

# May the Nitrosative and Carbonyl Stress Promote Inflammation in Patients with Colorectal Cancer?

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**Purpose:** Overproduction of reactive nitrogen species (RNS) causes the nitrosative stress, which plays a vital role in the development of metabolic, inflammatory, and cancerous diseases. However, the role of nitrosative and carbonyl stress in the biology of colorectal cancer (CRC) is still not well understood. Therefore, this study evaluated nitrosative stress, protein and DNA oxidation/glycooxidation, and pro- and anti-inflammatory cytokines in CRC patients compared with healthy controls.

**Patients and Methods:** Fifty-five CRC patients (21 women, 34 men) and 55 healthy controls matched for sex and age were included in the experiment. Nitrosative stress parameters (nitric oxide (NO), peroxyntirite, S-nitrosothiols, and nitrotyrosine), protein oxidation (total thiols) and glycooxidation products (kynurenine N-formylkynurenine, dityrosine, Amadori products, and amyloid), and DNA damage markers (8-hydroxydeoxyguanosine (8-OHdG)), as well as levels of pro- and anti-inflammatory cytokines, were measured in serum or plasma samples.

**Results:** The levels of NO, peroxyntirite, S-nitrosothiols, nitrotyrosine, total thiols, kynurenine, N-formylkynurenine, dityrosine, Amadori product, amyloid, and 8-OHdG, as well as IL1 $\alpha$ , IL1 $\beta$ , IL6, IL10, and TNF- $\alpha$ , were significantly higher in CRC patients than in controls. Oxidation and glycooxidation products were positively correlated with pro-inflammatory (IL1 $\alpha$ , IL1 $\beta$ , IL6, TNF $\alpha$ ) and anti-inflammatory cytokines (IL10), indicating that redox damages may promote inflammation in CRC patients. Many redox biomarkers differentiate patients with CRC from healthy individuals with high sensitivity and specificity.

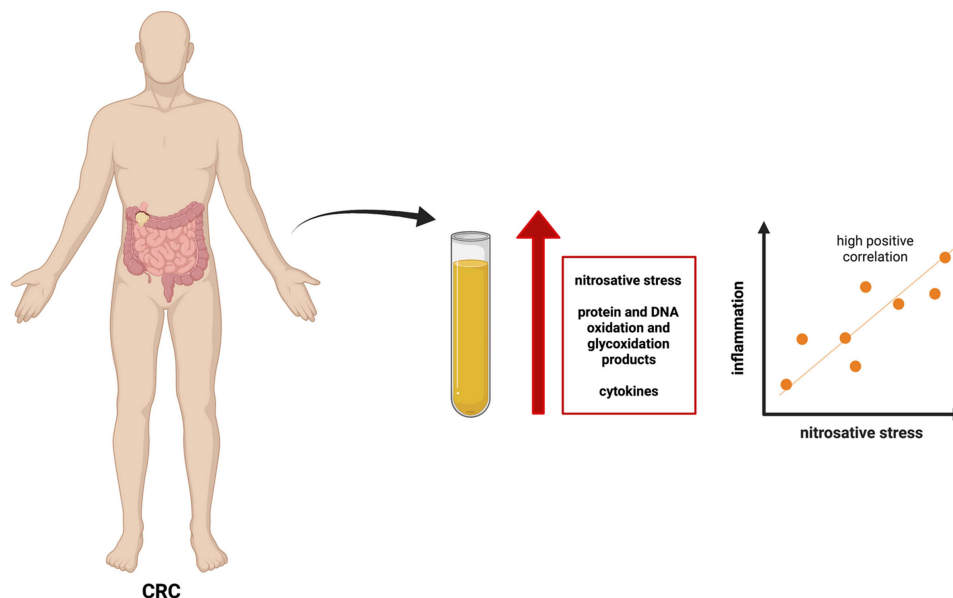
**Conclusion:** Correlations of chosen oxidative products with pro-inflammatory (IL1 $\alpha$ , IL1 $\beta$ , IL6, TNF $\alpha$ ) and anti-inflammatory cytokines (IL10) suggest that redox damages may promote inflammation in CRC patients. Thus, our research is the first point for further clinical trials focusing on the evaluation of the diagnostic utility of nitrosative stress biomarkers in a larger group of CRC patients.

**Keywords:** nitrosative stress, cytokines, inflammation, colorectal cancer

## Introduction

Although screening tests such as stool examinations and colonoscopy are widely available, colorectal cancer (CRC) remains one of the cancers with the worst prognosis.<sup>1</sup> According to the database published by Global Cancer Observatory (GLOBOCAN), the total number of deaths due to colorectal cancer in 2020 was 579,858, whereas the number of new cases was 1,148,515. This classified CRC in third place of the most common cancers globally.<sup>1</sup> Simultaneously, GLOBOCAN predicts that the number of new cases will increase by 58.8% to 1.92 million within twenty years, and the number of deaths will increase by 71.9% to 991,332.<sup>1</sup> Over 20% of CRC patients have an IV stage with metastasis at diagnosis, which precludes adequate surgical resection. In patients with metastasis, only 70–75% survive one year, 30–35% over three years, and about 20% of CRC patients survive beyond five years from the time of diagnosis.<sup>2</sup> Therefore, to improve these unfavorable statistics, new diagnostic methods would be helpful in the diagnosis of colorectal cancer. There is still a great need for additional trials on identifying new non-invasive biomarkers associated with the early events of colorectal

## Graphical Abstract



carcinogenesis and the development of new, more effective therapies in CRC treatment. The basis for further studies is knowledge increasing CRC biology and finding a connection between biochemical alterations in cancerous tissues and blood.

One of the factors associated with carcinogenesis (also in the colon) is oxidative and nitrosative stress reflecting the lack of balance between the production of reactive oxygen (ROS) and nitrogen (RNS) species and the ability to detoxify/remove free radicals by antioxidants both enzymatic and non-enzymatic.<sup>3</sup> It is well known that increased production of ROS and RNS leads to proteins, lipids, and DNA damages. Overproduction of free radicals is also responsible for chronic inflammation.<sup>4,5</sup> The effect of inflammation is the induction of immune cells to the secretion of cytokines and chemokines.<sup>6</sup> Exposure of colon cells to cytokines may induce inflammatory pathways such as JAK-STAT, NF- $\kappa$ B, or MAPKs cascades, increase the expression of pro-inflammatory enzymes, and induce the production of pro-inflammatory mediators (such as interleukin  $1\beta$  or tumour necrosis factor  $\alpha$ ).<sup>7</sup> It has been observed that TNF- $\alpha$  derived by activated macrophages and T-cells may promote inflammation and initiate carcinogenesis by inducing DNA damage.<sup>8</sup>

It is well known that inflammation may lead to carcinogenesis and then modulate cancer cells by stimulating or inhibiting their growth. The activity of cells associated with inflammation and inflammation-regulating factors may modulate the balance between their pro- and antitumour events.<sup>9</sup> Acute inflammation in the early stage of cancer may show anticancer action, and in some types of cancers, the ability to induction of controlled acute inflammatory process may be used as an element of therapy. Nevertheless, in chronic inflammation, inflammatory cells promote the cancer cells growth and stimulate proliferation and survival.<sup>10</sup> Understanding the mechanisms linking inflammation with carcinogenesis may provide significant benefits both in the clinical and scientific aspects linked to developing new diagnostic and therapeutic methods.

Therefore, our study's main aim was to assess the relationship between nitrosative stress and pro-inflammatory mediators in colorectal cancer patients in comparison with healthy controls. We also assessed the diagnostic utility of nitrosative stress and pro-inflammatory mediators using the receiver operating characteristic (ROC) analysis.

## Materials and Methods

### Patients

The study was approved by the Bioethics Committee of the Medical University of Bialystok, Poland (permission number APK.002.99.2021). After a detailed description of the aim of the research and the possible risk, all patients qualified to

the study agreed in writing for participation in the experiment. The study was performed in accordance with the World Medical Association Declaration of Helsinki for ethical principles for medical research involving human subjects.

The study included 55 patients (21 women and 34 men) treated surgically due to colorectal cancer in the 2nd Clinical Department of General and Gastroenterological Surgery at the Medical University of Białystok Clinical Hospital in 2017–2021. Patients were qualified based on the following criteria: men and women without coexistence of other systemic diseases who did not receive radio- or chemotherapy before the surgery. Exclusion criteria for patients with colorectal cancer were as follows: acute inflammatory diseases, infectious diseases (HIV/AIDS, hepatitis A, B, or C), autoimmune diseases (Crohn's and Hashimoto's disease, ulcerative colitis, rheumatoid arthritis, and psoriasis), cardiovascular diseases, metabolic diseases, such as osteoporosis, type 1 diabetes, mucopolysaccharidosis and gout, digestive, respiratory or genitourinary systems diseases. Additionally, smokers and patients taking drugs (antibiotics, non-steroidal anti-inflammatory drugs, glucocorticosteroids, vitamins, and dietary supplements) for the three months before the surgery were excluded from the study.

The time from cancer diagnosis to the surgical resection ranged from a minimum of two days to a maximum of four weeks. The study material was taken from all patients before surgery.

The control group included 55 healthy patients (selected by sex and age to match the study group) attending follow-up visits at the Department of Restorative Dentistry at the Medical University of Białystok from January 2018 to January 2020. To the control group were qualified patients with normal results of complete blood count (CBC) and biochemical blood tests (Na<sup>+</sup>, K<sup>+</sup>, creatinine, AST, ALT).

The number of patients in the study and in the control group was set based on a previously conducted pilot study. The power of the study was set at 0.8.

## Histopathological Analysis

Histopathological diagnosis was determined using hematoxylin and eosin (H+E) staining and included the following parameters: histological type, the grade of histological malignancy according to the World Health Organization (WHO) guidelines<sup>11</sup> and tumour stage according to the TNM classification standard of the Union for International Cancer Control.<sup>12</sup>

Each tumour was cut along a line that was parallel to the longest tumour axis. In this way, 4 to 8 slices contained cancer and adjacent macroscopically unchanged tissues of 1–1.5 cm in size were taken. Tissues were fixed in 10% buffered formalin but no longer than 24 hours. The specimens were embedded in paraffin at a temperature of 56 °C. Paraffin blocks were cut into 4 µm thick sections on a microtome Microm H340. The obtained sections were stained with hematoxylin-eosin and reviewed by two independent pathologists on a microscope Olympus CX22 under 200× and 400× magnification.

## Blood Collection

Fasting venous blood (10 mL) was taken from all patients on an empty stomach, upon overnight rest, using the S-Monovette<sup>®</sup> K3 EDTA and S-Monovette<sup>®</sup> serum collection system (Sarstedt, Germany). Immediately after collection, blood was centrifuged at 1500 x g for 10 min at +4°C (MPW 351, MPW Med. Instruments, Warsaw, Poland) to separate plasma or serum from erythrocytes. The top layer (plasma or serum) was then taken. About 0.5 M butylated hydroxytoluene (20 µL/ 2 mL plasma or serum) was added to prevent sample oxidation. Until redox determinations, all samples were stored at –80°C.

## Determination of Redox Markers

All the determinations were conducted in duplicate in the plasma or serum samples. The Infinite M200 PRO Multimode Microplate Reader (Tecan) was using for measurement of absorbance/fluorescence. The results were standardized to 100 mg of total protein. The total protein content was estimated colorimetrically at 562 nm wavelength with the bicinchoninic acid (BCA) method. A commercial kit was used according to the manufacturer's instructions (Thermo Scientific PIERCE BCA Protein Assay; Rockford, IL, USA).

## Nitrosative Stress

Nitric oxide (NO) concentration was determined indirectly by measuring its stable decomposition products NO<sup>2-</sup> and NO<sup>3-</sup> using the Griess reaction. For this purpose, sulfanilamide and NEDA·2 HCl (N-(1-naphthyl)-ethylenediamine dihydrochloride) were used.<sup>32,33</sup> The absorbance was analyzed at 490 nm.<sup>13,14,15</sup>

The peroxyxynitrite concentration was determined spectrophotometrically based on peroxyxynitrite-mediated nitration resulting in the nitrophenol formation.<sup>16</sup> The absorbance was assessed at 320 nm.

The concentration of S-nitrosothiols was analyzed spectrophotometrically using Griess's assay based on the reaction with Cu<sup>2+</sup> ions.<sup>17</sup> The absorbance was assessed at 490 nm.

The concentration of nitrotyrosine was determined spectrophotometrically using an ELISA kit (Immundiagnostik AG; Bensheim, Germany) according to the manufacturer's instructions.

## Protein Glycoxidation

A fluorescence evaluation of oxidative protein products was carried out. Dityrosine, kynurenine, and N-formylkynurenine levels were determined fluorimetrically. Immediately before measurement, serum samples were diluted in 0.1 M H<sub>2</sub>SO<sub>4</sub> (1:5, v/v).<sup>18</sup> The characteristic fluorescence at 330/415, 365/480, 325/434, and 295/340 nm, respectively, was measured in 96-well black-bottom microplates.<sup>19</sup> The results were expressed in arbitrary fluorescence units (AFU)/mg protein.

The formation of amyloid cross- $\beta$ structure was measured colorimetrically using thioflavin T.<sup>20</sup> The characteristic fluorescence was measured at 435/485 nm.

The formation of the Amadori product was analyzed colorimetrically using nitro blue tetrazolium (NBT) assay.<sup>21</sup> The absorbance was measured at 525 nm, and an extinction coefficient of 12,640 cm<sup>-1</sup>mol<sup>-1</sup> L for monoformazan was used.

## Oxidative Damage Products

The concentration of total thiols was measured colorimetrically at 420 nm using Ellman's reagent.<sup>22</sup> The concentration of thiol groups was calculated from the calibration curve using reduced glutathione (GSH) as a standard.

The 8-hydroxydeoxyguanosine (8-OHdG) level was determined colorimetrically with commercial ELISA kits (Cayman Chemical, Miami, USA; USCN Life Science, Wuhan, China, respectively), according to the manufacturer's instructions.

## Inflammatory Cytokines

The concentrations of interleukin 1 alpha (IL1 $\alpha$ ), interleukin 1 beta (IL1 $\beta$ ), interleukin 6 (IL6), interleukin 10 (IL10), and tumour necrosis factor alpha (TNF- $\alpha$ ) were analysed using commercial ELISA kits from EIAab, Wuhan, China, according the instructions provided by the manufacturer.

## Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, La Jolla, USA). The Shapiro–Wilk test was used to determine the normality of distribution. For a normal distribution, the Student's *t*-test was used. In the case of the lack of normal distribution, the Mann–Whitney *U*-test was used. The results were presented as median (minimum-maximum), and the value of *p* < 0.05 was considered statistically significant. Spearman correlation coefficient was used to evaluate the relationships between redox and clinicopathological parameters describing the study group. In order to identify factors that determine the levels of redox biomarkers, we performed multiple regression analyses. Tumour's location, tumour's size, histological type, pT, pN, and pM were included as independent variables; 95% confidence intervals (CI) were noted along with regression parameters. Receiver Operating Characteristic (ROC) analysis was used to assess the diagnostic utility of the redox biomarkers. AUC (area under the curve) and optimal cutoff values were determined for each parameter, ensuring high sensitivity and specificity.

## Results

### Clinical Findings

The study group consisted of 55 patients with colorectal cancer, and the control group included 50 healthy people matched by age and sex with the study group. The tumour differentiation grade in all patients with CRC was G2 (moderately differentiated). Tumour location was divided into two groups: left-side tumours (descending colon, sigmoid colon, and rectum) and right-side tumours (caecum, ascending colon, transverse colon).<sup>23</sup> Right-side colorectal cancer

(RCRC) occurred in 40% of patients, whereas left-side (LCRC) in 60% of patients. Forty-four patients had adenocarcinoma, whereas 11 patients had mucinous adenocarcinoma. About 24% of patients had a pT1, 27% - pT2, 36% - pT3 and 13% had a pT4 stage of tumour. About 43% of patients have lymph nodes (N1+N2), and 15% have distant metastasis (M1). Most of the patients had a normal level of tumour markers – CEA, CA19-9, and CA72-4. Detailed patient characteristics are shown in [Table 1](#).

## Nitrosative Stress

The levels of NO, peroxynitrite, S-nitrosothiols, and nitrotyrosine were significantly higher in patients with CRC than in the control group ( $p = 0.0144$ ,  $p < 0.0001$ ,  $p < 0.0001$ ,  $p < 0.0001$ ) ([Figure 1](#)). ROC analysis was performed for evaluation of the diagnostic value of nitrosative stress parameters in the diagnostics of colorectal cancer ([Table 2](#)). Determination of NO, peroxynitrite, S-nitrosothiols, and nitrotyrosine showed a very high diagnostic value (AUC = 0.6545,  $p = 0.01423$ , AUC = 0.9176,  $p < 0.0001$ ; AUC = 0.7771,  $p < 0.0001$ ; AUC = 0.8740,  $p < 0.0001$ ) in differentiating the group of patients with CRC from healthy people. The cutoff values with sensitivity and specificity values for determining nitrosative stress parameters have been shown in [Table 2](#).

## Protein Glycoxidation

We observed increased fluorescence of glycoxidation products (dityrosine, kynurenine, N-formylkynurenine, Amadori product, amyloid) in patients with CRC in comparison with control group. The differences were statistically significant, and p-values were followed:  $<0.0001$ ,  $<0.0001$ ,  $<0.0001$ ,  $<0.0001$ ,  $p = 0.0163$  ([Figure 2](#)). AUC for dityrosine was 0.9171 with cutoff value  $>632.4$  AFU/100mg protein, 87.78% sensitivity and 88.0% specificity, for kynurenine was 0.8707 with cutoff value  $>964.4$  AFU/100mg protein, 80.49% sensitivity and 80.0% specificity whereas AUC for N-formylkynurenine was 0.8873 with cutoff value  $>744.4$  AFU/100mg protein, 82.93% sensitivity and 82.0% specificity. We also demonstrated a very high diagnostic value of amyloid (AUC = 0.6492,  $p = 0.01615$ ) and Amadori products (AUC = 0.9985,  $p < 0.0001$ ) determination in the colorectal cancer patients ([Table 2](#)).

## Oxidative Damage Products

We also assessed total thiols and 8-OHdG levels as a DNA and protein oxidation damages marker. There was a significant increase in total thiols and 8-OHdG levels in patients with colorectal cancer compared to healthy people ( $p < 0.05$ ,  $p < 0.0001$ ) ([Figure 2](#)). We demonstrated a very high diagnostic value of total thiols (AUC = 0.6333,  $p = 0.03802$ ) and 8-OHdG (AUC = 0.8594,  $p < 0.0001$ ) in differentiation of CRC patients from healthy control ([Table 2](#)).

## Inflammatory Cytokines

All the analyzed cytokines (IL1 $\alpha$ , IL1 $\beta$ , IL6, IL10, TNF- $\alpha$ ) were significantly higher in the study group than in control. The differences between both groups in all analyzed parameters were statistically significant ([Figure 3](#)). ROC analysis demonstrated that each of the analyzed cytokines (IL1 $\alpha$ , IL1 $\beta$ , IL6, IL10, TNF- $\alpha$ ) might be useful for differentiation patients with colorectal cancer. AUC for IL1 $\alpha$  was 0.7299, whereas the p-value was 0.0001054; for IL1 $\beta$ , these values were the following 0.7002 and 0.0009468, for IL6 AUC was 1.000 and p-value  $<0.0001$ . AUC for IL10 was 0.9564 and p-value  $<0.0001$  and for TNF- $\alpha$  AUC was 0.9857 and p-value  $<0.0001$  ([Table 2](#)).

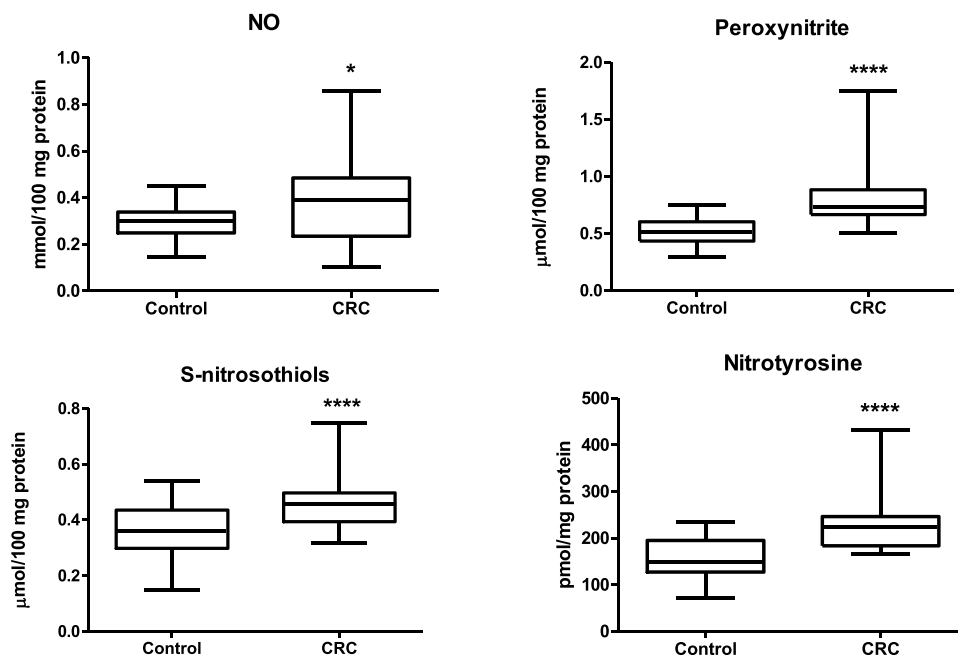
## Correlations

All statistically significant associations are presented in [Figures 4](#) and [5](#). The analysis of the clinical parameters and analysed parameters revealed positive correlations between peroxynitrite ( $p = 0.015$ ,  $R = 0.383$ ) and nitrotyrosine ( $p = 0.018$ ,  $R = 0.370$ ) and tumour location. Interestingly, we demonstrated a negative correlation between IL1 $\alpha$  and tumour size ( $p = 0.034$ ,  $R = -0.263$ ) and positive correlation between IL6 and histological type of the tumour ( $p = 0.043$ ,  $R = 0.299$ ). We observed correlations between CEA and dityrosine ( $p = 0.022$ ,  $R = 0.484$ ), total thiols ( $p = 0.05$ ,  $R = 0.369$ ), kynurenine ( $p = 0.026$ ,  $r = 0.471$ ), N-formylkynurenine ( $p = 0.036$ ,  $R = 0.448$ ), and NO ( $p = 0.015$ ,  $R = 0.511$ ). Moreover, the level of dityrosine ( $p = 0.044$ ,  $R = 0.342$ ), kynurenine ( $p = 0.005$ ,  $R = 0.588$ ), and N-formylkynurenine ( $p = 0.006$ ,  $R = 0.562$ ) correlated positively with CA19-9 ([Figures 4](#) and [5](#)). IL1 $\alpha$  was positively correlated with Amadori products ( $p =$

**Table I** Characteristics of Study Group

Parameter	n (%)
<b>Age</b>	
<60	12 (22.0%)
>60	43 (78.0%)
<b>Sex</b>	
Male	34 (62.0%)
Female	21 (38.0%)
<b>Histological type</b>	
Adenocarcinoma	44 (80.0%)
Mucinous adenocarcinoma	11 (20.0%)
<b>Tumor location</b>	
Left-side	33 (60.0%)
Right-side	22 (40.0%)
<b>Tumor's size</b>	
<3cm	17 (31.0%)
>3cm	38 (69.0%)
<b>pT – depth of invasion</b>	
T1	13 (24.0%)
T2	15 (27.0%)
T3	20 (36.0%)
T4	7 (13.0%)
<b>pN – lymph node metastasis</b>	
N0	32 (58.0%)
N1	14 (25.0%)
N2	9 (17.0%)
<b>pM – distant metastasis</b>	
M0	47 (85.0%)
M1	8 (15.0%)
<b>Stage at diagnosis</b>	
I	12 (22.0%)
II	18 (32.0%)
III	19 (35.0%)
IV	6 (11.0%)
<b>CEA level (ng/mL)</b>	
0–5.0	39 (71.0%)
>5.0	16 (29.0%)
<b>CA 19–9 (U/mL)</b>	
0–35	44 (80%)
>35	11 (20%)
<b>CA 72–4 (U/mL)</b>	
0–6, 9	39 (70%)
>6, 9	16 (30%)

0.0013,  $R = 0.4852$ ), total thiols ( $p = 0.0434$ ,  $R = 0.317$ ), kynurenine ( $p = 0.045$ ,  $R = 0.313$ ) and nitrotyrosine ( $p = 0.007$ ,  $R = 0.410$ ). Positive correlations were also present between IL1 $\beta$  and Amadori products ( $p = 0.001$ ,  $R = 0.488$ ), dityrosine ( $p = 0.075$ ,  $R = 0.280$ ), total thiols ( $p = 0.009$ ,  $R = 0.401$ ), kynurenine ( $p = 0.036$ ,  $R = 0.328$ ), S-nitrosothiols



**Figure 1** Nitrosative stress parameters in patients with colorectal cancer and the control group. The data are presented as median (minimum - maximum). \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ .

**Abbreviation:** NO, nitric oxide.

( $p = 0.085$ ,  $R = 0.273$ ), and nitrotyrosine ( $p = 0.001$ ,  $R = 0.479$ ). We showed positive correlations between IL6 and Amadori products ( $p = 0.003$ ,  $R = 0.441$ ), total thiols ( $p = 0.026$ ,  $R = 0.347$ ), and nitrotyrosine ( $p = 0.017$ ,  $R = 0.369$ ). Moreover, there was a positive correlation between IL10 and Amadori products ( $p = 0.004$ ,  $R = 0.438$ ), total thiols ( $p = 0.029$ ,  $R = 0.340$ ), kynurenine ( $p = 0.027$ ,  $R = 0.346$ ) and nitrotyrosine ( $p = 0.004$ ,  $R = 0.434$ ). The significant associations were also found between  $TNF\alpha$  and Amadori products ( $p = 0.0004$ ,  $R = 0.522$ ), dityrosine ( $p = 0.029$ ,  $R = 0.340$ ), total thiols ( $p = 0.005$ ,  $R = 0.425$ ), kynurenine ( $p = 0.02$ ,  $R = 0.360$ ), and nitrotyrosine ( $p = 0.002$ ,  $R = 0.453$ ).

## Multiple Regression Analysis

In order to find a relationship between level of evaluated nitrosative stress parameters, protein and DNA oxidation/glycooxidation products, and pro- and anti-inflammatory cytokines and clinicopathological parameters including tumour's location, tumour's size, histological type, pT, pN and pM, we developed multiple linear regression models. The redox biomarkers and cytokine levels were used as independent/input variables and clinicopathological parameters were used as output/target/dependent variable. Multiple regression analysis showed that the right-side tumour was associated with the higher peroxynitrite level ( $p = 0.05$ ). Moreover, the association between NO ( $p = 0.035$ ) and amyloid ( $p = 0.028$ ) concentration and presence of mucinous adenocarcinoma was also observed. Significant associations were also noticed between high stage of depth of tumour invasion (pT3) and NO ( $p = 0.045$ ), kynurenine ( $p = 0.042$ ), N-formylkynurenine ( $p = 0.049$ ), dityrosine ( $p = 0.015$ ), and 8-OHdG ( $p = 0.049$ ) (Table 3).

## Discussion

For many years, scientists have tried to explain the significance of redox disturbances in malignant transformation. However, current reports do not indicate the impact of nitrosative stress in developing colorectal cancer. It is well known that nitrosative stress is caused by excessive RNS production induced by endogenous systems and exposure to different physicochemical or pathological factors like UV radiation, chemical carcinogens, lifestyle, diet, or chronic inflammatory conditions.<sup>24</sup> Numerous, clinical and epidemiological research strongly support the thesis about the dual role of ROS and RNS.<sup>25</sup> In high concentrations, RNS cause protein damages to amino acids, post-translational protein modifications, and eNOS-mediated Ras activation by S-nitrosylation.<sup>26</sup> RNS also directly oxidizes polyunsaturated fatty acids in membrane

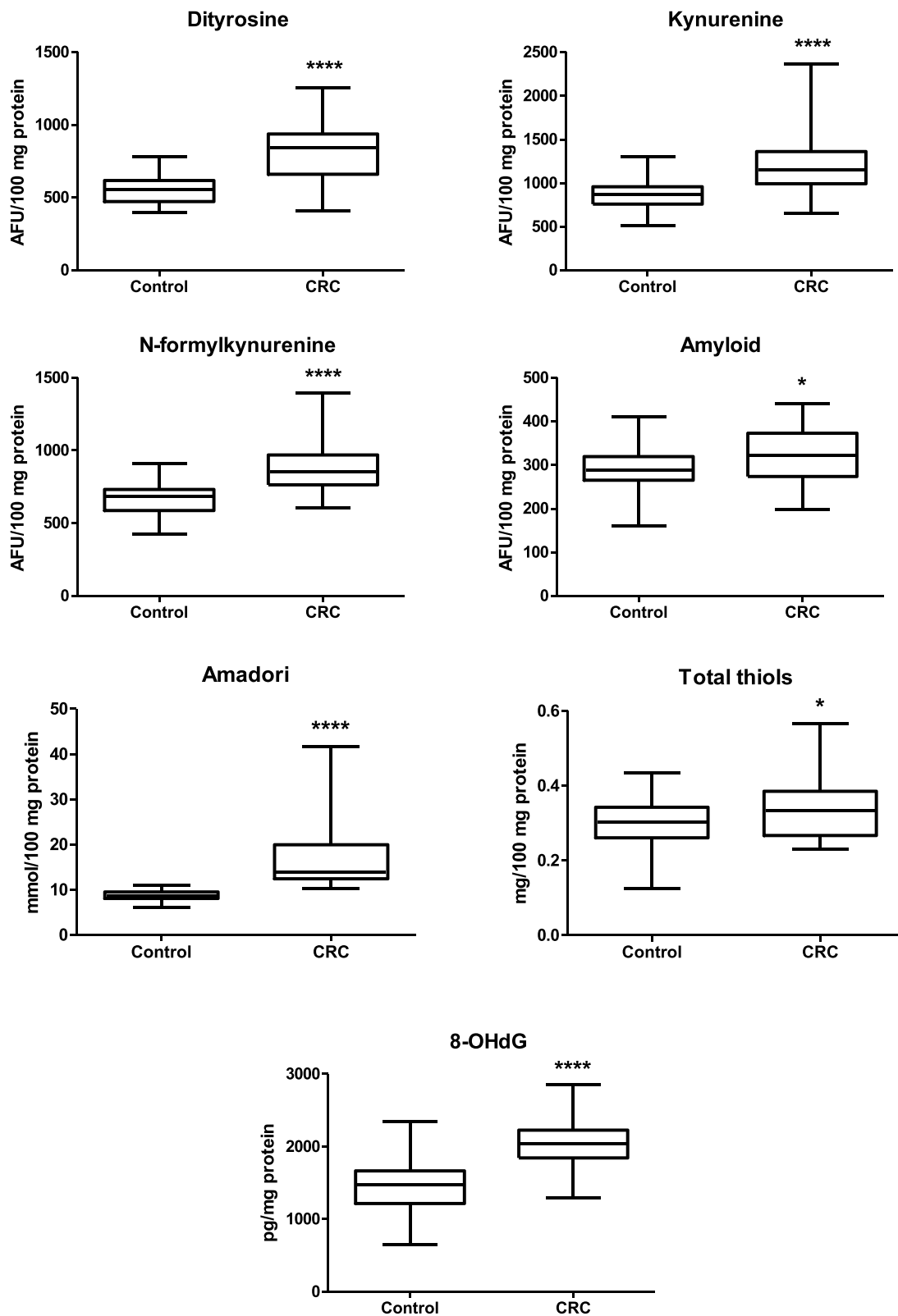
**Table 2** ROC Analysis of Nitrosative Stress Parameters, Protein and DNA Oxidation/Glycoxidation Products, and Pro- and Anti-Inflammatory Cytokines Between Patients with Colorectal Cancer and the Healthy Controls

Parameter	AUC	p-value	Cut-Off	Sensitivity (%)	Specificity (%)	95% Confidence Interval
NO	0.6545	0.01423	> 0.3116	61.90	62.79	0.5277 to 0.7813
Peroxynitrite	0.9176	< 0.0001	> 0.6149	85.37	86.00	0.8628 to 0.9723
S-nitrosothiols	0.7771	< 0.0001	> 0.4182	63.41	64.00	0.6836 to 0.8705
Nitrotyrosine	0.8740	< 0.0001	> 185.5	73.81	72.92	0.8050 to 0.9430
Kynurenine	0.8707	< 0.0001	> 964.4	80.49	80.00	0.7957 to 0.9457
N-formylkynurenine	0.8873	< 0.0001	> 744.4	82.93	82.00	0.8208 to 0.9539
Dityrosine	0.9171	< 0.0001	> 632.4	87.78	88.00	0.8527 to 0.9814
Amyloid	0.6492	0.01615	> 305.9	64.10	64.00	0.5325 to 0.7659
Amadori product	0.9985	< 0.0001	> 10.86	97.56	98.00	0.9949 to 1.002
Total thiols	0.6333	0.03802	> 0.3149	53.49	53.85	0.5130 to 0.7535
8-OHdG	0.8594	< 0.0001	> 1759	78.13	78.33	0.7803 to 0.9385
IL-1 $\alpha$	0.7299	0.0001054	> 381.9	70.21	71.43	0.6180 to 0.8418
IL-1 $\beta$	0.7002	0.0009468	> 51.54	65.96	66.67	0.5794 to 0.8211
IL-6	1.000	< 0.0001	> 13.17	100.0	100.0	1.000 to 1.000
IL-10	0.9564	< 0.0001	> 48.99	90.91	90.00	0.9101 to 1.003
TNF- $\alpha$	0.9857	< 0.0001	> 196.7	91.30	92.00	0.9690 to 1.002

lipids and inhibits lipid synthesis, fatty acid desaturation, or lipase activation.<sup>27</sup> Reactive oxygen and nitrogen species are also responsible for nucleic acids oxidation, which is associated with the inability to repair DNA damage.<sup>28</sup> Free radicals may cause point mutations in oncogenes associated with cancer, including p53, V-Raf murine sarcoma viral oncogene homolog hB (BRAF), or adenomatous polyposis coli (APC). RNS can also induce chromosomal abnormalities, DNA adduct formation, methylation, and acetylation, which may favor the transformation of normal cells into cancer cells.<sup>24</sup>

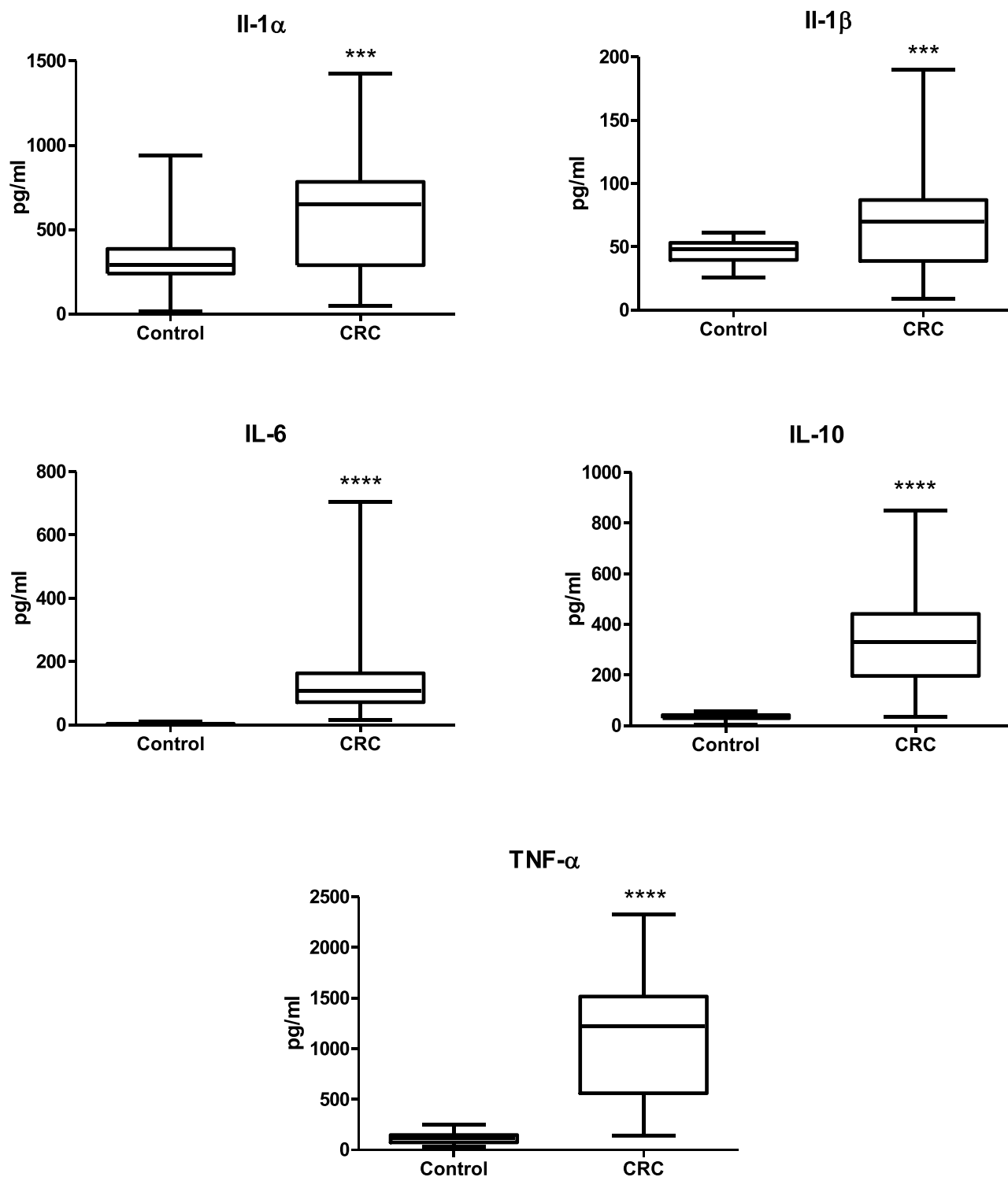
The primary source of nitrogen for RNS production in vivo is nitric oxide. NO is a product of the catalytic action of the nitric oxide synthase (NOS) on L-arginine. It can also be formed from nitrite derived from food or bacterial metabolism. NOS occur in three isoforms: neuronal (nNOS; type I NOS) and endothelial NOS (eNOS; type III NOS), which are regulated by the interaction of Ca<sup>2+</sup> with calmodulin and the third isoform, inducible-NOS (iNOS; type II NOS) activated in response to inflammation, infection, or trauma. Moreover, iNOS is induced by different cells in response to cytokines and endotoxins, such as IL1, IFN $\gamma$ , and TNF $\alpha$ .<sup>29</sup> Although NO may contribute to the anti-inflammatory and antioxidant properties,<sup>30</sup> at high concentrations NO promotes oxidation reactions and the formation of reactive nitrogen species.<sup>31</sup> NO, and their metabolites like peroxynitrite can mediate the nitration of tyrosine to form 3-NT.<sup>32</sup> NO via antiapoptotic and genotoxic mechanisms, and inhibition of host immune response against cancer may take part in malignant transformation.<sup>31</sup> NO also participates in induction and promotion of angiogenesis and metastasis or suppression of proliferation and infiltration of leukocytes to the tumour site. Although NO's role in many cancers has been described, few reports about its importance in colorectal carcinogenesis. Therefore, we determined NO concentration and other nitrosative stress parameters (peroxynitrite S-nitrosothiols, nitrotyrosine). We observed statistically and significantly increased NO, peroxynitrite, S-nitrosothiols, and nitrotyrosine concentration in the serum of patients with CRC compared to healthy control. It may be explained that cancer cells are characterized by enhanced oxygen consumption and its insufficient supply, which causes hypoxia. Therefore, tumour cells exposed to hypoxia contain





**Figure 2** Oxidation and glycooxidation products in patients with colorectal cancer and the control group. The data are presented as median (minimum - maximum). \* $p < 0.05$ , \*\*\*\* $p < 0.0001$ .

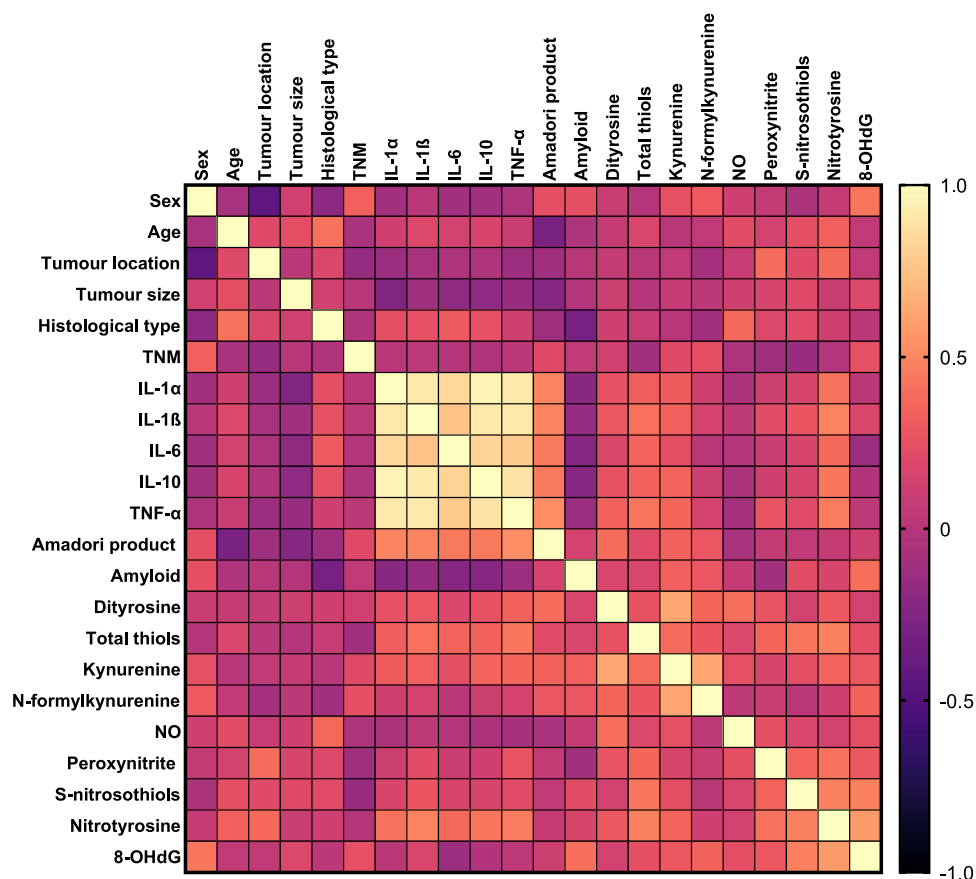
**Abbreviation:** 8-OHdG, 8-hydroxydeoxyguanosine.



**Figure 3** Inflammatory cytokines in patients with colorectal cancer and the control group. The data are presented as median (minimum - maximum). \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

**Abbreviations:** IL1 $\alpha$ , interleukin 1 alpha; IL1 $\beta$ , interleukin 1 beta; IL6, interleukin 6; IL10, interleukin 10; TNF- $\alpha$ , tumor necrosis factor alpha.

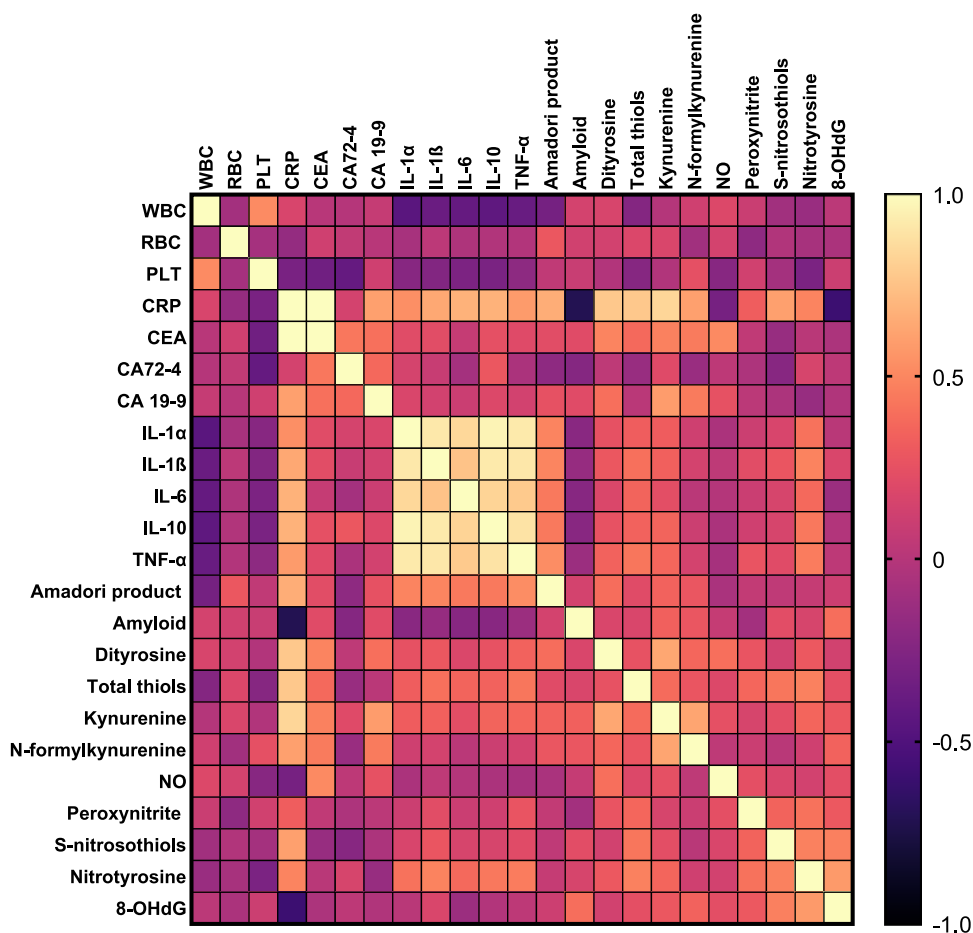
higher NO levels and its metabolites.<sup>33,34</sup> It is well known that NO may react rapidly to form peroxynitrite. Peroxynitrite may oxidize DNA and cause single-strand DNA breaks via an attack on the sugar-phosphate backbone, whereas other NO metabolites – N<sub>2</sub>O<sub>3</sub> may take part in nitrosation amines to form N-nitrosamines and then alkylate DNA. Nitrosation of primary amines in DNA bases leads to the deamination and DNA-crosslinks.<sup>35</sup> It may be confirmed that increased



**Figure 4** Heat map of correlations redox and inflammatory parameters with chosen clinicopathological parameters.

levels of reactive nitrogen species play an active role in colorectal carcinogenesis via numerous mechanisms, including DNA injury. On the other hand, increased concentration of S-nitrosothiols in CRC patients may be the body's adaptive response expressed as cellular storage of NO.<sup>36</sup> It is well-known that S-nitrosothiols preserve cells against the destructive effects of NO<sub>2</sub>, NO<sup>+</sup>, NO<sup>-</sup> and peroxynitrite.<sup>13</sup> We also observed that NO concentration increased with CEA level, which may be a valuable tool in the early non-invasive diagnosis of CRC or monitor tumour recurrence in these patients after tumour resection.

Disturbances in ROS and RNS production and insufficient antioxidant barrier result in oxidative and nitrosative damages to proteins, lipids, and nucleic acids. Although ROS and RNS react with most biomolecules, proteins are the main target of action of free radicals. Protein oxidative modifications include mainly protein carbonylation and tyrosine nitration, known as irreversible modifications. In contrast, reversible oxidation includes mainly cysteine modifications such as sulfenic acid, nitrosothiols, protein disulfides, and s-glutathione.<sup>37</sup> Total thiols belong to the group of organic compounds consisted of sulfhydryl group (-SH). Plasma thiols include the protein sulfhydryl groups and protein-mixed disulfides with homocysteine, cysteinyl glycine, and glutathione. Our work observed significantly higher concentrations of total thiols in patients with colorectal cancer. It may be an adaptive response to the enhanced production of free radicals. Moreover, the oxidation reaction of thiols with ROS/RNS leads to the formation of reversible disulfide bonds, which is the earliest indicator of radical-mediated protein oxidation. When oxidative/nitrosative stress condition ends, disulfide bond structures may be reduced to the thiol groups again. Thus, dynamic thiol-disulfide balance is maintained. Thiol/disulfide balance takes part in organizing antioxidant defense, apoptosis, detoxification, and cellular signalling.<sup>38</sup> As stated above, ROS and RNS production may also cause nucleic acid damages. The 8-OHdG is the most common DNA damage because it is relatively quickly formed and has promutagenic properties.<sup>39,40</sup> Therefore, it is not surprising that we observed a considerable increase of 8-OHdG in CRC patients. This indicates that cancer is inextricably linked



**Figure 5** Heat map of correlations redox and inflammatory parameters with chosen laboratory tests.

with DNA oxidative damages. It has been observed that one of the causes associated with modulation of oxidative DNA damage is impairment of DNA repair mechanisms. Research-based on cultured cells and mice models revealed that overexpression of Ogg1 (mammalian glycosylases responsible for the initiation of the base excision repair pathway) increased the repair rate by several fold but did not decrease the steady-state level or the spontaneous mutation rate.<sup>39</sup> Therefore, 8-OHdG seems to play a significant role in spontaneous mutagenesis accompanying malignant transformation.

We also demonstrated an increased level of protein glycoxidation products (kynurenine, N-formylkynurenine, dityrosine, Amadori products, amyloid) in patients with colorectal cancer compared to healthy controls. N-formylkynurenine and kynurenine are primary products of tryptophan metabolism, and as has been observed, colon cancer cells show greater tryptophan uptake and processing than normal colonic cells and tissues; therefore, the level of its metabolites was increased. It is known that kynurenine is a ligand for the transcription factor – AHR (aryl hydrocarbon receptor), and through the binding to this receptor, it may regulate growth-promoting genes in cancer cells. Some of the reports suggest that blocking the binding of kynurenine to AHR and inhibiting kynurenine production via tryptophan conversion may decrease the growth of cancer cells. Therefore, kynurenine may act as an oncometabolite.<sup>41</sup>

Glycoxidation products also can initiate the pro-inflammatory NF- $\kappa$ B pathway, which enhances the expression of pro-inflammatory cytokines and chemokines, adhesion molecules, growth factors, and nitric oxide synthases (mainly iNOS).<sup>13</sup> Our work showed a relationship between pro-inflammatory (IL1 $\alpha$ , IL1 $\beta$ , IL6, TNF $\alpha$ ) and anti-inflammatory cytokines (IL10) with oxidation and glycoxidation products. Levels of pro-inflammatory and anti-inflammatory cytokines were considerably higher in patients with colorectal cancer than in the control group, indicating the intensification of inflammatory processes in patients with cancer. Chronic inflammation caused by NF- $\kappa$ B activation can be one of the

**Table 3** Multiple Regression Analysis of the Nitrosative Stress Parameters, Protein and DNA Oxidation/Glycooxidation Products, and Pro- and Anti-Inflammatory Cytokines in Patients with Colorectal Cancer

Parameter	$\beta$ 1: Tumour's Location			$\beta$ 2: Tumour's Size			$\beta$ 3: Histological Type			$\beta$ 4: pT			$\beta$ 5: pN			$\beta$ 6: pM		
	Estimate	95% CI	P-value	Estimate	95% CI	P-value	Estimate	95% CI	P-value	Estimate	95% CI	P-value	Estimate	95% CI	P-value	Estimate	95% CI	P-value
<b>NO</b>	0.092	-0.06002 to 0.2443	0.225	-0.014	-0.1669 to 0.1384	0.849	0.217	0.01648 to 0.4169	0.035	0.174	0.003806 to 0.3442	0.045	-0.141	-0.2993 to 0.01683	0.078	0.129	-0.1220 to 0.3795	0.302
<b>Peroxy nitrite</b>	0.2075	-0.00029 to 0.4152	0.05	0.1196	-0.08881 to 0.3280	0.249	0.0514	-0.2219 to 0.3248	0.703	-0.07013	-0.3025 to 0.1622	0.541	0.0509	-0.1649 to 0.2667	0.633	-0.0538	-0.3962 to 0.2885	0.749
<b>S-nitrosothiols</b>	0.02868	-0.0516 to 0.1090	0.471	0.01103	-0.0695 to 0.0916	0.781	-0.02155	-0.1273 to 0.08419	0.679	0.03516	-0.05472 to 0.1250	0.429	-0.06087	-0.1443 to 0.0226	0.146	-0.04978	-0.1822 to 0.08265	0.448
<b>Nitrotyrosine</b>	42.84	-28.20 to 113.9	0.227	-2.736	-73.99 to 68.52	0.938	-52.68	-146.1 to 40.78	0.258	58.04	-21.41 to 137.5	0.146	-49.1	-122.9 to 24.68	0.184	-37.25	-154.3 to 79.80	0.519
<b>Kynurenine</b>	156.6	-104.6 to 417.9	0.230	127.9	-206.7 to 317.4	0.669	98.1	-245.6 to 441.8	0.563	304.4	12.21 to 596.6	0.042	-121	-392.3 to 150.3	0.368	73.35	-357.1 to 503.9	0.730
<b>N-formylkynurenine</b>	58.77	-77.80 to 195.3	0.386	13.48	-123.5 to 150.5	0.841	20.06	-159.6 to 199.8	0.821	149.2	-3.511 to 302.0	0.049	-23.37	-165.2 to 118.5	0.738	-24.87	-249.9 to 200.2	0.226
<b>Dityrosine</b>	72.02	-67.38 to 211.4	0.299	18.25	-121.6 to 158.1	0.791	46.91	-136.5 to 230.3	0.605	198.4	42.48 to 354.3	0.015	-55.26	-200.1 to 89.54	0.441	-109.9	-339.6 to 119.8	0.336
<b>Amyloid</b>	37.36	-16.59 to 91.32	0.167	-10.63	-64.76 to 43.49	0.690	-80.63	-151.6 to -9.635	0.028	38.03	-22.32 to 98.38	0.207	-46.86	-102.9 to 9.184	0.097	-51.4	-140.3 to 37.52	0.246
<b>Amadori product</b>	0.043	0.6510 to 0.7360	0.901	-0.374	-1.069 to 0.322	0.281	-0.0595	-0.9720 to 0.8529	0.134	0.657	-0.1185 to 1.433	0.094	-0.511	-1.231 to 0.209	0.157	-0.442	-1.585 to 0.701	0.435
<b>Total thiols</b>	0.01961	-0.04705 to 0.08627	0.552	-0.03877	-0.1056 to 0.02811	0.245	0.001197	-0.08651 to 0.08890	0.978	0.02446	-0.05010 to 0.09902	0.507	-0.05267	-0.1219 to 0.01657	0.130	-0.00687	-0.1167 to 0.1030	0.898
<b>8-OHdG</b>	54.35	-272.6 to 381.4	0.734	126.4	-210.5 to 463.3	0.445	-39.87	-472.0 to 392.3	0.85	374.1	-0.9979 to 749.3	0.049	-206.9	-578.7 to 164.9	0.261	-152.6	-1005 to 699.9	0.714
<b>IL-1<math>\alpha</math></b>	-151.5	-372.0 to 69.13	0.172	-170.3	-401.8 to 61.24	0.144	185.6	-82.89 to 454.1	0.169	58.39	-178.4 to 295.2	0.619	-62.42	-289.6 to 164.8	0.580	161	-536.9 to 117.3	0.201
<b>IL-1<math>\beta</math></b>	-13.9	-38.82 to 11.02	0.265	-6.118	-32.27 to 20.03	0.638	19.51	-10.82 to 49.84	0.199	3.772	-22.97 to 30.52	0.776	-4.297	-29.96 to 21.37	0.735	-21.68	-58.63 to 15.27	0.242
<b>IL-6</b>	-23.31	-127.6 to 81.01	0.652	-92.09	-201.6 to 17.39	0.097	112.6	-14.34 to 239.6	0.080	-33.85	-145.8 to 78.12	0.543	0.978	-106.5 to 108.4	0.985	-70.9	-225.6 to 83.81	0.358
<b>IL-10</b>	-67.58	-203.6 to 68.42	0.319	-79.13	-221.9 to 63.60	0.268	120.1	-45.47 to 285.6	0.149	16.27	-129.7 to 162.2	0.822	-36.09	-176.2 to 104.0	0.604	-124.1	-325.7 to 77.60	0.219
<b>TNF-<math>\alpha</math></b>	-307	-719.2 to 105.2	0.139	212.9	-683.4 to 181.9	0.247	355.5	-146.2 to 857.3	0.159	49.8	-392.7 to 492.3	0.820	-86.8	-511.4 to 337.8	0.680	-366.9	-978.2 to 244.4	0.231

essential promoters of tumour development. Inflammation may influence cancer initiation and progression by vascularization and remodeling the tumour microenvironment, crucial in tumour cell survival.<sup>42</sup> Inflammatory tissues also release cytokines that activate NF- $\kappa$ B, which induces LOX, COX2, and iNOS, leading to overproduction of ROS and RNS. ROS and RNS-mediated MAPK (mitogen-activated protein kinases) contribute to the production of inflammatory cytokines, including pro-inflammatory mediators like TNF $\alpha$  and IL6, IL1 $\beta$ , or TGF $\beta$ . It has been confirmed that the NF $\kappa$ B pathway, oxidative/nitrosative stress, and the JAK-STAT pathway cooperate and intensify each other in cancer progression. However, NF- $\kappa$ B plays a vital role in promoting cancer by inducing abnormal cellular proliferation, cell survival, cell cycle regulation, and the development of resistance to drug therapies.<sup>43</sup> Interestingly, mice with loss of NF- $\kappa$ B function in colonic epithelial cells developed fewer colon tumours after AOM/DSS application and had higher epithelial cell apoptosis without decreasing inflammation. In contrast, mice that lost function of NF- $\kappa$ B function in myeloid cells developed fewer tumours and tumours of smaller size and had decreased expression of cytokines and chemokines, especially IL1, IL6, and TNF $\alpha$  that are responsible for inflammation.<sup>8</sup>

In conclusion, we have shown that protein and DNA oxidation and glycoxidation products, nitrosative stress markers, and cytokines are significantly increased in patients with CRC. Therefore, nitrosative stress, protein oxidation, and carbonyl stress may play a significant role in colorectal carcinogenesis. Oxidation and glycoxidation products were positively correlated with pro-inflammatory (IL1 $\alpha$ , IL1 $\beta$ , IL6, TNF $\alpha$ ) and anti-inflammatory cytokines (IL10), indicating that redox damages may promote inflammation in CRC patients. Many redox biomarkers differentiate CRC patients from healthy individuals with high sensitivity and specificity. Thus, our research is a primary step for further clinical trials focusing on the evaluating the diagnostic utility of redox biomarkers in a larger population of colorectal cancer patients. Evaluation of the association between the intensity of nitrosative stress and the survival rate of CRC patients also seems to be advisable.

In the end, it is also worth considering some of limitations of our study. We carried out a research on a small group of patients; therefore, further study is required on a greater group of patients with CRC. The concentration of chosen redox parameters was assessed only in blood samples (serum/plasma), giving our results an approximate value. We evaluated only the chosen redox biomarkers, so we cannot completely describe the oxido-reductive disturbances in CRC patients. Nevertheless, the undoubted advantage of our work is a carefully selected group of CRC and control patients without any comorbidities.

## Data Sharing Statement

All data generated or analysed during this study are included in this published article.

## Ethics Approval and Consent to Participate

The study was approved by the Bioethics Committee of the Medical University of Bialystok, Poland (permission number APK.002.99.2021). After a detailed explanation of the purpose of our research and the possible risk, all the qualified patients agreed in writing to participate in the experiment.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no competing interests.

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