no | Number of analyzed cells | Comet classification | GDI (%) |
|-------------|--------------------------|----------------------|---------|
| 1           | 100                      | 29 20 28 16 7        | 1.52    |
| 2           | 100                      | 17 11 26 20 26       | 2.27    |
| 3           | 100                      | 39 8 23 7 23         | 1.67    |
| 4           | 100                      | 26 9 24 9 32         | 2.12    |
| 5           | 100                      | 38 10 23 13 16       | 1.59    |
| 6           | 100                      | 34 15 17 9 25        | 1.76    |
| 7           | 100                      | 29 10 20 10 31       | 2.04    |
| 8           | 100                      | 33 14 25 5 23        | 1.71    |
| 9           | 100                      | 29 7 30 7 27         | 1.96    |
| 10          | 100                      | 17 9 29 16 29        | 2.29    |
| 11          | 100                      | 28 16 31 6 19        | 1.72    |
| 12          | 100                      | 34 7 36 7 16         | 1.64    |
| 13          | 100                      | 21 15 29 10 25       | 2.03    |
| 14          | 100                      | 9 5 32 26 28         | 2.59    |
| 15          | 100                      | 26 10 24 6 34        | 2.12    |
| 16          | 100                      | 15 18 34 21 12       | 1.97    |
| 17          | 100                      | 25 15 33 15 12       | 1.74    |
| 18          | 100                      | 20 15 36 18 11       | 1.85    |
| 19          | 100                      | 29 20 28 16 7        | 1.52    |
| 20          | 100                      | 17 11 26 20 26       | 2.27    |
| 21          | 100                      | 39 8 23 7 23         | 1.67    |
| 22          | 100                      | 26 9 24 9 32         | 2.12    |
| 23          | 100                      | 38 10 23 13 16       | 1.59    |
| 24          | 100                      | 34 15 17 9 25        | 1.76    |

(b)

| Patients no | Number of analyzed cells | Comet classification | GDI (%) |
|-------------|--------------------------|----------------------|---------|
| 1           | 100                      | 6 8 1 2             | 0.33    |
| 2           | 100                      | 3 5 4 1             | 0.29    |
| 3           | 100                      | 5 7 3 3             | 0.39    |
| 4           | 100                      | 4 9 3 2             | 0.39    |
| 5           | 100                      | 18 4 1 1            | 0.33    |
| 6           | 100                      | 16 3 1 —            | 0.25    |
| 7           | 100                      | 20 5 2 —            | 0.36    |
| 8           | 100                      | 20 2 2 —            | 0.30    |
| 9           | 100                      | 29 4 — —            | 0.37    |
| 10          | 100                      | 3 2 2 —             | 0.30    |
| 11          | 100                      | 13 5 3 — —          | 0.29    |
| 12          | 100                      | 11 4 4 4 —          | 0.47    |
| 13          | 100                      | 8 9 3 1 —           | 0.38    |
| 14          | 100                      | 6 5 2 4 —           | 0.38    |
| 15          | 100                      | 77 12 6 1 4         | 0.43    |

GDI, genetic damage index.

Table 1. Degree of DNA damage in peripheral blood lymphocytes of COVID-19 patients (a) and control subjects (b) measured by comet assay

shown between the two groups of participants (Figure 2B). No significant differences in the number of erythrocytes (4.49 ± 0.66 vs. 4.79 ± 0.32 × 10¹², P = 0.083) and platelets (254.91 ± 100.11 vs. 251.00 ± 48.64 × 10⁹, P = 0.873), as well as in hemoglobin concentration (133.95 ± 13.68 vs. 141.46 ± 10.39 g/l, P = 0.079) were observed between COVID-19 patients and control subjects. But COVID-19 patients had statistically lower hematocrit values compared with the controls (0.39 ± 0.05 vs. 0.42 ± 0.03 l/l, P = 0.026).

Additionally, PLR was calculated in the study population. We found that COVID-19 patients had a significantly higher value of PLR compared to control subjects (300.54 ± 134.09 vs. 117.76 ± 37.79, P < 0.001) (Figure 3).

In further investigation, we analyzed some laboratory parameters in COVID-19 patients and control subjects (Table 2) and their relationship with GDI (Table 3). Bivariate correlation analysis confirmed the existence of a statistically significant positive relationship of GDI and IL-6 concentration (Spearman r = 0.711, P = 0.032) as well as a statistically significant negative relationship of GDI and platelet count (Pearson r = −0.314, P = 0.012) in COVID-19 patients. The correlation between the concentration of GDI and CRP did not reach statistical significance (Pearson r = 0.402, P = 0.051). Besides, there was a significant positive correlation between GDI and age in the patients with COVID-19 (Pearson r = 0.452, P = 0.026). No statistical significance was observed between GDI and laboratory parameters in the control group of subjects.

When it comes to gender differences, we showed that female patients with COVID-19 had a slightly higher GDI value compared to the males, but without significant differences (1.92 ± 0.37 vs. 1.84 ± 0.25, P = 0.561). Although, no significant differences between females and males in the concentration of CRP (45.68 ± 34.58 vs. 36.51 ± 26.14 mg/l, P = 0.474) and procalcitonin (0.09 ± 0.11 vs. 0.09 ± 0.07 ng/ml, P = 0.677) were shown, it was estimated that GDI positively correlated with the degree of inflammation in females (Pearson r = 0.913, P = 0.002 for CRP; Spearman r = 0.886, P = 0.019 for procalcitonin). Besides, it was noted that female subjects had more pronounced hemostasis abnormalities (significantly lower average platelet count and longer aPTT) (Table 4), which also statistically correlated with GDI. There was a negative relationship of GDI and platelet count (Pearson r = −0.687, P = 0.005) and positive relationship of GDI and aPTT (Spearman r = 0.698, P = 0.004 for aPTT). We did not observe statistically significant differences in the rest of the analyzed parameters between females and males.

Finally, we performed regression analysis to evaluate the potential impact of the applied diagnostic and therapeutic agents on DNA damage (Table 5). The administration of two antibiotics, fluoroquinolone (β = 0.425, P = 0.039) and macrolides (β = 0.449, P = 0.028), daily dose of corticosteroid (β = 0.756, P < 0.001), as well as anticoagulant (β = 0.851, P < 0.001) and antiviral (β = 0.510, P = 0.011) therapy, and chest X-rays (β = 0.819, P < 0.001) were identified to increase DNA damage in patients with COVID-19. Similar smoking habit was also shown to be associated with the increase of DNA damage (β = 0.471, P = 0.020). Contrary, oxygen therapy exhibited the protective role on genetic material of COVID-19 patients (β = −0.407, P = 0.048).

Discussion

Our study examined the differences in DNA damage, expressed as GDI, in hospitalized COVID-19 patients and healthy controls. Besides, we elucidated the relationships between DNA damage and certain laboratory parameters as well as the relationships between DNA damage and the applied diagnostic and therapeutic agents in COVID-19 patients.
DNA damage in COVID-19

COVID-19 is a severe acute respiratory syndrome caused by the beta-coronavirus SARS-CoV-2, for the first time observed in Wuhan, Hubei province of China in December 2019 [22, 23]. Although a primarily respiratory pathogen, SARS-CoV-2 could induce a number of complications with the signs of cardiovascular, gastrointestinal, hepatobiliary, urinary, and central nervous system involvement [24–26]. Typical laboratory features detected during COVID-19 include neutrophilia, lymphopenia, high levels of inflammation-related parameters, liver transferase enzymes, lactate dehydrogenase, creatinine kinase, as well as the disturbances of coagulation factors [24, 27–29].

Accordingly, in our study, we have shown that COVID-19 patients had significantly higher degree of DNA damage compared with control subjects. The absolute number of neutrophil leukocytes was statistically higher, while the absolute number of lymphocytes was statistically lower in COVID-19 patients compared with control subjects. It has been demonstrated that the markers of systemic inflammation (NLR, PLR, and SIRI) were considerably greater in COVID-19 patients than in controls. No more pronounced differences in the other blood count parameters were observed between the two groups of participants. The analysis of the relationship between DNA damage and laboratory parameters indicated that GDI was positively correlated with IL-6 concentration and negatively with platelet count in COVID-19 patients. Although without greater differences in the values of GDI between male and female patients, DNA damage level was slightly higher in women, and it positively correlated with the inflammatory markers (CRP and procalcitonin). It was observed that female subjects had more pronounced hemostasis abnormalities (significantly lower average platelet count and longer aPTT), which also statistically correlated with GDI. Additionally, smoking habit, applied therapy (antibiotics, corticosteroid, anticoagulant, and antiviral therapy), and chest X-rays were also shown to be associated with the increase of DNA damage in COVID-19 patients.

Previous studies have suggested that inflammation and pro-inflammatory cytokines in different pathological conditions stimulate the formation of ROS, which in turn might cause DNA damage [12, 13]. On the other hand, oxidative stress, resulting from the release of ROS by the immune cells to an inflammatory stimulus, contribute to further immune dysregulation and the development of uncontrolled inflammatory response, so-called ‘cytokine storm syndrome’ [30–33]. This implies the existence of the phenomenon in which the effect of ROS is potentiated by some cytokines, and vice versa.

In general, oxidative stress and inflammation seen with a viral infection lead to DNA damage and genome instability due to the diminished DNA repair capacity [11, 34]. The lack of DNA damage response and the appropriate DNA repair regulation result in the accumulation of somatic mutations in

![Figure 2. The differences in white blood cells count (A) and the markers of inflammation (B) between COVID-19 patients and control subjects.](image-url)
different types of cells [11, 35]. On the other side, DNA damage in COVID-19 patients might be caused by many factors, including diagnostic and therapeutic agents.

Here, we did not study the oxidative stress. On the other hand, it was shown that the degree of DNA damage positively correlated with the concentration of IL-6 in COVID-19 patients. Also, the higher degree of inflammation, detected on the basis of increased concentrations of CRP and procalcitonin, corresponded to a higher degree of DNA damage in the female population.

To the best of our knowledge, this is the first study about DNA damage in COVID-19 patients. Earlier, we estimated the increased DNA damage in patients with autoimmune diseases [36], which could be triggered by viral infections [37, 38]. Indeed, Lorente et al. analyzed DNA and RNA oxidative damage in COVID-19 patients by measuring the concentration of oxidized guanine species and mortality prediction [39]. In this study, we used the alkaline version of the comet assay for detection the damage of genetic material not only because of oxidative stress but also to assess the total genotoxic pressure in severe COVID-19 patients. Our results are consistent with previously published data on DNA damage in severely ill patients with multiple trauma [21] and sepsis [40], in which the level of multiple organic dysfunctions has been shown to positively correlated with DNA damage. Similar, Pinto concluded that SARS-CoV-2 virus could promote mutagenesis by increasing in micronuclei-bearing cells in buccal mucosa of 11 patients with COVID-19 [41].

Concerning laboratory parameters in our patients, we determined considerably high values of inflammatory markers (NLR, PLR, and SIRI), which previously have been analyzed in COVID-19 and found to play an important role in disease prognosis and the development of multiorgan failure in SARS-CoV-2 infection [42–44]. It seems that there is a prolonged activation of neutrophils with the production of pro-inflammatory mediators [45], which might induce the immune system impairment, through the bone marrow suppression, and decline in lymphocytes count in COVID-19 [46].

Our investigation also indicated that COVID-19 patients showed markedly deviations in the concentrations of ferritin,

Figure 3. Platelet-to-lymphocyte ratio in COVID-19 patients (A) and control subjects (B).
DNA damage in COVID-19

LDH, AST, ALT, CK, pro-BNP, urea, and creatinine. This is in line with the results of previous studies that dealt with the diagnostic and prognostic value of laboratory findings in COVID-19 patients [8, 47, 48]. The aggregated inflammatory response in SARS-CoV-2 infection causes multiorgan dysfunction that is reflected through a remarkable increase in different laboratory parameters. However, the mentioned parameters did not show a significant relationship with DNA damage, nor the hemostasis parameters. Namely, we found that lower values of platelet count, as well as the higher values of aPTT correspond to a more pronounced DNA damage in females.

Table 2. The laboratory parameters in COVID-19 patients and control subjects

| Parameter     | COVID-19 patients | Control subjects | Significance* |
|---------------|-------------------|------------------|---------------|
|               | Ý ± SD Min–Max    | Ý ± SD Min–Max   |               |
| CRP           | 39.57 ± 28.79     | 1.55 ± 0.44      | P < 0.001     |
| Procalcitonin | 0.092 ± 0.082     | 0.033 ± 0.003    | P = 0.007     |
| IL-6          | 251.53 ± 466.20   | 2.02 ± 0.66      | P = 0.043     |
| PT            | 16.74 ± 10.19     | 12.31 ± 0.43     | P < 0.001     |
| aPTT          | 33.67 ± 9.31      | 28.63 ± 1.62     | P = 0.024     |
| D-dimer       | 0.93 ± 1.21       | 0.30 ± 0.07      | P = 0.002     |
| Fibrinogen    | 5.17 ± 1.25       | 2.73 ± 0.55      | P < 0.001     |
| Ferritin      | 1103.95 ± 1361.38 | 115.09 ± 85.28   | P < 0.001     |
| LDH           | 794.65 ± 256.57   | 725.00 ± 23.17   | P < 0.001     |
| AST           | 47.67 ± 42.49     | 15.80 ± 5.26     | P = 0.026     |
| ALT           | 59.00 ± 59.16     | 9.40 ± 4.03      | P < 0.001     |
| CK            | 132.78 ± 95.53    | 73.80 ± 44.62    | P = 0.032     |
| pro-BNP       | 695.82 ± 990.60   | 36.28 ± 33.36    | P = 0.014     |
| Urea          | 7.46 ± 2.80       | 4.91 ± 1.37      | P = 0.010     |
| Creatinine    | 89.25 ± 43.47     | 87.52 ± 12.37    | P = 0.238     |

CRP, C-reactive protein; IL-6, interleukin 6; PT, prothrombin time; aPTT, activated partial thromboplastin time; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CK, creatine kinase; pro-BNP, pro-brain natriuretic peptide.

*P values correspond to independent sample T test or Mann–Whitney test (depending on distribution); statistically significant differences are bolded.

Table 3. Relationships of genetic damage index with age and biochemical/hemostasis parameters in COVID-19 patients and control subjects

| Parameter     | Genetic damage index | Significance |
|---------------|----------------------|-------------|
|               | Pearson/Spearman coefficient Significance | Pearson/Spearman coefficient Significance |
| Age           | r = 0.452 P = 0.026 | r = 0.436 P = 0.104 |
| CRP           | r = 0.402 P = 0.051 | r = 0.433 P = 0.517 |
| Procalcitonin | r = 0.300 P = 0.186 | r = 0.202 P = 0.576 |
| IL-6          | r = 0.711 P = 0.032 | r = 0.396 P = 0.258 |
| Platelets     | r = -0.514 P = 0.012 | r = -0.164 P = 0.650 |
| PT            | r = 0.015 P = 0.947 | r = 0.045 P = 0.902 |
| aPTT          | r = 0.297 P = 0.180 | r = 0.145 P = 0.689 |
| D-dimer       | r = 0.040 P = 0.858 | r = 0.265 P = 0.458 |
| Fibrinogen    | r = 0.157 P = 0.521 | r = 0.301 P = 0.398 |
| Ferritin      | r = -0.066 P = 0.775 | r = 0.114 P = 0.755 |
| LDH           | r = 0.077 P = 0.746 | r = 0.333 P = 0.347 |
| AST           | r = -0.031 P = 0.895 | r = -0.073 P = 0.840 |
| ALT           | r = -0.390 P = 0.066 | r = 0.124 P = 0.716 |
| CK            | r = 0.366 P = 0.123 | r = 0.082 P = 0.821 |
| pro-BNP       | r = 0.328 P = 0.199 | r = 0.567 P = 0.112 |
| Urea          | r = 0.199 P = 0.351 | r = 0.422 P = 0.225 |
| Creatinine    | r = 0.345 P = 0.098 | r = 0.541 P = 0.106 |

CRP, C-reactive protein; IL-6, interleukin 6; PT, prothrombin time; aPTT, activated partial thromboplastin time; LDH, lactate dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CK, creatine kinase; pro-BNP, pro-brain natriuretic peptide.

*P values correspond to bivarite correlation test; statistically significant values are bolded.
COVID-19 occur due to the endothelial dysfunction induced by hypoxia and an uncontrolled inflammatory reaction [49]. The damage of vascular endothelium might lead to the excessive activation of coagulation cascade with subsequent disseminated intravascular coagulation and a decrease in platelet count [50, 51]. Thus, we have shown that the degree of coagulopathy directly corresponds to the degree of DNA damage. At the same time, the existence of a correlation between DNA damage and inflammatory markers (CRP and procalcitonin) supports the theory that inflammation plays a role in the development of hemostasis disorders. The final result of hemostasis disorders in COVID-19 is multiorgan failure [51, 52].

Keeping in mind that comet assay has been described as a biomarker of exposure of DNA molecules to various damaging agents [53, 54], we also analyzed the possible influence of therapeutic agents and chest X-rays on DNA damage in our COVID-19 patients. Thus, the administration of some antibiotics (fluoroquinolone and macrolides), corticosteroids, anticoagulant, and antiviral agents has been shown to increase the level of DNA damage in COVID-19 patients. This is in line with the available literature data about genotoxic effects of certain medications like erythromycin and/or lincomycin [55], metronidazole and dimetridazole [56], and glucocorticoid receptor agonists [57]. Likewise, our results correspond to previously conducted studies related to genotoxic potential of oral anticoagulant [58] and antiviral [59] therapy. The last one indicated that the antiviral nucleoside analog drugs could induce genome error catastrophe in SARS-CoV-2 via lethal mutagenesis.

It was shown earlier that once applied diagnostic X-ray examination in 20 children with pulmonary diseases significantly increased the level of DNA damage (observed by alkaline comet assay) [60]. Similarly, He et al. [61] confirmed the genotoxic effects of X-rays on human lymphocytes isolated from the irradiated whole-blood samples. Our results agree with the mentioned data which imply that diagnostic imaging by X-rays could induce measurable DNA damage in lymphocytes of exposed subjects.

In conclusion, COVID-19 patients have significantly higher level of DNA damage than control subjects. There is a positive correlation between DNA damage and inflammatory markers and negative correlation between the degree of DNA damage and platelet count, respectively. DNA damage in female COVID-19 patients corresponds to the level of hemostasis abnormalities. Smoking habit, applied therapy (antibiotics, corticosteroid, anticoagulant, and antiviral therapy) as well as chest X-rays express a genotoxic potential that contributes to a higher level of DNA damage in COVID-19 patients. Further in vivo and in vitro analyses are needed to assess the influence of complex interaction of various pharmacological and environmental agents on DNA in-

### Table 4. The differences in the parameters of hemostasis between female and male patients with COVID-19

| Parameter | COVID-19 patients | Significance* |
|-----------|------------------|--------------|
|           | Females | Males | n = 8 | n = 16 |
| PT        | 21.66 ± 17.60 | 14.45 ± 2.31 | P = 0.945 |
| aPTT      | 41.64 ± 12.92 | 29.95 ± 3.46 | P = 0.032 |
| D-dimer   | 0.54 ± 0.30  | 1.10 ± 1.42  | P = 0.319 |
| Fibrinogen| 5.12 ± 1.45  | 5.19 ± 1.21  | P = 0.908 |
| Platelets | 194.00 ± 65.77| 287.40 ± 101.65| P = 0.029 |

PT, prothrombin time; aPTT, activated partial thromboplastin time. *P values correspond to independent sample T test or Mann–Whitney test (depending on distribution).

### Table 5. Influence of smoking, applied therapy, and chest X-rays on genetic damage index in COVID-19 patients

| Parameter                    | Genetic damage index | Significance* |
|------------------------------|----------------------|--------------|
|                              | Unstandardized coefficients | Standardized coefficient | t  |
|                              | B                   | Standard error | β   |  |
| Smoking                      | 0.268               | 0.107        | 0.471 | 2.506 | P = 0.020 |
| Oxygen therapy               |                      |              |      |      |      |
| Flow rate, l/min            | −0.001              | 0.002        | −0.121 | −0.571 | P = 0.574 |
| Duration/in days/before sampling | −0.069     | 0.033        | −0.407 | −2.090 | P = 0.048 |
| Antibiotic therapy          |                      |              |      |      |      |
| Fluoroquinolone             | 0.255               | 0.116        | 0.425 | 2.201 | P = 0.039 |
| Cephalosporines             | 0.133               | 0.118        | 0.235 | 1.134 | P = 0.269 |
| Macrolides                  | 0.279               | 0.119        | 0.449 | 2.354 | P = 0.028 |
| Carbapenem                  | 0.107               | 0.064        | 0.188 | 0.900 | P = 0.378 |
| Tetracyclines               | 0.096               | 0.161        | 0.126 | 0.598 | P = 0.556 |
| Duration/in days/before sampling | 0.068     | 0.018        | 0.626 | 3.767 | P = 0.001 |
| Corticosteroid therapy      |                      |              |      |      |      |
| Daily dose                  | 0.303               | 0.056        | 0.756 | 5.414 | P < 0.001 |
| Duration/in days/before sampling | 0.021     | 0.029        | 0.156 | 0.739 | P = 0.468 |
| Anticoagulant therapy/duration | 0.116     | 0.015        | 0.851 | 7.595 | P < 0.001 |
| Antiviral drug/Favipiravir   | 0.387               | 0.139        | 0.510 | 2.778 | P = 0.011 |
| Chest X-rays                | 0.362               | 0.054        | 0.819 | 6.691 | P < 0.001 |

*Statistically significant P values obtained by Linear regression test are bolded.
DNA damage in COVID-19 patients with different clinical features. Besides, the association of oxidative stress with the specific DNA damage measured by enzyme-modified comet assay could be performed in order to additionally elucidate DNA damage in COVID-19 patients.

**Funding**

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant nos. III41010 and ON175069).

**Conflict of Interest Statement**

None declared.

**References**

1. Aguilera A, Gómez-González B. Genome instability: a mechanistic view of its causes and consequences. Nat Rev Genet 2008;9:204–217.
2. Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature 2009;461:1071–1078.
3. Schwarz KB. Oxidative stress during viral infection: a review. Free Radical Biol Med 1996;21:641–649.
4. Xu LH, Ghosal G, Chen J. DNA damage tolerance: a double-edged sword guarding the genome. Transl Cancer Res 2013;2:107–129.
5. Su YC, Hong JR. RNA viruses: ROS-mediated cell death. Int J Cell Biol 2014;2014:467452.
6. Ashok K, Rita S, Jaskaran K, et al. Wuhan to world: the COVID-19 pandemic. Front Cell Infect Microbiol 2021;11:242.
7. Li S, Ma F, Yokota T, et al. Metabolic reprogramming and epigenetic changes of vital organs in SARS-CoV-2 induced systemic toxicity. JCI Insight 2021;6:e145027.
8. Yang A-P, Li H-M, Tao W-Q, et al. Infection with SARS-CoV-2 causes abnormal laboratory results of multiple organs in patients. Aging 2020;12:10059–10069.
9. Ciaccio M, Agnello L. Biochemical biomarkers alterations in Coronavirus Disease 2019 (COVID-19). Diagnosis 2020;7:365–372.
10. Ryan EL HR, Grand RJ. Activation of the DNA damage response by RNA viruses, Biomolecules 2016;6:2.
11. Vig J, Dong X, Milholland B, et al. Genome instability: a conserved mechanism of aging? Essays Biochem 2017;61:305–313.
12. Chapple ILC. Reactive oxygen species and antioxidants in inflammatory diseases. J Clin Periodontol 1997;24:287–296.
13. Valko M, Leibfritz D, Moncol J, et al. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44–84.
14. Katsuyama M. NOX/NADPH oxidase, the superoxide generating enzyme: its transcriptional regulation and physiological roles. J Pharmacol Sci 2010;114:134–146.
15. Jiang H, Mei Y-F. SARS-CoV-2 spike impairs DNA damage repair and inhibits V(D)J recombination in vitro. Viruses 2021;13:2056.
16. Huang M FS, Liu DX. Coronavirus infection induces DNA replication stress partly through interaction of its nonstructural protein 13 with the p125 subunit of DNA polymerase delta. J Biol Chem 2011;286:39356–39359.
17. World Health Organization. (2020) Laboratory testing strategy recommendations for COVID-19: interim guidance. https://apps.who.int/iris/handle/10665/331309.
18. Zhanaetov AK, Moroz VV, Durnev AD, et al. DNA damage and cell death assessment in patients with severe multiple trauma using comet assay. Healthb 2010;2:412–417.
19. Singh NP, McCoy MT, Tice RR, et al. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–191.
20. Collins A, Duinská M. Oxidation of cellular DNA measured with the comet assay. Methods Mol Biol 2002;186:147–159.
21. Pitárque M, Vaglenov A, Nosko M, et al. Evaluation of DNA damage by the comet assay in shoe workers exposed to toluene and other organic solvents. Mutat Res 1999;441:115–127.
22. Wu Z, McGoogan JM. Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese Center for Disease Control and Prevention. JAMA 2020;323:1239–1242.
23. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet 2020;395:497–506.
24. Guan WJ, Liang WH, Zhao Y, et al.; China Medical Treatment Expert Group for COVID-19. Comorbidity and its impact on 1590 patients with Covid-19 in China: a nationwide analysis. Eur Respir 2020;55:547. doi:10.1183/13993003.00547-2020.
25. Zhang JJ, Dong X, Cao YY, et al. Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. Allergy 2020;75:1730–1741.
26. Munster VJ, Koopmans M, van Doremalen N, et al. A novel coronavirus emerging in China—key questions for impact assessment. N Engl J Med 2020;382:692–694.
27. Terpos E, Ntanasis-Stathopoulos I, Elalamy I, et al. Hematological findings and complications of COVID-19. Am J Hematol 2020;95:834–847.
28. Yao Z, Zheng Z, Wu K, et al. Immune environment modulation in pneumonia patients caused by coronavirus: SARS-CoV-MERS-CoV and SARS-CoV-2. Aging (Albany NY) 2020;12:7639–7651.
29. Chen G, Wu D, Guo W, et al. Clinical and immunological features of severe and moderate coronavirus disease 2019. J Clin Invest 2020;130:2620–2629.
30. Nasi A, McArdle S, Gaudernack G, et al. Reactive oxygen species as an initiator of toxic innate immune responses in retort to SARS-CoV-2 in an ageing population, consider N-acetylcysteine as early therapeutic intervention. Toxicol Rep 2020;7:768–771.
31. Wu J. Tackle the free radicals damage in COVID-19. Nitric Oxide 2020;102:39–41.
32. Cecchini R, Cecchini AL. SARS-CoV-2 infection pathogenesis is related to oxidative stress as a response to aggression. Med Hypotheses 2020;143:110102.
33. Qin C, Zhou L, Hu Z, et al. Dysregulation of immune response in patients with COVID-19 in Wuhan, China. Clin Infect Dis 2020;71:762–768.
34. Di Micco R, Krizhanovsky V, et al. Cellular senescence in ageing: from mechanisms to therapeutic opportunities. Nat Rev Mol Cell Biol 2020;22:75–95.
35. Zhang L, Dong X, Lee M, et al. Single-cell whole-genome sequencing reveals the functional landscape of somatic mutations in B lymphocytes across the human lifespan. Proc Natl Acad Sci USA 2019;116:9014–9019.
36. Mihaljevic O, Zivanovic-Simonovic S, Milosevic-Djordjevic O, et al. Apoptosis and genome instability in children with autoimmune diseases. Mutagenesis 2018;33:351–357.
37. Lufing MA. Viruses and the DNA damage response: activation and antagonism. Annu Rev Virol 2014;1:605–625.
38. Hussein HA, Rahala EA. The role of viral infections in the development of autoimmune diseases. Crit Rev Microbiol 2019;45:394–412.
39. Lorente L, Martin MM, González-Rivero AE, et al. DNA and RNA oxidative damage and mortality of patients with COVID-19. Am J Med Sci 2021;361:585–590.
40. Wabha AY, Elwassi M, Magdy M, et al. Association between DNA damage and serum levels of copper, zinc, and selenium in full-term neonates with late-onset sepsis. J Pediatr Infect Dis 2020;15:299–306.
41. Pinto TG, Alpire MES, Ribeiro DA. Cytogenetic biomonitoring in buccal mucosa cells of COVID-19 patients: preliminary findings. Int J Cell Clin 2021;15:299–306.
42. Liu Y, Du X, Chen J, et al. Neutrophil-to-lymphocyte ratio as an independent risk factor for mortality in hospitalized patients with COVID-19. *J Infect* 2020;81:6–12.

43. Lagunas-Rangel FA. Neutrophil-to-lymphocyte ratio and lymphocyte-to-C-reactive protein ratio in patients with severe coronavirus disease 2019 (COVID-19): a meta-analysis. *Med Virol* 2020;92:1733–1734.

44. Adil M, Baig ZF, Amir M, et al. Neutrophil to lymphocyte ratio vs platelets to lymphocyte ratio: biomarkers to predict severity of disease and their comparasion in patients of COVID-19. *Pak Armed Forces Med J* 2020;70:1609–1615.

45. Laforge M, Elbim C, Frère C, et al. Tissue damage from neutrophil-induced oxidative stress in COVID-19. *Nat Rev Immunol* 2020;20:515–516.

46. Abdelaal Ahmed Mahmoud M, Alkhatip A, Kamel MG, et al. The diagnostic and prognostic role of neutrophil-to-lymphocyte ratio in COVID-19: a systematic review and meta-analysis. *Expert Rev Mol Diagn* 2021;21:505–514.

47. Mallick P, Patel U, Mehta D, et al. Biomarkers and outcomes of COVID-19 hospitalisations: systematic review and meta-analysis. *BMJ Evid Based Med* 2021;26:107–108.

48. Moorothy S, Koshy T, Kumar M, et al. Role of inflammatory and liver function markers in assessing the prognosis of patients with COVID-19. *World Acad Sci J* 2021;3:52.

49. Ulanowska M, Olas B. Modulation of hemostasis in COVID-19: blood platelets may be important pieces in the COVID-19 puzzle. *Pathogens* 2021;10:370.

50. Miesbach W, Makris M. COVID-19: coagulopathy, risk of thrombosis, and the rationale for anticoagulation. *Clin Appl Thromb Hemost* 2020;26.

51. Iba T, Levy JH. Inflammation and thrombosis: roles of neutrophils, platelets and endothelial cells and their interactions in thrombus formation during sepsis. *J Thromb Haemost* 2018;16:231–41.

52. Garcia-Olivé I, Sintes H, Radua J, et al. D-dimer in patients infected with COVID-19 and suspected pulmonary embolism. *Respir Med* 2020;169:106023.

53. Kuchafová M, Hronek M, Rybaková K, et al. Comet assay and its use for evaluating oxidative dna damage in some pathological states. *Physiol Res* 2019;68:1–15.

54. Möller P, Stopper H, Collins AR. Measurement of DNA damage with the comet assay in high-prevalence diseases: current status and future directions. *Mutagenesis* 2020;35:5–18.

55. Rocci L, Peluso C, Stingo V. Micronucleus test and comet assay for the evaluation of zebrafish genomic damage induced by erythromycin and lincomycin. *Environ Toxicol* 2012;27:598–604.

56. Ré JL, M, éo MP de, et al. Evaluation of the genotoxic activity of metronidazole and dimetridazole in human lymphocytes by the comet assay. *Mutat Res* 1997;375:147–155.

57. Hayes JE, Doherty AT, Coulson M, et al. Micronucleus induction in the bone marrow of rats by pharmacological mechanisms: I: glucocorticoid receptor agonism. *Mutagenesis* 2013;28:227–232.

58. Wingert NR, Arbo MD, Göethel G, et al. In vitro toxicity assessment of rivaroxaban degradation products and kinetic evaluation to decay process. *Drug Chem Toxicol* 2019;42:509–518.

59. Waters MD, Warren S, Hughes C, et al. Human genetic risk of treatment with antiviral nucleoside analog drugs that induce lethal mutagenesis: the special case of molnupiravir. *Environ Mol Mutagen* 2022;63:37–63.

60. Milkovic D, Garaj-Vrhovac V, Ranogajec-Komor M, et al. Primary DNA damage assessed with the comet assay and comparison to the absorbed dose of diagnostic X-rays in children. *Int J Toxicol* 2009;28:405–416.

61. He JL, Chen WL, Jin LF, et al. Comparative evaluation of the in vitro micronucleus test and the comet assay for the detection of genotoxic effects of X-ray radiation. *Mutat Res* 2000;469:223–231.