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Article

Alterations of the antioxidant and inflammatory response of the peripheral blood granulocytes to SARS-CoV-2 infection in the deceased COVID-19 patients

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Abstract: It is assumed that upon SARS-CoV-2 infection granulocytes can undergo potentially destructive oxidative burst. Therefore, the aim of this study was to evaluate some parameters of redox and inflammatory signaling in granulocytes of recovered and of deceased COVID-19 patients. Granulocytes were isolated from the blood of 32 COVID-19 patients on admission to the hospital (16 survived and 16 died within a week). The levels of proteins (immunoassay), eicosanoids (UPLC-MS) and antioxidants activity (spectrophotometry) were examined. Enhanced activation of Nrf2 and NFκB and the levels of heme oxygenase and proinflammatory cytokines where found in granulocytes of all COVID-19 patients, while Cu,Zn-SOD and Mn-SOD activities were decreased, especially in deceased patients. Moreover, in patients who died increased levels of pro-inflammatory eicosanoids (PGE2 and TXB2) and decreased of anti-inflammatory (15d-PGJ2 and 5-HETE) were observed. However TXB2 was decreased, and IL-2 and IL-10 levels were increased in survivors, if compared both to healthy subjects and deceased patients, who did not change their cytokine generation. Therefore, it seems that by triggering transcription factors granulocytes activate redox signaling, leading to the production of pro-inflammatory eicosanoids, while reducing cellular antioxidant capacity via SOD, they express altered response to COVID-19, which might result in the onset of the vicious cycle of systemic oxidative stress in deceased patients.

Keywords: granulocytes; COVID-19; antioxidants; inflammation; eicosanoids; receptors-coupled G protein; SOD

1. Introduction

Recent studies indicate that the immune response to the presence of SARS-CoV-2 virus plays a key role in the pathogenesis and clinical symptoms of COVID-19, as the severe course of COVID-19 is associated with a strong impairment of the immune system. On one hand COVID-19 infection leads to a high level of pro-inflammatory cytokines and an increase in the number of neutrophils, but on the other it also leads to lymphopenia resulting in an increased ratio of neutrophils to lymphocytes as a hallmark severe course of COVID-19 [1,2]. Granulocytes, mostly neutrophils, are among the first leukocytes to be recruited to the site of infection as cells of the innate immune system and play a key role in shaping an early response to infection as well as mediating the innate immune system and the acquired response. However, if granulocytes do not undergo adequate regulation, the inflammatory functions of these cells, aimed at killing pathogens, can lead to tissue damage [3].

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After entering the body, the virus is recognized by the cells of the immune system, in which tool-like receptors (TLR 7/8) present on granulocytes lead to the activation of these cells. As a result, the innate response is activated, promoting the activation of many inflammatory mediators and disturbance of the balance in the intracellular environment. This is mainly due to the overproduction of reactive oxygen species (ROS) generated by granulocytes to remove pathogens. In this context, the greatest importance is attached to neutrophils, which in the process of "oxidative burst" produce a significant amount of free superoxide anion radicals and hydrogen peroxide, which are important for the elimination of pathogens [4]. However, activation of TLR 7/8 also stimulates the release of ROS outside the inflammatory cells. As a result, ROS can cause oxidative modifications of the major biomolecules, including lipids, resulting in damage to cell membranes, including erythrocytes, which leads to the release of iron ions catalyzing free radical reactions with the generation of superoxide anion radicals and hydrogen peroxide, which promote the onset of systemic oxidative stress, as observed in the course of COVID-19 [5]. Moreover, activation of granulocytes as well as monocytes/macrophages, leads to spread of oxygen burst in response to SARS-CoV-2 infection, by inducing excessive ROS production, it can cause oxidative modification of cellular and extracellular bioactive molecules thus contributing to the aggravation of severe disease and chronic inflammation [6]. This is due to the fact that overproduction of ROS leads to redox imbalance, which promotes the activation of the NFκB pathway, which results in increased expression of cytokines and immunoglobulin G and further dysfunction of granulocytes and lymphocytes [7]. Severe COVID-19 cases show an increase in inflammatory cytokines indicative of a "cytokine storm", seen especially in cases of extremely severe disease, resulting in disseminated intravascular coagulation [8]. This can initiate viral sepsis and inflammation of the lungs, leading to ARDS, respiratory failure, shock, and multiple organ failure and death [9].

However, a "cytokine storm" is not a single event if aggressive COVID-19. Namely, the active elements of both the intracellular and extracellular tissue components are bioactive lipids, which participate in the functioning of these systems, e.g. as basic components of biological membranes and a source of energy [10]. On the other hand, in pathophysiological processes, innate immune cells, including granulocytes and monocytes/macrophages, are recruited to the site of infection and there they increase the production of ROS, which increases the release of bioactive lipids [11]. Thus, polyunsaturated fatty acids and their metabolites play a key role in the development of infections, including COVID-19 [12]. Therefore, it is also believed that susceptibility to SARS-CoV-2 is strongly associated with pre-existing conditions characterized by dysregulation of the metabolome, including the lipidome [13]. Consequently, infection with COVID-19 causes massive cell death, which in turn triggers a "eicosanoid storm" of bioactive endogenous lipid mediators that are involved in the various stages of infection [14,15].

Therefore, the aim of this study was to link changes in the level of pro-inflammatory parameters, including those related to antioxidant signaling and lipid mediators, in the granulocytes of COVID-19 patients depending on the severity of the disease.

2. Materials and Methods

2.1. Samples collection

Blood samples were taken from a group of 16 COVID-19 survivors (7 women and 9 men), mean age: 61 (49-71) and 16 COVID-19 patients who died (8 women and 8 men), mean age 72 (age 60-79). Blood samples were collected upon admission to the hospital from patients treated at the Dubrava Clinical Hospital in Zagreb, which served as the national COVID-19 center, thus providing medical care to patients suffering from COVID-19, in particular patients with severe disease. The control group consisted of 16 healthy donors (7 women and 9 men, mean age 37 years (28-53) (Table 1), which were of younger age than COVID-19 patients, among which those who passed away were older than survivors. The results of basic blood laboratory parameters of healthy people and patients with COVID-19 are presented in Table 2. This study was conducted after obtaining the approval of the ethics committee 2020-1012-13 of the Dubrava Clinical Hospital in Zagreb, while patients included in the study have signed informed consent.

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Obtained blood samples were collected in test tubes with ethylenediaminetetraacetic acid (EDTA) and centrifuged in two stages. In the first step, in order to separate the plasma, buffy coat and erythrocytes, the sample was centrifuged at 3000xg (4◦C). To acquire homogeneous fraction of granulocytes, the obtained buffy coat was loaded onto Gradisol G (Aqua-MedZPAM – KOLASA, Łódź, Poland) and centrifuged for 25 minutes (300xg at room temperature). The obtained granulocytes were washed three times with PBS (3 min centrifugation at 300 x g) and resuspended in PBS. Purity of the obtained cell fraction was examined microscopically (Nikon Eclipse Ti, Nikon Instruments Inc., New York, NY,USA). Butylated hydroxytoluene (BHT) as an antioxidant was added to all samples to prevent oxidation. The samples were stored at -80°C until analyses.

Table 1. Demographic and clinical characteristics of patients with COVID-19 compared to healthy subjects.

	Healthy control	COVID-19 recovered	COVID-19 deceased
Age (years)	36.9 ± 9.4	61.1 ± 7.2[a]	72.1 ± 9.0*
Sex	7F + 9M	7F + 9M	8F + 8M
Body Mass Index	26.6 ± 4.7	30.3 ± 2.5	28.5 ± 3.3

* – significant to the healthy control group

Table 2. Comparison of some laboratory data of patients with COVID-19 in respect to normal values and the outcome of the disease.

	Normal range	COVID-19 recovered	COVID-19 deceased
WBC [10^3/μL]	4.00 - 10.00	11.17±3.44	11.43±3.52
Neutrophils [%]	40.0 - 72.0	80.67±5.54	86.60±4.62*
Platelets [10^3/μL]	150 - 400	292.50±105.14	226.61±57.37
Blood oxygen saturation [%]	> 95%	91.17±5.65	90.67±5.33
Ferritin [◎g/L]	11 - 336	913±475	943±436
PCT [ng/mL]	< 0.1	0.38±0.35	1.04±1.09
LDH [U/L]	140 - 280	420±130	358±140
CRP [mg/L]	0.00 - 5.00	139.01±74.08	185.14±59.37
IL-6 [pg/ml]	0 - 43.5	87±45	185±104*

*- significant ($p<0.05$) difference to the values of recovered patients

Methods

2.2. Determination of superoxide dismutases activity

The activity of cytosolic superoxide dismutase dependent on copper and zinc ions (Cu,Zn–SOD–EC.1.15.1.1) in granulocytes was determined spectrophotometrically (480 nm) according to the method of Misra and Fridovich [16] modified by Sykes [17]. One unit of Cu,Zn–SOD was defined as the amount of the enzyme which inhibits epinephrine oxidation to adrenochrome by 50%. Enzyme specific activity was expressed as units per mg of protein.

Manganese-dependent superoxide dismutase (Mn-SOD–EC.1.15.1.1) activity was measured by the method described by Galler & Winge [18]. To inhibit to about 90% the activity of Cu,Zn-SOD, the granulocytes were treated with 1mM KCN. Mn-SOD activity was not inhibited. One unit of SOD activity was defined as the amount of the enzyme required to inhibit the oxidation of epinephrine to adenochrome by 50%. Enzyme specific activity was expressed as units per mg of protein.

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2.3. Determination of expression of the proteins analysed

Measurement of protein expression in granulocytes was performed using enzyme-linked immunosorbent assay (ELISA) [19]. Lysates of granulocytes were applied to ELISA plate wells (Nunc Immuno MaxiSorp, Thermo Scientific, Waltham, MA, USA). Plates with attached proteins were incubated (4^0C) for 3h with blocking solution (5% fat-free dry milk in carbonate binding buffer). After washing with PBS supplemented with 0.1% Tween 20, samples were incubated at 4^0C overnight with appropriate primary antibody against Nrf2, HO-1, TNFα, NFκB p52 (host: rabbit), Keap1, TRPV1 (host: mouse) (Sigma-Aldrich, St. Louis, MO, USA); CB1, CB2, NFκB p65, IL-10 (host:mouse) (Santa Cruz Biotechnology, CA, USA); p-Nrf2, PPARγ (host: rabbit), IL-2 (host: mouse) (Invitrogen, Waltham, MA, USA), IκBα (host: mouse) (Abcam, Cambridge, UK). Next, following washing (PBS supplemented with 0.1% Tween 20), plates were incubated for 30 min with peroxidase blocking solution (3% hydrogen peroxide, 3% fat free dry milk in PBS) at room temperature. As a secondary antibody goat anti-rabbit/mouse EnVision+ Dual Link/HRP solution (1:100) (Agilent Technologies, Santa Clara, CA, USA) was used. After 1 h of incubation at room temperature, secondary antibodies were removed and plates were incubated with chromogen substrate solution (0.1 mg/mL TMB, 0.012% H_2O_2) for 40 min. The reaction was stopped by adding 2 M sulfuric acid and absorption was read within 10 min at 450 nm and automatically recalculated from standard curves for each protein (NFκBp52, TRPV1: Lifespan Biosciences, Seattle, WA, USA; NFκBp65: OriGene Technologies, Rockville, USA; TNFα; Merck, Darmstadt, Germany; PPARγ, IL-10, IL-2: Fine, Test Wuhan, Hubei, China; CB1, IκBα: Abcam, Cambridge,UK; CB2: Abnova, Taipei, Taiwan; Keap1: Sino Biological, Beijing, China; HO-1: Enzo Life, Farmingdale, NY, USA; Nrf2, p-Nrf2: MyBioSource, San Diego, CA, USA into the protein level in the samples.

2.4. Determination of the level of eicosanoids

Eicosanoids analysis was performed using an Shimadzu UPLC system (Nexera X2) coupled with an electrospray ionization source (ESI) to an Shimadzu 8060 Triple Quadrupole system (Shimadzu, Kyoto, Japan) operating in negative mode [20]. Analyte separation was performed using a Eclipse Plus C18 analytical column (2.1 × 100 mm, 1.8 µm particle size) with 3 µL injection volume. The mobile phase consisted of (A) 0.1 % acetic acid in MilliQ water and (B) acetonitrile. The following gradient was employed: 0.0–1.0 min 25–40 % B, 1.0–2.5 min 40–42 % B, 2.5–4.5 min 42 -50 % B, 4.5–10.5 min 50-65 % B; 10.5–12.5 min 65-75 % B; 12.5-14.0 min 75-85 % B; 14.0-14.5 min 85-95 % B; 14.5-15 min 95-25 % B; 15.0-16.0 min 25 % B. Briefly, CSF samples were thawed on ice and spiked with 10 µL internal standard solution (100 ng/mL TXB_2- d_4, PGD_2-d_4, 15-d-PGJ_2-d_4 and 15-HETE-d_8) and then applied to pre-washed and conditioned solid phase extraction SPE cartridges. After loading the sample the cartridges were washed, dried under high vacuum and eluted. Eluates were concentrated and reconstituted in ACN/H_2O (8:2) with 0.1 % acetic acid and vortexed (if necessary centrifuged to remove any residuals). Solutions were then transferred to LC vials with low-volume inserts and LC–MS/MS analysis was performed immediately. The precursor to the product ion transition was as follows: m/z 351.3→271.2 for PGE_2, m/z 315.2→271.2 for 15-d-PGJ_2, m/z 369.3→169.1 for TXB_2, m/z 319.2→257.2 for 5-HETE, m/z 355.0→275.3 for PGD_2-d_4, m/z 373.0→173.1 for TXB_2-d_4 m/z 319.3→275.2 for 15-d-PGJ_2-d_4 and 327.0→226.2 for 15-HETE-d_8. Level of eicosanoids was expressed in pmol/mg protein.

All parameters examined in PMNs are expressed per mg of protein (as determined according to the Bradford method [21].

2.5. Statistical Analysis

Data were expressed as mean ± SD, and were analyzed by one-way analysis of variance (ANOVA) followed by a post hoc Tukey testing using Statistica software (Statistica 13.3, StatSoft, Poland). Laboratory results were compared using Mann-Whitney U test and Wilcoxon signed-rank test.

3. Results

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Viruses-induced infections are known to contribute to an increase in the number of granulocytes, especially neutrophils, which was also seen in our COVID-19 patients (Table 2). It is known that this situation, on the one hand, increases ROS generation, but also may promote changes in the antioxidant capacity of granulocytes, which leads to the formation of oxidative stress and, consequently, dysregulation of the immune response and disease progression [22].

Antioxidant response to SARS-CoV-2 infection

The results of this study show that the granulocytes of COVID-19 patients, especially those who have died from the disease, underwent significant changes in antioxidant defense (Figures 1-2). It was found that in the course of infection there is a change of the antioxidant capacity already at the level of transcription of cytoprotective proteins, especially antioxidant ones, which was caused by changes of the level of the transcription factor - Nrf2. There was a tendency to increase the expression of this factor in the granulocytes of patients, especially with the severe form of the disease (Figure 1), which was visible in both women and men (supplementary Figure 1A), in contrast, to the level of pNrf2 in the granulocytes of patients who recovered. Namely, the analysis of changes in women and men showed an increased level in women who died as a result of COVID-19 (Supplementary, Figure 1A). At the same time an increase of the level of the cytosolic inhibitor Nrf2, the protein Keap1, was detected in the granulocytes of COVID-19 survivors. The changes in Nrf2 expression were accompanied by increased levels of heme oxygenase (HO-1) in the granulocytes of deceased patients. The observed changes corresponded to the direction of Keap1 level modification in particular in men (Supplementary, Figure 1A). HO-1 is considered to be a reliable marker of the transcriptional efficiency of Nrf2, and which is an antioxidant enzyme. Opposite to the changes of the level of HO-1, the activity of the basic antioxidant enzymes responsible for the dismutation of the superoxide anion that is generated mainly in the mitochondrial respiratory chain reactions, including both superoxide dismutases, cytosolic (Cu, Zn-SOD) and mitochondrial (Mn-SOD) was significantly decreased in the granulocytes of patients with COVID-19 (Figure 2). The observed direction of changes in the activity of both enzymes in the entire population of COVID-19 patients corresponded to changes in the granulocytes of both women and men (Supplementary, Figure 2A).

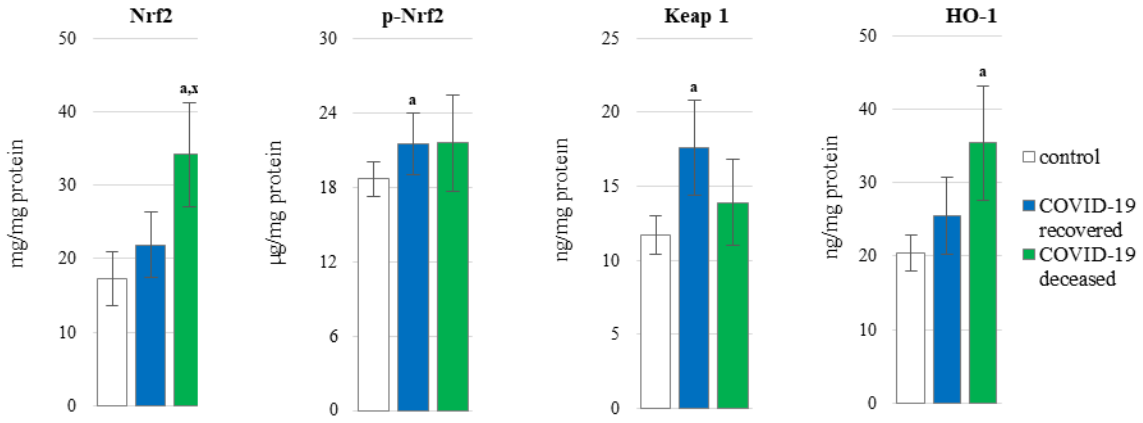

Figure 1. The levels of Nrf2, phosphorylated Nrf2 (p-Nrf2) and its inhibitor Kelch-like ECH-associated protein 1 (Keap1) as well as heme oxygenase 1 (HO-1) in the granulocytes of patients with COVID-19, including those who recovered (n = 16) and those who deceased (n = 16) as well as healthy subjects (n = 16). Data points represent the mean ± SD; a - significantly different from healthy subjects, $p < 0.05$; x - significantly different from recovered patients with COVID-19, $p < 0.05$.

Thus, increased level of the antioxidant enzyme HO-1 was opposite to the reduced activities of essential antioxidant enzymes (Cu,Zn-SOD and Mn-SOD), which may lead to oxidative modifications of some biologically active proteins, including Keap1 and SODs. Therefore the overproduction of ROS and the consequent redox imbalance may have changed the immune response and were responsible for disease progression in SARS-CoV-2 infection, in particular for patients with the lethally aggressive COVID-19.

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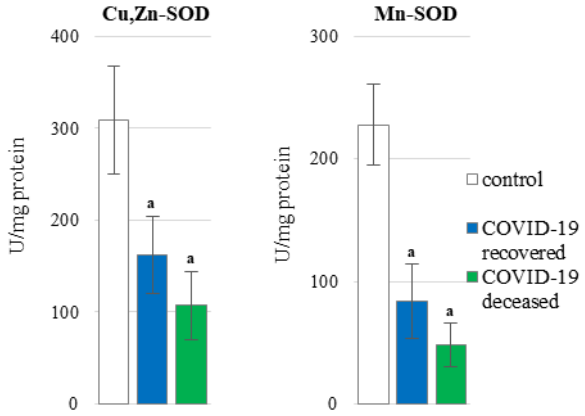

Figure 2. The activity of superoxide dismutase (cytosolic - Cu,Zn-SOD and mitochondrial - Mn-SOD) in granulocytes of patients with COVID-19, including those who recovered (n=16) and those who deceased (n=16) as well as healthy subjects (n=16). ata points represent the mean ± SD; a - significantly different from healthy subjects, $p < 0.05$; x - significantly different from recovered patients with COVID-19, $p < 0.05$.

Inflammatory response to SARS-CoV-2 infection

Accordingly, the SARS-CoV-2 infection could have caused metabolic dysregulation not only at the level of antioxidant efficacy, but also in respect to inflammation manifested by disturbances at the level of the metabolic pathway associated with the NFκB transcription factor. There was an increased expression of both NFκB subunits (p52 and p65) observed, especially in granulocytes of patients who did not survive the infection (Figure 3). The effect of these changes was a reduction in the expression of inhibitor IkBα in the granulocytes of both groups of patients with COVID-19. As a consequence, there was an increased level of pro-inflammatory interleukins, including IL2 in the granulocytes of COVID-19 patients who survived and IL6 in the group of deceased patients (Table 2, Figure 4). Despite the increased activity/level of pro-oxidative and pro-inflammatory parameters, also the level of the anti-inflammatory cytokine IL-10 in the granulocytes of COVID-19 survivors was elevated, suggesting the onset of a cytokine storm accompanying COVID-19, as described in the literature [22]. This trend was reduced in patients who died if compared with patients who survived. The observed direction of changes in parameters related to the inflammatory process (elements of the NFkB transcription factor and products of its activity) in COVID-19 patients was similar both in women and in men from (Supplementary, Figure 3A and 4A).

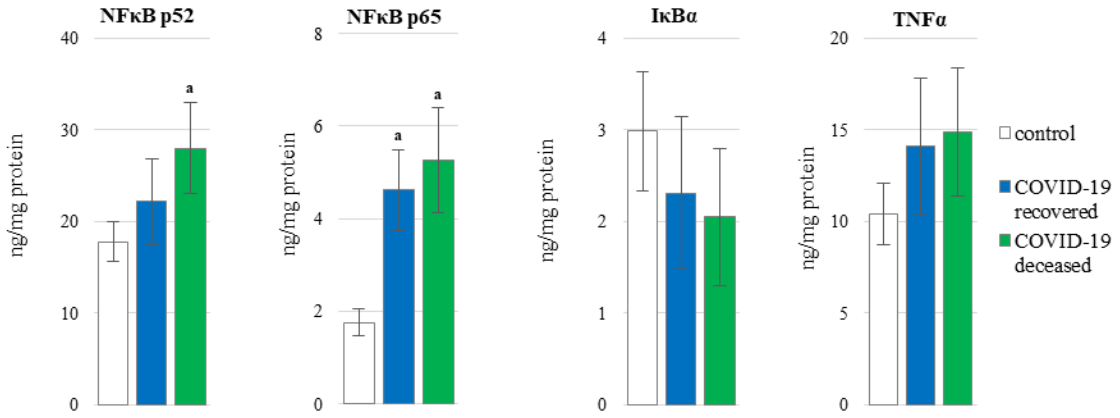

Figure 3. The level of proteins playing essential role in development of inflammation, such as two family members of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB p52 and NFκB p65) as well as tumor necrosis factor alpha (TNF-α), in the granulocytes of patients with COVID-19, including those who recovered (n=16) and those who deceased (n=16) as well as

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healthy subjects (n=16). Data points represent the mean ± SD; a - significantly different from healthy subjects, $p < 0.05$; x - significantly different from recovered patients with COVID-19, $p < 0.05$.

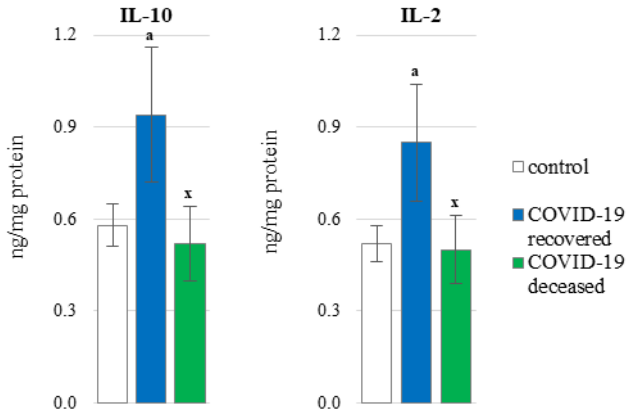

Figure 4. The level of anti-inflammatory interleukin 10 (IL-10) and pro-inflammatory interleukin 2 (IL-2) and interleukin 6 (IL-6) in the granulocytes of patients with COVID-19, including those who recovered (n=16) and those who deceased (n=16) as well as healthy subjects (n=16). Data points represent the mean ± SD; a - significantly different from healthy subjects, $p < 0.05$; x - significantly different from recovered patients with COVID-19, $p < 0.05$.

Our study also revealed that the redox imbalance observed in the granulocytes of patients with COVID-19 also contributed to the altered metabolism of PUFAs. As a result of enzymatic transformations of PUFAs in granulocytes of COVID-19 patients, the levels of a group of lipid mediators, such as eicosanoids, important for cell metabolism changed (Figure 5).

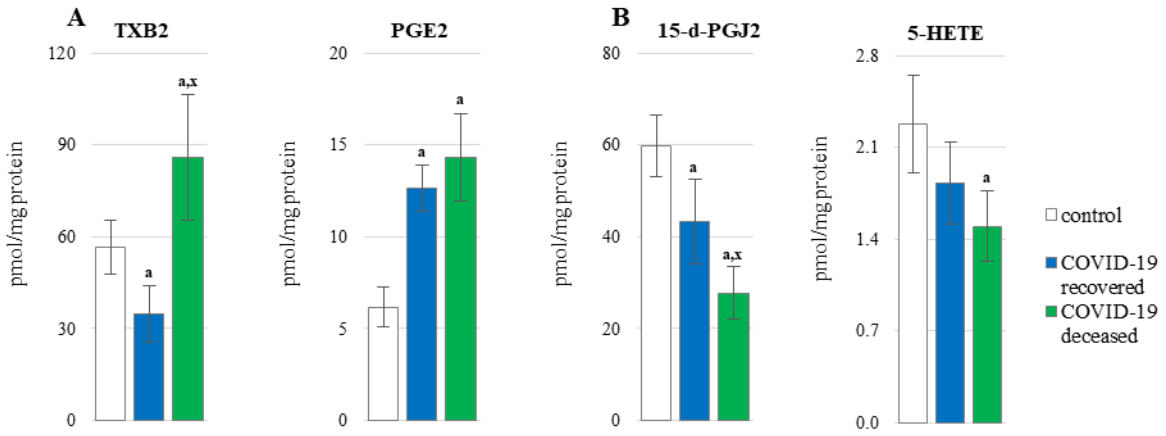

Figure 5. The level of pro-inflammatory eicosanoids (A): thromboxane B2 (TXB_2) and prostaglandin E2 (PGE_2) and anti-inflammatory eicosanoids (B): 15-deoxy-delta12,14-prostaglandin J2 (15d-PGJ2) and 5-hydroxyeicosatetraenoic acid (5-HETE) in the granulocytes of patients with COVID-19, including those who recovered (n=16) and those who deceased (n=16) as well as healthy subjects (n=16). Data points represent the mean ± SD; a - significantly different from healthy subjects, $p < 0.05$; x - significantly different from recovered patients with COVID-19, $p < 0.05$.

The obtained results indicate that SARS-CoV-2 infection caused an increase in the level of pro-inflammatory eicosanoids such as prostaglandin E2 (PGE2) and thromboxane B2 (TXB2) in the granulocytes of patients who eventually died as a result of COVID-19, while the granulocytes of recovered patients were characterized by a decrease in TXB2 and an increase in PGE2 levels. Moreover, in both groups of patients, a decrease in the level of pro-inflammatory eicosanoids was observed, including a statistically significant reduction in the anti-inflammatory eicosanoid 15-d-PGJ2, while the level of 5-HETE was significantly reduced only in patients who died. The observed di-

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rection of changes in the level of granulocyte eicosanoids in COVID-19 patients irrespective of their gender (Supplementary, Figure 5A).

These changes in phospholipid metabolism were accompanied by changes in the level of receptors coupled with protein G (Figure 6). The enhanced expression of CB_2 and PPARγ receptors, which have antioxidant and anti-inflammatory properties. was observed in both groups of patients. Additionally, increased expression of the CB_1 receptor in granulocytes of recovered patients was ~~observed~~/indicated, while the level of TRPV1 receptor did not change significantly. Changes in the expression of coupled with protein G receptors in patients of both sexes with COVID-19 followed the same trend as was seen in the entire patient population who survived or have died (Supplementary, Figure 6A).

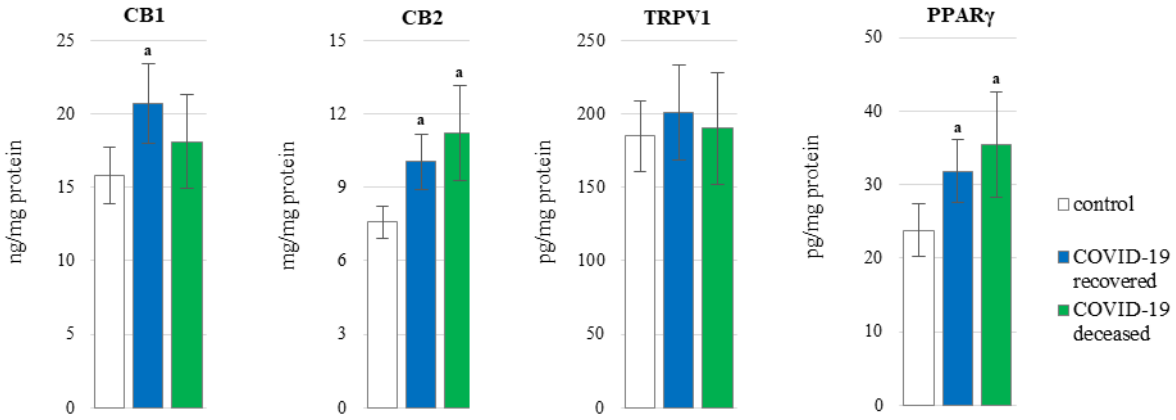

Figure 6. The level of receptors involved in oxidative and inflammatory reactions of granulocytes from patients with COVID-19, including those who recovered (n=16) and those who deceased (n=16) as well as healthy subjects (n=16). CB1 and CB2 - cannabinoid receptors 1 and 2; TRPV1 - the transient receptor potential cation channel subfamily V member 1; PPARγ - peroxisome proliferator-activated receptor gamma. Data points represent the mean ± SD; a - significantly different from healthy subjects, $p < 0.05$; x - significantly different from recovered patients with COVID-19, $p < 0.05$.

4. Discussion

In the immune response to the penetration of the pathogen into the human body, both the acquired immunity mechanisms, where lymphocytes are the effector cells, and innate immunity, associated especially with phagocytes (monocytes/macrophages and granulocytes), are activated. However, innate response develops first, with granulocytes, and especially neutrophils playing a major role in this kind of response, [2]. Although their main mechanisms of action are associated with the response to bacterial infections, they are also activated in the case of viral infections. However, while in the case of bacterial infections the action of cytotoxic factors such as proteolytical enzymes and ROS produced by neutrophils is largely limited to target ingested pathogenic bacteria, in case of viral infection the dominant mechanism of action of neutrophils is degranulation and the release of these toxic elements into the extracellular environment [23]. As a result, the surrounding tissues are damaged. It is especially intensified in the case of a significant increase in the number of neutrophils, as it is observed in the case of sepsis [24]. In our study, an increase in the percentage of neutrophils in COVID-19 patients was observed, especially in more severe cases, resulting in the patient's death. As a result, since it is known that neutrophils are recruited to the place where disease processes take place, in this case the lungs, the factors they produce may have affected the lung tissue, resulting in pulmonary damage, as observed in ARDS related to COVID-19 [25,26]. The mechanisms responsible for this are mainly those aimed at direct elimination of pathogens, including phagocytosis, oxidative burst, ROS release into the environment or neutrophil extracellular traps (NETosis), which in the case of COVID-19 lead to tissue damage [27]. Increased levels of NET components were observed in the lungs of COVID-19

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patients, and increased levels of myeloperoxidase, citrullinated histone H3 and DNA were identified in the serum of COVID-19 patients [28]. However, in addition to acting directly, neutrophils also modulate the immune response. This is based on the release of cytokines such as the IL-2, IL-6 and Il-10 observed in this study.

As a result of SARS-COV-2 infection, there is also an oxidative burst in granulocytes with an increased generation of superoxide radical anion and hydrogen peroxide, which favors the intensification of oxidative conditions [2]. Therefore, the observed reaction of granulocytes to overproduction of ROS is the activation of the nuclear factor associated with E2 factor (Nrf2), which is responsible for the induction of biosynthesis of cytoprotective proteins, including those that protect cells against oxidative and/or electrophilic stress [29]. Under physiological conditions, Nrf2 functions in the cytoplasm in the form of a complex with its inhibitor, the protein Keap1, which directs Nrf2 to ubiquitination-dependent degradation [30]. On the other hand, under oxidative conditions resulting from viral replication, an indicator of which can be observed, an increased level of the lipid peroxidation product - 4HNE in the plasma of the same patients with COVID-19 [31] may lead to modifications, by ROS or electrophilic compounds, of the critical sulfhydryl groups of the cysteine of Keap1, the consequence of which is the dissociation of the Nrf2-Keap1 complex and the migration of Nrf2 to the nucleus, where it binds to a DNA sequence known as an antioxidant response element (ARE), in the promoter regions of target genes coding mainly antioxidant enzymes [32], thus increasing the transcription of these genes. The Nrf2 gene also contains the ARE sequence in its promoter [33], enhancing its expression in the positive feedback loop. A significant increase of free Keap1 was observed especially in the granulocytes of patients who survived COVID-19, which confirms the possibility of increased transcriptional effects of Nrf2 and, consequently, increased antioxidant defense in the granulocytes of these patients. In general, oxidative stress and inflammation overexpress Nrf2 to protect cells from the effects of overproduction of ROS and pro-inflammatory cytokines [34]. A particularly significant increase in the level of Nrf2 and its phosphorylated form was observed in COVID-19 patients who died, which also corresponds to elevated levels of heme oxygenase (OH-1), which is a key antioxidant generated by the transcriptional effects of Nrf2 [35]. Upregulation of the Nrf2-transcriptional target HO-1, has been linked to an antiviral response against many viruses including influenza virus and respiratory syncytial virus [36]. Moreover, HO-1 is known to be involved in the degradation of heme into three products, such as biliverdin, Fe^{2+} and CO, and it is suggested that each of the products is active against SARS-CoV-2 [37]. It is believed that biliverdin can inhibit the 3CL-Pro and PLpro proteases necessary for SARS-CoV-2 replication as it inhibits the proteases of other viruses with high homology to the above. Moreover, it is known that free iron (II) ions by binding to the HCV RdRp protein inhibits its enzymatic activity, hence the activity of a similar protein found in SARS-CoV-2 may be inhibited [38].

On the other hand, CO induces response against viruses with a positive single-stranded RNA such as E71, and this effect is phenocopied by the CO donor, which is associated with the activation of guanylate cyclase and, consequently, protein kinase G, which inhibits the activity of NADPH oxidase, the basic enzyme responsible for the generation of the superoxide anion in the cytosol [38]. As a consequence, if the presented mechanisms work for SARS-COV-2, which is also a positive single-strand RNA virus, the activation of the Nrf2/HO-1 pathway observed in granulocytes could lead to amelioration of the disease. However, it acts in opposite way, being associated with the fatal outcome of SARS-CoV-2 infection. It can therefore be suggested that, since both CO and biliverdin have a pro-inflammatory effect, this effect is dominant and decisive for the fate of patients.

Studies by other authors also show that overexpression of HO-1 in endothelial cells inhibits TNF-α-induced expression of pro-inflammatory adhesion molecules (E-selectin and VCAM-1), which takes place at the mRNA level by interfering with the transcription rate [37]. Such relationships indicate that the Nrf2 pathway interacts with the NFκB pathway, inhibiting its activation by increasing HO-1 expression and thus reducing cytokine release. This is especially evident in the case of the use of the Nrf2 activator, which caused a reduction in the level of 36 genes that code for cytokines, which resulted in a reduction in the level of cytokines in COVID-19 [39]. On the other hand, NF-κB can

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also regulate the expression of genes regulated by ARE, a regulatory element contained in the promoter of many proteins with a cytoprotective nature [40]. It has been shown that the canonical NF-κB subunit - p65 antagonizes the formation of the Nrf2-ARE junction, which reduces the transcription of ARE dependent genes, decreases the level of CBP and increases the recruitment of HDAC3 (histone 3 deacetylase) to the ARE region [40]. The potential contribution of Nrf2 to the development of COVID-19 is also evidenced by the fact that activators of Nrf2 - 4-OI and DMF suppress inflammation in COVID-19, including peripheral blood mononuclear cells, with a similar response observed in the case of infection with other viruses such as Herpes Simplex-1/-2, vaccinia virus and Zika virus [41]. In addition, it is believed that the induction of Nrf2 may alleviate the dysfunction of regulatory T lymphocytes and, consequently, autoimmune disorders in COVID-19 [38], as has been found that patients with pre-existing susceptibility to autoimmune diseases are at greater risk of aggressive COVID-19.

The genes regulated by Nrf2 include the genes of SOD, catalase, peroxiredoxin and glutathione peroxidase [42], while the transformation of the first reactive form of oxygen generated in the mitochondrial process - superoxide anion to hydrogen peroxide involves the cytosol (Cu,Zn-SOD) and mitochondrial (Zn,Mn-SOD) superoxide dismutases. The results of this study indicate that in the granulocytes of COVID-19 patients there is a dramatic reduction in the activity of both cellular superoxide dismutase isoforms, especially in the group of patients who died as a result of COVID-19. This may suggest that despite the conditions for increased biosynthesis, as indirectly evidenced by the increase in Nrf2 and HO-1 levels, the enzyme was inactivated, presumably as a result of oxidative modification by electrophiles generated during infection, such as ROS, 4-HNE or 15d-PGJ2. However, given the observed results of the Nrf2 antioxidant response in the lung biopsies of COVID-19 patients, which indicate the suppression of genes associated with the Nrf2 antioxidant response, and in vitro experiments that have shown that the expression of Nrf2 inducible proteins is also reduced, it is likely that the suppression of this metabolic pathway occurs in COVID-19 patients [41]. These data are in agreement of the decreased SOD1 and SOD2 activities observed in current study, reflecting also decrease of the SOD levels recently revealed in the plasma of COVID-19 patients [43]. In contrast, the inverse granulocyte response to COVID-19 with respect to HO-1 may be a specific gene response of that protein or the type of cell whose antioxidant response was analyzed. Similarly in vitro experiments on human follicular cells infected with other respiratory viruses such as influenza A virus (IAV H1N1), have shown reduction in SOD1 levels as a result of proteasomal degradation of the transcription factor protein specificity 1 (Sp1), which participates in the expression of the SOD1 gene [44]. In another study, H5N1 infection decreased Cu, Zn-SOD expression in lung epithelial cells at mRNA and protein levels, while forced SOD1 expression significantly inhibited H5N1-induced increase in ROS levels and decreased proinflammatory response and viral replication [45].

ROS generated during infection also regulate the activation of NF-KB by altering the phosphorylation of IkBα kinase, the primary inhibitor of NFkB, what leads to the release of p50 and p65 dimers and their translocation to the nucleus for the transcription of inflammatory genes, including TNF-α [46,47]. Among them, the degradation of IκBα plays an important role in the activation of NFκB signaling pathways, which was seen in our study in the granulocytes of COVID-19 survivors, as shown by decreased IκBα level. It is also known that the complex of the Nrf2 inhibitor, Keap1/Cul3, can target the IκBα element to ubiquitination and degradation [48]. Thus, the increased level of Keap1 in the granulocytes of COVDI-19 survivors supports this possibility. In addition, Keap1 can negatively regulate NFκB functionality by preventing HSP90 from binding to IKKβ which triggers its autophagous degradation, and further degrades this inhibitor.

Moreover, one of the inhibitors of the NFκB-IκB complex is major bioactive product of PUFA peroxidation 4-HNE, the level of which increases under oxidative stress [48,49] and was found to be associated with systemic vascular stress in COVID-19 patients, in particular in those who passed away [31,51]. However, it is known that NFκB is essential for maintaining immune homeostasis and preventing autoimmunity caused by COVID-19 [46]. It has already been shown that viruses inducing respiratory infections such as SARS-CoV-2 simultaneously contribute to the activation of the NFκB pathway

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[46], but the activation of this transcription factor in granulocytes from COVID-19 patients has not been assessed before. The increases in the levels of the two NFκB subunits, p52 and p65, observed in our study may indicate that this is the result of oxidative stress and the expression of cytokines, including TNFα. It may be suggested that COVID-19 activates the NFκB pathway in granulocytes, especially in patients with severe, fatal disease, as evidenced by increases in TNFα, IL-10 and IL-2. The NFκB influences the induction of innate immune response leads to activation of cells involved in adaptive immunity, notably T-cells in connection with autoimmune inflammation and inflammasome release [52].

In recent years endogenous lipids and their metabolites are gaining attention as likely the strongest mediators of inflammation. The PUFAs, especially arachidonic acid and its metabolites, play a key role in the response to viral infection [53,54]. In granulocytes of our COVID-19 patients, the biosynthesis of pro-inflammatory eicosanoids was enhanced, and the anti-inflammatory effect was reduced. Increased levels of pro-inflammatory eicosanoids such as PGE2 and TXB2 have been found in the granulocytes of COVID-19 patients who died. Literature data show that significantly increased levels of pro-inflammatory PGE2, TXB2, 12-HHTrE and LTB4 were found in BAL fluids of COVID19 patients [55]. Increased PGE2 levels may be one of the main factors leading to increased COVID-19 infection [56] through the cascade release of pro-inflammatory cytokines [56]. Increased PGE2 levels have also been detected in obese patients, which increases the risk of exacerbation of COVID-19 disease [58]. Moreover, it is known that PGE2 may aggravate intravascular thrombosis [59], which is another challenge in COVID-19 patients. In our study, elevated levels of TXB2 were found, particularly in patients who died as a result of COVID-19. Similar relationships were found in patients with COVID-19-associated pneumonia patients [60,61]. TXB2 is a biologically inactive catabolite of TXA2 thromboxan, which is a potent activator of platelet aggregation, thus participating in the blood coagulation process. This is consistent with the diffused microthrombosis observed in COVID-19 [60,61]. Moreover, IL-1 also causes thrombogenicity of platelets by stimulating the formation of thromboxane A2, which is released into the inflamed environment in patients with COVID-19 disease [62]. The above changes in pro-inflammatory lipid mediators in granulocytes of COVID-19 patients were also accompanied by a significant decrease in the level of anti-inflammatory prostaglandin 15-d-PGJ2, which inhibits COX2 activity in inflammatory conditions [63]. Moreover, 15d-PGJ2, as an electrophilic molecule, forms adducts with proteins, changing their structure and functions, which allows for modification of both inflammation and oxidative stress, while 15d-PGJ2 directly inhibits multiple steps in the NFκB signaling pathway and the expression of NFκB dependent genes [63]. In addition, 15d-PGJ2 activates Nrf2 signaling and induces the transcription of antioxidant enzyme genes, including HO-1 [64], which was seen in granulocytes of our COVID-19 patients.

At the same time, 15d-PGJ2 may exert an anti-inflammatory effect by stimulating PPARγ receptors. In the event of a COVID-19 related cytokine storm, PPARγ agonists are considered as potential candidates for treatment [65]. However, there is no clinical trial of 15d-PGJ2 in SARS-CoV-2 infection so far. Moreover, the moderate and severe forms of the disease were characterized by higher levels of 5-HETE, suggesting that PUFAs metabolites may play a key role in COVID-19 infection [66]. The reduction of the 5-HETE level may be related to the decreased activity of the 5-LOX as a result of the action of the prostaglandin PGE2 [67]. Activation of CB2 receptors also causes anti-inflammatory effects by inhibiting leukocyte recruitment, reducing the synthesis and release of chemokines, adhesion molecules, prostanoids, eicosanoids, ROS and pro-inflammatory cytokines [68,69], supporting our findings.

5. Conclusions

In granulocytes of COVID-19 patients, a decrease in antioxidant activity was observed, associated with the activation of the Nrf2 antioxidant pathway as an attempt to compensate for the reduction in antioxidant activity, which was more intense in patients who passed away. These changes were probably supported by the increased expression of the anti-inflammatory and antioxidant CB2 receptor, as was observed in our patients. On the other hand, the redox-dependent NFκB pathway, activated by ROS, was acting

in the opposite way. In patients who have deceased, the expression of both of its subunits was increased, while in those who survived, only NFκB p65 was increased, but less than in the granulocytes of the deceased patients. Higher activation of the above-mentioned redox dependent pathways may suggest increased oxidative stress in patients who died. However, for cytokines, the situation is different, as levels of both pro-inflammatory and anti-inflammatory were elevated only in survivors, suggesting the malfunction of the immune system in the patients who eventually died. The levels of other inflammatory modulators, the eicosanoids, were also altered in COVID-19. In the granulocytes of patients who did not survive COVID-19, anti-inflammatory eicosanoids levels were reduced, while pro-inflammatory eicosanoids level was increased, indicating activation of immune cells eventually causing a more severe course of COVID-19. Finally, it should be emphasized that changes in the expression/activity of antioxidant and inflammatory parameters did not significantly differentiate patients in respect to their gender.

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