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Original Scientific Paper

SERUM ACTIVITY OF ANTIOXIDATIVE ENZYMES AND CONCENTRATION OF MALONDIALDEHYDE AS PREDICTORS OF COLORECTAL CANCER STAGE IN CROATIAN PATIENTS

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SUMMARY – One of the factors involved in the colorectal cancer development is intracellular oxidative stress and antioxidative imbalance. The study aimed to explore the link between oxidative stress measured by the activity of antioxidative markers and lipid peroxidation product malondialdehyde (MDA) as possible cancer predictors of colorectal cancer by using several statistical methods. The study included 50 adult colorectal cancer patients of both genders. MDA level, the activity of antioxidative markers superoxide dismutase and catalase, and glutathione concentration were determined in patient sera. There was no age difference between male and female patients (p=0.579), and no gender differences according to cancer site (p=0.995), stage (p=0.083), and size (p=0.245). There were no differences in the levels of studied enzymes. Correlation analysis of antioxidative markers and MDA with cancer size and patient age revealed strongest individual correlation between the MDA and cancer size variables (r=-0.56).

Key words: *Colorectal cancer; Oxidative stress; Antioxidative markers; Malondialdehyde*

Introduction

Colorectal cancer (CRC) is an intestinal tissue neoplasm and 'the first runner-up' on the prevalence scale of fatal malignancies. Based on the annual number of newly diagnosed patients, it is the third most prevalent human malignancy in the world[1]. In 2020, more than 1.9 million new CRC cases and more than 930,000 deaths due to CRC were estimated worldwide. The incidence rates were highest in Europe, Australia and New Zealand[2]. The most common causes of cancer deaths in European Union member states in 2020 were lung cancer (20.4% of all cancer deaths),

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followed by colon and rectal cancer (12.4%), breast (7.3%) and pancreatic cancer (7.1%)[3]. The mortality rates were highest in Eastern Europe[2]. Morgan *et al.*[2] predict that by 2040, the numbers of CRC cases will increase to up to 3.2 million new cases *per* year (which is a 63% increase) and 1.6 million deaths *per* year (a 73% increase). Incidence rates of CRC have been decreasing in high-income countries, largely as a result of effective screening programs[2].

In the Republic of Croatia, the most common newly diagnosed cancer in 2020 was colon and rectal cancer (3,706 new cases). In terms of mortality, it ranked second with 2,320 noted cases[3]. The national program aiming at early detection of colon cancer has been carried out in Croatia since 2008. Colon cancer can be detected in its early stage, in which the curing odds are high[4]. Detection leans on testing for occult bleeding. Even though as many as 3,000 new patients are identified annually, in more than half of them, the disease had already spread[4]. According to Ljubičić *et al.*[5], screening primarily addresses high-risk groups that include persons with endoscopically detected and removed colon polyps, patients surgically treated for colon cancer, individuals with a positive family history of CRC, patients with inflammatory bowel diseases, individuals and families with hereditary disorders or genetic mutations, where the risk of the disease is several times greater.

Cancer initiation and progression involves a variety of factors, among others including reactive oxygen species (ROS) effects. However, the precise mechanisms underlying the induction of oxidative stress and the role of ROS in cancer progression have not yet been fully elucidated[6]. There is indicative evidence that oxidative stress damages DNA molecules, thus contributing to CRC development. As a result, an adenomatous polyp gradually turns into CRC. It is believed that such a transformation takes place within a 10-year timeframe[7]. The ROS molecules include superoxide anion ($\cdot O_2^-$), hydroxyl radical (·OH), peroxide radical (ROO·), alkoxy radical (RO·), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorite acid (HOCl), ozone (O_3), and peroxide nitrite anion ($ONOO^-$)[8-10]. In order to prevent oxidative damage due to ROS, cells have an array of mechanisms for neutralization of free radicals of both exogenous and endogenous origin, such as bilirubin conjugates, thiols, NADPH and NADH, ubiquinone, metal-binding proteins, antioxidative enzymes or food molecular ROS scavengers such as vitamins, polyphenols, etc. Among cellular endogenous antioxidative defense systems, the most prominent role is played by superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH), which are the usual markers of the antioxidative defense and oxidative stress in tissues and cells[8,9]. Oxidative stress can be perceived as a disbalance between pro- and antioxidants present in the human body[11] and could increase under physiological and pathological circumstances[12]. Although the use of lipid peroxidation markers or antioxidative markers has a great diagnostic potential in oncology, further research is needed before their introduction as diagnostic tools[8,9,12].

The aim of this work was to explore the link between oxidative stress measured by the activity of antioxidative enzymes and lipid peroxidation product malondialdehyde (MDA) in CRC using several predictive models. Several studies have documented that in CRC patients, the level of antioxidative enzymes is generally lowered, while MDA level rises[8,13-16], which was also a basis for the hypothesis of this work to establish whether similar changes occurred in the population of Croatian patients. Additionally, we wanted to create a statistically based prediction model and also hypothesized that differences in the values of some of the analyzed predictors (carcinoma size, MDA, SOD, CAT and GSH) may indicate a difference in CRC stages.

Patients and Methods

The study included 50 adult (age 18-90 years) CRC patients of both genders (30 men and 20 women) treated at the Department of Oncology, Sestre milosrdnice University Hospital Center in Zagreb, Croatia. The study was conducted before any oncologic treatment. Before study enrolment, the patients signed an informed consent form. The study was approved by the Boards of Ethics of both the University of Applied Health Sciences (approval no. 251-379-21-18-02) and Sestre milosrdnice University Hospital Center (approval no. EP-4669118-2).

Inclusion criteria were signed an informed consent form; adult age (≥18 years); and patients undergoing preoperative treatment due to confirmed colon cancer. Exclusion criteria were any malignant process in the last 5 years; patients with other severe uncontrolled medical disorders, non-malignant systemic diseases, or

active, uncontrolled infections; immunocompromised patients, e.g., serologically positive human immunodeficiency virus (HIV) patients; and patients previously treated with neoadjuvant chemoradiotherapy.

Following the histopathologic diagnosis, the patients were divided into cancer stage-based groups (0-IV) by the TNM classification system most often used for CRC. This system is based on the size of tumor (T) (extent of intestinal wall infiltration); N (lymph node metastasis); and M (metastasis to distant sites). In CRC, stage 0 is cancer localized on the bowel lining, also known as cancer *in situ*; stage 1, cancer has grown through the muscularis mucosa into the submucosa. It has not spread to nearby lymph nodes (N0) or to distant sites (M0); stage 2, cancer has spread into the outer wall of the bowel or into tissue or organs next to the bowel. It has not spread to the lymph nodes or distant parts of the body; stage 3, cancer has spread to nearby lymph nodes but has not spread to distant body parts; and stage 4, cancer has spread to distant body parts such as the liver or lungs[17].

Blood samples were obtained for laboratory analysis on hospital admission. Blood samples were collected from peripheral vein into serum tube with a clot activator and gel (BD Vacutainer system), and centrifuged at 3500 rpm for 10 min. After centrifugation, serum was separated into a small tube without additive and frozen at -80 °C until analysis. The level of lipid peroxidation product MDA, the activity of antioxidative enzymes SOD and CAT, and the concentration of GSH were determined in patient sera. Serum protein concentration was used to express all parameters as units *per* mg of protein, determined according to Lowry *et al.*[18]. Bovine serum albumin (BSA, Sigma Aldrich, Darmstadt, Germany) was used as the standard. The analysis was performed at the Faculty of Science, University of Zagreb and University of Applied Health Sciences, Zagreb.

Laboratory methods for analysis of MDA concentration, SOD and CAT enzyme activity and serum GSH concentration

Malondialdehyde was determined by a modified method according to Jayakumar *et al.*[19], in reaction of 100 μL of serum with 750 μL of 0.81% thiobarbituric acid (TBA) in 20% acetic acid solution (pH 3.5). The mixture was incubated for 60 min at 95 °C. The resulting chromogen was measured spectrophotometrically at 532 nm (Libra S22 spectrophotometer, Biochrom, UK). MDA concentration was calculated using the following formula:

$$c_{(MDA)} = A \times V_{sample}(mL) / e \times V_{reactive\ mixture}\ (mL) \times c_{proteins}\ (mg\ mL^{-1})$$

Molar absorption coefficient (e) for malondialdehyde-thiobarbituric acid (MDA-TBA) complex = $1.56 \times 10^5\ M^{-1}cm^{-1}$. MDA concentration was expressed in nmol of MDA/mg of proteins.

Superoxide dismutase activity was measured by virtue of inhibition of cytochrome C reduction within the xanthin/xanthin oxidase system according to the modified Flohé & Ötting technique[20], in 3-minute reaction monitored spectrophotometrically (Libra S22 spectrophotometer, Biochrom, UK) at 450 nm. SOD activity was expressed in U mL^{-1} according to the following equation:

SOD act (U/mL) =10^((% inhibition +12.757)/30.932)

Catalase enzyme activity was determined spectrophotometrically according to the Aebi protocol[21], monitoring the absorbance reduction as a result of enzymatic decomposition of hydrogen peroxide over 1 minute at 240 nm. CAT activity was expressed through the H_2O_2 extinction coefficient (ε = 39.4 $mM^{-1}cm^{-1}$, i.e., as μmol of H_2O_2 decomposed *per* minute *per* mg of proteins (μmol of H_2O_2/min/mg of proteins), which corresponds to CAT units *per* mg of proteins (U/mg of proteins).

Total glutathione concentration was determined according to the modified Tietze technique[22], based on the GSH thiol group (-SH) and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, the Ellman reagent) reaction and addition of 20 U/mL of glutathione reductase which removes the chromogen specifically from reduced glutathione over 3 minutes, measured spectrophotometrically at 412 nm (Plate Reader, Biorad, Germany). GSH concentration was expressed in μg GSH/mg of proteins.

Statistics

Gender differences were tested using the Mann-Whitney U test, χ^2-test, and Fisher exact test. To control for the inflation of type I error due to multiple testing, Benjamini-Hochberg correction was used. Correlations between continuous variables were calculated using Spearman's correlation test. Given that the criterion variable (cancer stage) is ordinal, ordinal logistic regression was used. Upon model compilation and prior to its interpretation, model assumptions, i.e.,

multicollinearity and proportional ratios, were checked. The most appropriate model among those proposed was selected based on the credibility-ratios test results. Akaike Information Criterion (AIC) and residual model deviance (RD) were also compared. Statistical analysis was completed using the R Software, Version 4.0.3. The analysis employed the *brant* R package.

Results

There was no statistically significant difference in the mean age between male and female patients (Mann-Whitney U test; p=0.579). There were no differences between genders according to cancer site (χ^2-test; p=0.995), cancer stage (Fisher exact test; p=0.083), and cancer size (Mann-Whitney U test; p=0.245) (Table 1).

Between genders, the corrected p-values of Mann-Whitney U test for SOD, CAT, MDA, and GSH were 0.052, 0.428, 0.188, and 0.442, indicating no statistically significant differences in the levels of any of the measured enzymes (Table 2).

The cancer size and MDA and SOD levels correlated with cancer stage (Table 3).

In the logistic regression model, multicollinearity was absent as evidenced by low variance inflation factor. The Brant test showed that proportional intra-model ratios could be assumed (Table 4).

Two predictor variables turned out to be significant, i.e., cancer size and MDA level. Provided that all other predictors are under control, should the cancer grow by 1 cm, the chance of its advancing into the higher stage is 2.45-fold increased. Should MDA level rise by 1 (again, provided that all other predictors are under control), the likelihood of cancer progression to a more advanced stage is 1.22-fold higher.

Given that cancer size represents a much stronger predictor than MDA level, two additional models were compiled and compared. One of them is fairly simple, making use of cancer size as the sole predictive variable, while the other takes both predictors proven relevant by the precedent model, that is to say, cancer size and MDA level. The models referred to above were compared using not only the credibility-ratios test, but also the AIC and RD (Table 5). As illustrated by Table 5, the models significantly differ from each other. The Cancer Size * MD model has lower AIC and RD, indicating better explanation of changes in cancer stage.

Figure 1 illustrates the change in the likelihood of accurate cancer staging based on cancer size and MDA level. Although the model demonstrates the link between changes in MDA levels and the likelihood of accurate cancer staging, cancer size itself was proven more significant.

Table 1. Demographic, clinical, and pathologic data

	Male (n=30)	Female (n=20)	Total (N=50)
Age			
Median (min-max)	63.5 (46.0, 85.0)	65.5 (47.0, 91.0)	64.5 (46.0, 91.0)
Cancer site			
Rectum	10 (33.3%)	6 (30.0%)	16 (32.0%)
Colon	20 (66.7%)	14 (70.0%)	34 (68.0%)
Cancer stage			
Stage 0	3 (10.0%)	2 (10.0%)	5 (10.0%)
Stage I	6 (20.0%)	4 (20.0%)	10 (20.0%)
Stage II	7 (23.3%)	4 (20.0%)	11 (22.0%)
Stage III	3 (10.0%)	8 (40.0%)	11 (22.0%)
Stage IV	11 (36.7%)	2 (10.0%)	13 (26.0%)
Cancer size (cm^2)			
Median	4.5 (3-6)	5.25 (3.12-6.38)	4 (2.75-5.5)

All values are median and interquartile range (Q1-Q3)

Table 2. Levels of laboratory parameters

	Male (n=30)	Female (n=20)	Total (N=50)
SOD (U/mL)	7.54 (6.35-9.8)	9.9 (8.41-12.4)	8.66 (6.9-10.65)
CAT (U/mg)	43.6 (28.1-101.7)	57.3 (34.4-118.4)	51.6 (28.1-110.1)
MDA (nmol/mg)	5.18 (3.49-9.02)	8.01 (4.9-11.6)	6.81 (3.49-9.34)
GSH (μg/mg)	87.8 (72.7-113.3)	84.9 (72.0-97.9)	86.1 (72.5-109.2)
Missing	3 (10.0%)	1 (5.0%)	4 (8.0%)

All values are median and interquartile range (Q1-Q3); SOD = superoxide dismutase; CAT = catalase; MDA = malondialdehyde; GSH = reduced glutathione

Table 3. Correlation between tested laboratory parameters and pathologic data

Variable 1	Variable 2	ρ	LCI	UCI	p
Size (cm^2)	Stage	0.58	0.35	0.74	**<0.001***
MDA	Size (cm^2)	-0.56	-0.72	-0.33	**<0.001***
SOD	Size (cm^2)	-0.35	-0.57	-0.08	**0.012***
SOD	MDA	0.23	-0.05	0.48	0.106
CAT	MDA	0.21	-0.07	0.46	0.144
SOD	Stage	-0.20	-0.46	0.08	0.156
MDA	GSH	0.14	-0.16	0.41	0.350
GSH	Size (cm^2)	-0.13	-0.41	0.17	0.389
GSH	Stage	0.10	-0.19	0.38	0.504
SOD	CAT	0.09	-0.20	0.36	0.547
MDA	Stage	-0.07	-0.34	0.22	0.644
CAT	Size (cm^2)	-0.06	-0.34	0.22	0.666
CAT	GSH	0.04	-0.25	0.33	0.774
SOD	GSH	-0.03	-0.32	0.26	0.825
CAT	Stage	<0.01	-0.28	0.28	0.988

*statistically significant correlation, LCI = lower confidence interval; UCI = upper confidence interval; SOD = superoxide dismutase; CAT = catalase; MDA = malondialdehyde; GSH = reduced glutathione

Table 4. Ordinal logistic regression (outcome: cancer stage)

Predictor	β	SE	Exp(β)	95% CI	VIF
Size*	0.89	0.22	2.45	1.63-3.86	2.01
MDA*	0.20	0.09	1.22	1.03-1.46	1.78
SOD	0.04	0.09	1.04	0.87-1.26	1.21
CAT	<0.01	0.06	1.00	0.99-1.01	1.03
GSH	0.01	0.02	1.01	0.99-1.03	1.04

*significant predictor; β = standardized regression coefficient; SE = standard error β; Exp(β) = exponent value; 95% CI = 95% confidence interval of Exp(β); VIF = variance inflation factor; SOD = superoxide dismutase; CAT = catalase; MDA = malondialdehyde; GSH = reduced glutathione

Table 5. Inter-model comparison

Model predictor	df	LR	p	AIC	RD
Cancer size	41	13.10	<0.01	138.33	128.33
Cancer size * MDA	39			129.35	115.35

df = degrees of freedom; LR = likelihood ratio; AIC = Akaike information criterion; RD = residual deviation; MDA = malondialdehyde

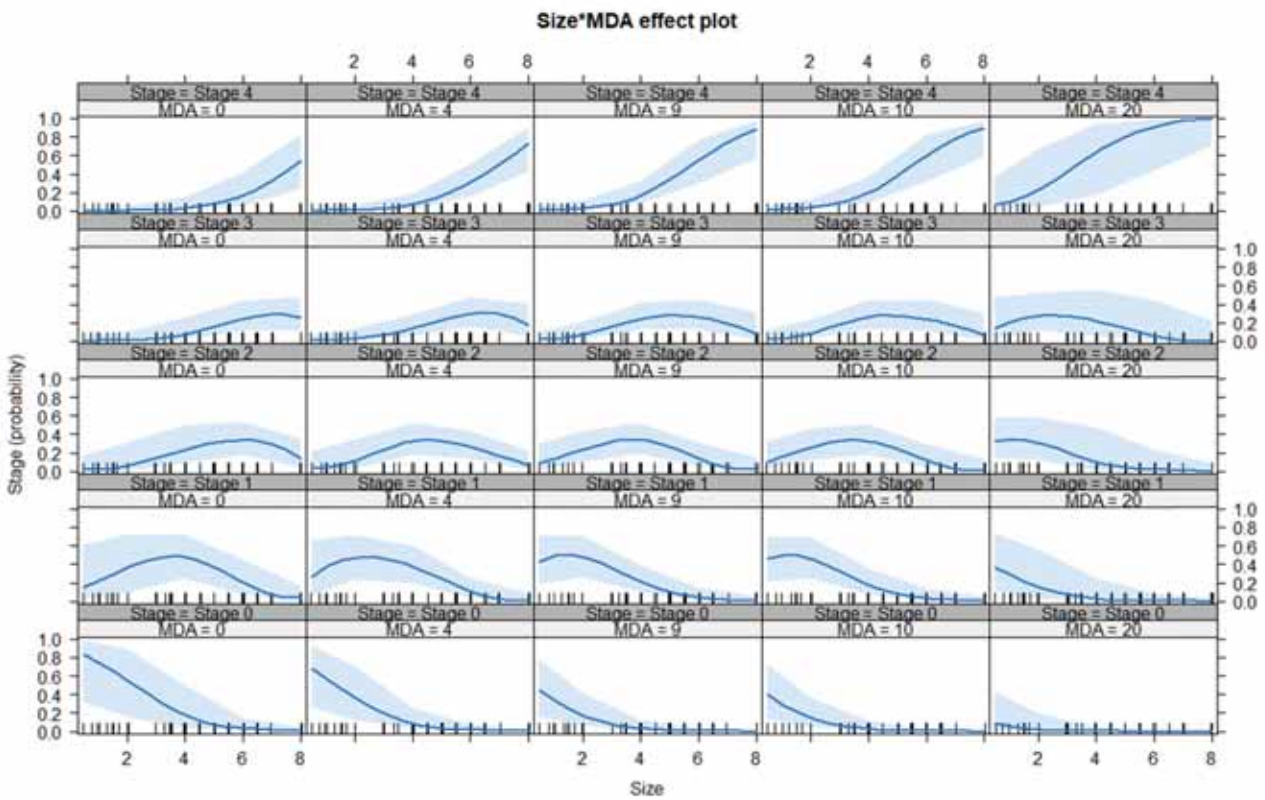

Fig. 1. Plotted effect of the predictors on the criterion.

Discussion

According to study results, MDA determination could be a candidate marker for further investigation as an indicator of oxidative stress intensity or lipid peroxidation-inflicted damage in CRC. Increase in MDA level may result from cellular damage, reaction with free amino groups belonging to proteins and nucleic acids, as well as from mutagenicity targeted at the guanin site of the DNA sequence[1], but also a consequence of rapid metabolism of cancer cells *in situ*, contributing to their release in blood.

As a proof to that, especially interesting was the result that showed that two predictor variables turned out to be significantly correlated, i.e., cancer size and MDA level. The results point to a relationship where, if the cancer grows by 1 cm, then the chance of its advancing into the higher stage is 2.45-fold increased, provided that all other predictors are under control. Thus, the results of this survey point to the mathematically estimated biological ratio that indicates that if MDA level rises by 1, the likelihood of cancer progression to a more advanced stage is 1.22-fold higher.

According to the ordinal logistic model elaborated above, significant tumor staging predictors are tumor size and MDA level. Correlogram (Fig. 1) reveals the correlation between MDA level and tumor stage to be weak, negative, and statistically nonsignificant, while within the OLR model it becomes statistically significant ($p \leq 0.05$) and positive. The latter should probably be attributed to the suppression effect. Namely, rather stronger correlations with tumor stage, as well as the correlation between cancer size and MDA probably increase the correlation between MDA level and cancer stage in an artificial manner[23].

Some of the latter studies have demonstrated a positive correlation between MDA and cancer stage, while others have tagged this correlation as negative.

The study by Rašić *et al.*[1] proved that MDA concentration increased with cancer advancing. Of note, serum MDA concentrations established across the control group did not statistically differ from those established in stage 1 cancer patients but were significantly higher in stage 2 cancer patients. In stage 3, MDA concentration was significantly higher than in stage 2 patients but not significantly different from that established in stage 4 patients[1]. The highest MDA concentrations were found in stage 4 cancer patients[1], which is in line with the results of the research published by Janion *et al.*[14]. In CRC, serum MDA concentrations were significantly higher in patients in whom the cancer had already spread than in those in whom metastases had not been proven yet. Serum MDA concentrations can be a useful tool for treatment effect monitoring[1].

The study by Janion *et al.*[14] failed to reveal any differences in total oxidant status, total antioxidant status, and serum MDA concentration at different clinical stages of the disease[14]. MDA concentration was significantly higher in CRC patients as compared to the control study arm[24-26]. The authors claimed this outcome to be of major importance in tumor development.

In cancer patients, the levels of antioxidative enzymes are generally lower, while the concentration of MDA tends to rise[8]. Gopčević *et al.*[27] documented lipid peroxidation enhancement in each CRC stage as compared to the control arm but failed to reveal any inter-stage differences.

Two most significant predictive variables yielded by our study were cancer size and MDA level. Of interest, cancer size as a predictive variable proved to be of higher statistical significance than MDA level, but their combination was statistically more accurate in predicting cancer stage. Based on the above, it is concluded that the type of research that employs statistical models represents a valuable contribution to the efforts in cancer research on the whole. When it comes to models, our study showed the model taking both cancer size and MDA level into account to be a rewarding research tool.

The remaining oxidative stress parameters analyzed in this study, antioxidative markers SOD, CAT and GSH, did not differ or correlate in the investigated CRC patients. Research conducted insofar also offered varying and contradictory results in this regard, although changes in antioxidative enzyme levels were mostly compared between CRC patients and control subjects rather than between various cancer stages. For example, the activity of antioxidative markers SOD, CAT and GSH was significantly lower in CRC patients as compared to controls[15]. In 2016, Veljković *et al.*[16] demonstrated a significantly lower CAT activity in the tumor as compared to healthy tissues of the same patients. The activity of antioxidative enzymes CAT and glutathione peroxidase was increased as compared to the control group, but statistically significant inter-

stage differences were not recorded. On the other hand, in comparison with the control study arm, SOD activity was lower in all CRC stages, most significantly in stage IV as compared to stage II[27]. Statistically significant increase in SOD and GSH activity was proven in CRC patients in comparison with control subjects, together with a significant drop in GSH level[25]. In CRC patients, SOD concentration was significantly higher as compared to controls, with significant concomitant reduction in CAT, GSH, glutathione peroxidase, and glutathione reductase activity[24]. Skrzydlewska *et al.*[6] also report on a significant increase in MDA, SOD, glutathione peroxidase, and glutathione reductase levels in cancer tissue as compared to controls, reaching their peak in stage IV. At the same time, there was a significant CAT activity reduction, reaching its lowest level in stage IV[6].

In conclusion, future research should include larger sample size, since according to the aims of this study, MDA was the most promising candidate for mathematically indicated relationship between MDA and the likelihood of 1.22-fold cancer progression to a more advanced stage, showing the possible link between MDA and cancer stage.

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Sažetak

SERUMSKA AKTIVNOST ANTIOKSIDACIJSKIH ENZIMA I KONCENTRACIJA MALONDIALDEHIDA KAO PREDIKTORI STADIJA KOLOREKTALNOG KARCINOMA U HRVATSKIH BOLESNIKA

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Jedan od čimbenika uključenih u proces nastanka kolorektalnog karcinoma je izloženost oksidacijskom stresu u stanicama kao i antioksidacijska neravnoteža. Cilj istraživanja bio je istražiti poveznicu između oksidacijskog stresa i kolorektalnog karcinoma mjerenjem aktivnosti antioksidacijskih biljega i pokazatelja lipidne peroksidacije malondialdehida (MDA) te pomoću nekoliko statističkih modela utvrditi postojanje mogućih prediktora stadija karcinoma. Istraživanje je provedeno na skupini od 50 odraslih bolesnika s kolorektalnim karcinomom obaju spolova. U serumu bolesnika određena je razina MDA, aktivnost antioksidacijskih biljega superoksid dismutase, katalaze te koncentracija glutationa. Nije utvrđena razlika između srednje dobi muškaraca i žena (p=0,579). Nema razlike između spolova po sijelu (p=0,995), stadiju karcinoma (p=0,083) niti prema veličini karcinoma (p=0,245). Također nije bilo razlika niti u jednom od mjerenih antioksidacijskih biljega. Analizom korelacije antioksidacijskih biljega i MDA s veličinom karcinoma i dobi bolesnika utvrđena je najveća pojedinačna korelacija između varijable MDA i varijable veličina karcinoma (ρ=-0,56).

Ključne riječi: *Kolorektalni karcinom; Oksidacijski stres; Antioksidacijski biljezi; Malondialdehid*