ORIGINAL ARTICLE

Markers of lipid peroxidation and antioxidant status in the serum and saliva of patients with active Crohn disease

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KEY WORDS

Crohn disease, lipid peroxidation, saliva, serum

ABSTRACT

INTRODUCTION Increased oxidative stress has been implicated in the pathogenesis of Crohn disease (CD). Except for C-reactive protein (CRP), good biological markers of CD activity are lacking.

OBJECTIVES We aimed to investigate the diagnostic usefulness of selected markers of oxidative stress in the serum and saliva of patients with active and inactive CD.

PATIENTS AND METHODS A total of 58 patients with confirmed CD (32 with active CD, 26 with inactive CD, and 26 healthy controls) were prospectively enrolled to the study. The markers examined were malondialdehyde (MDA), ferric reducing ability of plasma (FRAP), reduced glutathione (GSH), and catalase (CAT).

RESULTS MDA levels were higher in the serum and saliva of patients with active CD than in those with inactive CD and controls and were positively correlated with the Crohn's Disease Activity Index (r = 0.8, P <0.001) and CRP (P <0.001). Serum and saliva antioxidant indicators (FRAP and GSH) were decreased in both CD groups compared with controls and were negatively correlated with clinical activity and inflammation (FRAP, r = –0.5, P <0.001; GSH, r = –0.5, P <0.001; and CAT, r = –0.5, P <0.001).

CONCLUSIONS The increased lipid peroxidation and decreased antioxidant activity in serum and saliva confirm that CD patients are under oxidative stress. The positive correlations of MDA with the clinical activity and inflammation, as well as the comparison of the receiver operating characteristic curves for MDA and CRP, suggest that MDA could be a good diagnostic marker of CD.

INTRODUCTION Crohn disease (CD) is a chronic illness of the entire gastrointestinal tract and is characterized by recurrent granulomatous inflammation of unknown etiology.[1] The active disease manifests with symptoms such as abdominal pain, weight loss, chronic diarrhea, and fever and with severe complications such as malnutrition, intestinal strictures, fistulas, and intra-abdominal abscess.[1] Laboratory parameters evaluated in CD patients include C-reactive protein (CRP), a biochemical marker of disease activity; however, CRP is more useful for assessing or monitoring disease activity than for diagnosis.[2]

The precise etiopathogenesis of CD is unclear. Interactions among various factors, including genetics, the immune system, the intestinal microbiome, and the environment, play an important role in disturbing intestinal homeostasis, leading to a dysregulated inflammatory response in the gastrointestinal tract.[1,3,4] Oxidative stress, an imbalance between the release of reactive oxygen species (ROS) and reactive nitrogen species and neutralization activity, is an important pathogenetic factor in CD.[3,5,6] Hydrogen peroxide levels in immune cells of CD patients are correlated with indicators of inflammation (eg, tumor necrosis factor α [TNF-α], interleukin 1 [IL-1], IL-6, and IL-8).[7-11] ROS are generated by resident phagocytic cells, microvascular endothelial cells, and mucosal epithelial cells.[12-15] Moreover, activated cyclooxygenase and lipoxygenase generate ROS and are responsible for producing

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several proinflammatory cytokines (eg, TNF-α, IL-1, and IL-8) and other active molecules leading to cell damage.[7,16,17] Increased production of ROS damages double bonds in polyunsaturated fatty acids, inducing lipid peroxidation and oxidative damage.[18-20]

Lipid peroxidation is involved in the pathogenesis of numerous inflammatory diseases and malignancies.[16,21-23] Lipid peroxidation produces malondialdehyde (MDA) / thiobarbituric acid-reactive substances (TBARS), which are used to detect changes in oxidant status.[10,19,22,24] Antioxidant defenses, such as total antioxidant capacity measured using ferric reducing ability of plasma (FRAP) and glutathione (GSH), limit cell injury induced by ROS.[10,22,23] The persistence of oxidative stress in CD affects the course of the disease and is associated with some of its characteristic features and complications.[3,11,15,21,25-27]

The levels of oxidative stress markers can be determined with invasive techniques such as blood sampling; however, the use of human saliva is a noninvasive alternative. There is a correlation between salivary levels of inflammatory markers and the clinical severity of CD.[27-29] Saliva contains many biomarkers that reflect local and systemic diseases and may facilitate their diagnosis, prognosis, and therapeutic responsiveness.[20,22,30-32] Therefore, identifying oxidative stress markers in the saliva of CD patients may be of great clinical interest.

Except for CRP, there is a lack of good biologicals marker of CD activity.[33,34] We aimed to investigate the diagnostic usefulness of selected markers of oxidative stress in the serum and saliva of patients with active and inactive CD compared with healthy controls.

PATIENTS AND METHODS

Study population Fifty-eight adult patients (27 women, 31 men) diagnosed with CD were prospectively recruited between December 2014 and January 2016 from the gastroenterology clinic of the University Hospital in Kraków, Poland. The control group consisted of 26 healthy volunteers (12 women, 14 men). Patients were divided into 2 groups: with active CD (n = 32) and with inactive CD (in remission; n = 26). The diagnosis of CD was based on clinical, endoscopic, histopathological, and radiological criteria.[1] The severity of CD was determined using the Crohn's Disease Activity Index (CDAI).[1,35] A CDAI lower than 150 indicates diseases remission, and that of 150 or higher—active CD (150–219, mild exacerbation; 220–450, moderate; >450, severe exacerbation).[1,35] Patients were treated with azathioprine (2.0–2.5 mg/kg/d) according to the European Crohn's and Colitis Organisation guidelines.[36] Patients with inflammatory lesions in the colon were additionally treated with mesalamine (2 g/d).

The characteristics of the study groups are shown in TABLE 1. The exclusion criteria included severe systemic disease; diabetes; chronic inflammation; symptoms of acute illness; alcohol abuse; smoking; the use of antibiotics, antioxidants or anti-inflammatory medications in the past 6 months; pregnancy or lactation; periodontal disease; the presence of oral mucosal disease; and the use of orthodontic appliances. Exclusion criteria for CD patients were also the current treatment with biologic therapy, the presence of abdominal abscess, intestinal stricture, and active gastrointestinal bleeding.[37]

The Bioethics Committee of Jagiellonian University approved the study protocol (No. KBET/200/B/2014). The study was performed in accordance with the ethical principles of the 2008 Declaration of Helsinki. All subjects provided written informed consent before participating in the study.

Sample collection and storage Fasting blood samples were collected in tubes containing K_2-EDTA or free of anticoagulant. Blood was centrifuged at 3000 × *g* for 10 minutes at 4°C and serum was carefully separated. Serum samples were stored at –80°C for 6 months before the assessment of MDA, FRAP, and GSH concentrations as well as catalase (CAT) activity. The routine laboratory tests including complete blood count, platelet count, hemoglobin, and serum CRP levels were performed in the hospital laboratory using standard procedures.

Unstimulated whole saliva samples were collected in 10-ml sterilized and precooled polypropylene tubes from fasted participants between 8:00 AM and 10:00 AM. All subjects were asked to avoid oral hygiene measures, eating, drinking, or gum chewing for a minimum of 2 hours prior to sampling. The individuals rinsed their mouths with tap water and expectorated prior to the collection of saliva. The saliva samples were placed on ice and transported to the laboratory. Saliva was centrifuged at 1000 × *g* for 10 minutes at 4°C, and the supernatant was immediately aliquoted to sterile 1.5-ml test tubes and frozen at –80°C.

Laboratory analysis MDA levels were determined using the colorimetric method of Buege and Aust[20] based on thiobarbituric acid (TBA) reactivity, as described previously.[31] MDA was quantified by reaction with TBA and measurement of the pink chromophore produced. Aliquots (0.5 ml) of samples were mixed with 1 ml of reagent mixture prepared by diluting stock solution, containing 30 g of trichloroacetic acid (TCA; 15% final), 4.16 ml of concentrated HCl (0.2 N final), 0.74 g of TBA; 0.37% final), and H_2O, to 0.2 l. The stock solution (TCA/HCl/TBA) was heated to 70°C and dissolved in water 4 times to yield a working solution. Butylated hydroxytoluene in ethanol was added to a final concentration of 0.03%. After shaking, the tube was placed into a boiling water bath for 15 minutes. After cooling, the resulting chromogen was extracted with 3 ml of butyl alcohol by vigorous shaking for 1 minute. Separation of the organic phase was facilitated by centrifugation

at 2000 × *g* for 10 minutes. The TBA-MDA adduct was quantified using fluorescence emission at 553 nm with excitation at 532 nm, and the TBA-MDA concentration was calculated from a standard curve of 0 to 50 nmol MDA/sample using 1,1,3,3-tetramethoxypropane as the standard. The measurements were performed on a FLUOstar Omega spectrophotometer (BMG Labtech, Ortenberg, Germany). MDA concentrations were expressed as nmol/mg protein.

Total antioxidant capacity was measured using FRAP according to Benzie et al,[38] as described previously.[31] We obtained FRAP values by measuring ferric to ferrous ion reduction at low pH (0.3-M acetate buffer; pH, 3.6) coupled with 2,4,6-Tris(2-pyridyl)-s-triazine, whose absorbance was measured at a wavelength of 593 nm on a FLUOstar Omega spectrophotometer. The serum and saliva samples were centrifuged at 1000 × *g* for 15 minutes (0–4°C). Aliquots (0.1 ml) of the supernatants were mixed with 3 ml of reagent mixture containing 25 ml of acetate buffer (0.3 M; pH, 3.6), 0.25 ml 2,4,6-tri s(2-pyridyl)-s-triazine (5 mM TPTZ) in 40 mM of HCl, and 0.25 ml of ferric solution (20 mM $FeCl_3$) in distilled water. We calculated FRAP values by preparing an aqueous solution of known FeII concentration in the range of 0 to 1000 µM ($FeSO_4 \cdot 7H_2O$), and the blank contained FRAP reagent mixture. Reactions were performed for 5 minutes at 37°C. FRAP values were expressed as mmol/mg of protein.

GSH levels were determined using the Ellman method, as described previously.[31,39] The method is based on the reaction of thiols with the chromogen DTNB (5,5'-dithiobis-2-nitrobenzoic acid), whereby the formation of the yellow dianion of 5-thio-2-nitrobenzoic acid is measured.[40] Absorbance was read 5 minutes after the supernatant was introduced at room temperature at a wavelength of 412 nm on a FLUOstar Omega spectrophotometer (BMG Labtech). GSH concentrations were expressed as nmol/mg of protein.

Total protein levels were determined using the bicinchonic acid (BCA) method in accordance with the manufacturer's instructions (Sigma-Aldrich, city, state, United States), with bovine serum albumin as the standard, as described previously.[41] The BCA method entails reducing Cu^{2+} to Cu^{+}, whereby Cu^{+} ions react in an alkaline medium with BCA, which gives it a violet color. Absorbance was determined at a wavelength of 562 nm at 37°C on a FLUOstar Omega spectrophotometer. A calibration curve was plotted, and a simple linear regression equation derived. Protein concentrations were expressed in mg/ml.

To determine CAT activity in white blood cells, they were isolated from whole blood by centrifugation in a Histopaque density gradient of 1.077 g/ml; 5 ml of blood was gently layered onto 7 ml of Histopaque 1077 (Sigma-Aldrich) in a 15-ml conical tube and centrifuged at room temperature for 40 minutes at 230 × *g* with zero deceleration. Plasma rich in white blood cells (upper layer) was used for examination. White blood cells were diluted 1:0.5 v/v in culture medium RPMI 1640 (Gibco) with fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (1009 µg/ml) and incubated at 37°C. The cells were counted and the suspensions were standardized to obtain 10^6 white blood cells in 1 ml of RPMI. The cells were homogenized and centrifuged at 10 000 × *g* for 20 minutes at 4°C. A supernatant was used to test the catalase activity. The procedures were carried out on ice.

CAT activity was measured using the Catalase Activity Assay Kit (Randox Laboratories Ltd, London, United Kingdom) according to the manufacturer's instruction. Briefly, CAT present in white blood cells reacts with hydrogen peroxide (H_2O_2) to produce water and oxygen. In the presence of horseradish peroxidase, unconverted H_2O_2 reacts 1:1 with the substrate 10-acetyl-3,7-dihy droxyphenoxazine to form resorufin, which is measured fluorimetrically at the excitation and emission wavelength maximum of 535 nm and 587 nm, respectively, with a FLUOstar Omega spectrophotometer.

Statistical analysis Data were expressed as percentages for categorical variables, as mean with standard deviation (SD) for normally distributed continuous variables, and as median with interquartile range for nonnormally distributed continuous variables. The Shapiro–Wilk test was used to assess the normality of the data distribution. Results with a Gaussian distribution were analyzed by the *t* test. The nonparametric Mann–Whitney test and the χ^2 test were used to compare variables between groups. Oxidative stress marker levels were not normally distributed; therefore, we performed statistical comparisons of groups using the Kruskal–Wallis test, if the difference was significant; the post hoc test was used to assess significant pairwise differences. The associations between multiple variables were assessed using the Spearman rank correlation coefficient. The diagnostic usefulness of oxidant–antioxidant indicators was calculated by receiver operating characteristic (ROC) curves. The marker overall performance was expressed in terms of the area under the ROC curve (AUC) with 95% CI and *P* statistics for the difference between the calculated AUC and AUC of 0.5 (index with no discriminating power). A cut-off value corresponding to the highest accuracy was determined and the related sensitivities and specificities, summarized in the Youden index (sensitivity, specificity). We performed statistical analyses using Statistica 10.0 (StatSoft, Tulsa, Oklahoma, United States) and SPSS version 22.0 for Windows (SPSS, Chicago, Illinois, United States). The significance level was set at an α value of 0.05 for the 2-tailed test.

RESULTS The hemoglobin concentration was lower in patients with active CD than in inactive CD and controls ($P = 0.01$ and $P = 0.05$,

TABLE 1 Characteristics of patients with active and inactive Crohn disease and controls

Parameter		Active CD (n = 32)	Inactive CD (n = 26)	Control (n = 26)
Age, y, n (%)		34.6 (11.3)	36.1 (15.1)	33.8 (9.6)
Sex, male/female, n		19/13	17/9	14/12
Disease duration, y		8.3 (3.9)	9.9 (4.2)	NA
BMI, kg/m^2		22.4 (2.7)	24.6 (3.1)	23.8 (2.7)
Hemoglobin, g/l		112 (1.6)[a,b]	134 (1.3)	139 (1.6)
Red blood cell, × 10^{12}/l		3.8 (0.3)	4.2 (0.4)	4.8 (0.3)
White blood cell, × 10^9/l		8.7 (3.2)	6.8 (3.9)	5.2 (1.1)
Platelet count, × 10^3/µl, median (IQR)		397.4 (239.0–463.1)[b,c]	265.1 (240–286.3)	249.5 (223.4–296.8)
CRP, mg/l, median (IQR)		38.3 (18.5–49.2)[d]	1.8 (0.7–3.4)	1.6 (0.5–3.9)
CDAI, points		270.8 (31.2)[a]	67.2 (21.4)	NA
Localization of lesions, %	Small intestine	24%	18.2%	NA
	Large intestine	12%	18.2%	
	Ileocecal disease	64%	59.1%	
	Perianal disease	6%	8.2%	

Data presented as mean (SD) unless otherwise indicated.

a $P = 0.01$ compared with inactive CD; **b** $P = 0.05$ compared with control; **c** $P = 0.05$ compared with inactive CD; **d** $P = 0.001$ compared with inactive CD and control

Abbreviations: BMI, body mass index; CD, Crohn disease; CDAI, Crohn's Disease Activity Index; CRP, C-reactive protein; IQR, interquartile range; NA, not applicable

TABLE 2 Differences in the levels of selected biomarkers between patients with active and inactive Crohn disease and control group

Parameter		Active CD	Inactive CD	Control
MDA, nmol/g of protein	Serum	7.8 (2.0–15.7)[a]	4.1 (3.5–5.8)	2.6 (1.6–4.2)
	Saliva	9.6 (2.7–26.8)[a,b]	6.3 (4.9–32.7)[b]	4.5 (2.7–6.8)[b]
FRAP, mmol/g of protein	Serum	0.01 (0.01–0.02)[a]	0.02 (0.01–0.03)	0.02 (0.02–0.03)
	Saliva	0.04 (0.02–0.07)	0.03 (0.02–0.04)	0.05 (0.04–0.06)
GSH, µmol/g of protein	Serum	6.1 (3.4–8.9)[a]	8.7 (4.6–16.9)	10.1 (8.3–13.6)
	Saliva	6.8 (2.1–14.3)[a]	9.25 (3.5–10.1)	12.6 (9.3–16.8)
CAT, IU/mg protein		8.6 (5.5–14.4)[c]	14.6 (6.0–18.3)	13.7 (6.9–17.5)

Data are presented as median (IQR).

a $P < 0.01$ compared with inactive CD and control; **b** $P = 0.01$ compared with serum MDA; **c** $P = 0.001$ compared with inactive CD and control; Kruskal–Wallis test with post hoc analysis

Abbreviations: CAT, catalase; FRAP, ferric reducing ability of plasma; GSH, glutathione; MDA, malondialdehyde; others, see TABLE 1

respectively). The platelet count and serum CRP levels were higher in patients with active CD than in those with inactive CD and controls (both $P < 0.05$). The mean CDAI score of patients with active CD was also higher than in those with inactive disease ($P = 0.01$). The ileocecal region was the most frequent localization of CD lesions. Detailed data are presented in TABLE 1. Fourteen patients had mild CD activity (CDAI, 150–219 points), and 10 had moderate CD activity (CDAI, 220–450 points).

Serum and saliva MDA levels were elevated in active CD compared with inactive CD and controls ($P < 0.01$) (TABLE 2). MDA levels in saliva were higher than those in serum in active CD, inactive CD, and controls. Serum FRAP levels of patients with active CD were lower than in inactive CD and controls ($P < 0.01$) (TABLE 2). There were no differences in saliva FRAP levels between active CD and inactive CD; however, both FRAP concentrations were lower than those of controls. Saliva FRAP levels were higher than those in serum in all subjects. Serum and saliva GSH levels were lower in active CD patients compared with those with inactive CD and controls ($P < 0.01$) (TABLE 2). The distribution of CAT activity differed between active CD and controls ($P = 0.001$) and between active CD and inactive CD ($P = 0.001$) (TABLE 2).

The correlations between CD activity (assessed by CDAI), lipid peroxidation levels, and antioxidant parameters in serum and saliva are shown in TABLE 3. The correlations of MDA levels in serum

TABLE 3 Coefficients of correlation between the selected serum and saliva biomarkers and body mass index, Crohn's Disease Activity Index, C-reactive protein and hemoglobin levels, and platelet and white blood cell count in patients with active Crohn disease.

Biomarker		BMI	CDAI	CRP	Hemoglobin	Platelets	White blood cells
FRAP	Serum	$r = 0.3$, $P = 0.06$	$r = -0.8$ $P < 0.001$	$r = -0.5$ $P < 0.001$	$r = 0.6$ $P < 0.001$	$r = -0.5$ $P < 0.001$	$r = -0.1$ $P = 0.40$
	Saliva	$r = 0.3$ $P = 0.13$	$r = -0.4$ $P = 0.04$	$r = -0.4$ $P = 0.01$	$r = 0.2$ $P = 0.19$	$r = -0.5$ $P = 0.01$	$r = -0.3$ $P = 0.07$
MDA	Serum	$r = -0.40$ $P = 0.01$	$r = 0.8$ $P < 0.001$	$r = 0.6$ $P < 0.001$	$r = -0.6$ $P < 0.001$	$r = 0.6$ $P < 0.001$	$r = 0.2$ $P = 0.23$
	Saliva	$r = -0.4$ $P = 0.02$	$r = 0.8$ $P < 0.001$	$r = 0.7$ $P < 0.001$	$r = -0.5$ $P = 0.01$	$r = 0.6$ $P = 0.001$	$r = 0.4$ $P = 0.05$
GSH	Serum	$r = 0.3$ $P = 0.04$	$r = -0.8$ $P < 0.001$	$r = -0.5$ $P < 0.001$	$r = 0.5$ $P < 0.001$	$r = -0.6$ $P < 0.001$	$r = -0.2$ $P = 0.19$
	Saliva	$r = 0.1$ $P = 0.68$	$r = -0.5$ $P = 0.01$	$r = -0.6$ $P < 0.001$	$r = 0.3$ $P = 0.11$	$r = -0.5$ $P = 0.01$	$r = -0.3$ $P = 0.19$

Abbreviations: see TABLES 1 and 2

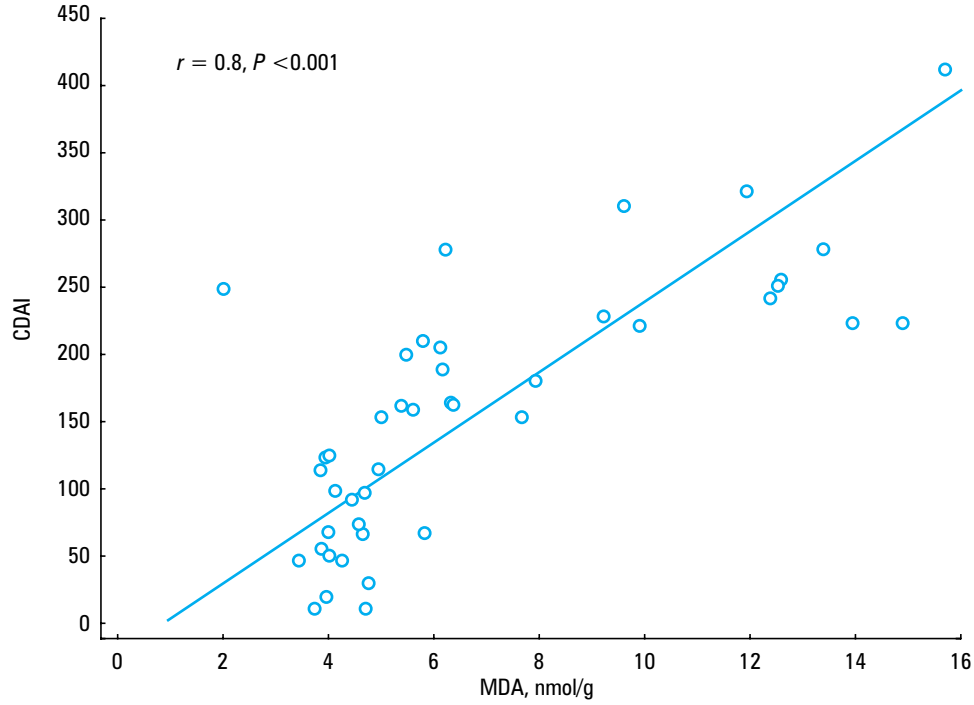

FIGURE 1 Spearman correlation of malondialdehyde (MDA) levels in the serum of patients with Crohn disease and the disease activity according to the Crohn's Disease Activity Index (CDAI)

and saliva of CD patients and CDAI are presented in FIGURES 1 and 2. There were strong positive correlations between MDA concentrations in serum and saliva and CDAI values. Negative correlations were observed between serum FRAP levels and and CDAI values. Negative correlations were also detected between serum GSH levels and saliva and CDAI values. There were significant positive correlations between MDA levels in serum and saliva of CD patients and CRP levels and platelet count, as well as negative correlations between serum and saliva MDA levels and body mass index and hemoglobin levels.

Positive correlations were detected between FRAP levels in serum and hemoglobin levels. Negative correlations were found between FRAP levels in serum and saliva and CRP levels and platelet count. Positive correlations were observed between GSH level in serum and BMI and hemoglobin levels. Negative correlations were detected between GSH levels in serum and saliva and CRP levels and platelet count. Data are presented in TABLE 3.

Correlations between CRP levels and CAT activity, and MDA and FRAP levels in serum and saliva of CD patients are shown in TABLE 4. MDA levels in serum and saliva positively correlated with CRP levels and negatively—with CAT activity. FRAP concentrations in serum and saliva negatively correlated with CRP levels. Serum FRAP levels positively correlated with CAT. GSH in serum and saliva negatively correlated with CRP levels; a positive correlation was observed between GSH in serum and saliva and CAT activity.

The results of the ROC curve analysis comparing CRP and MDA levels in serum are presented in FIGURE 3. The analysis showed diagnostic utility of CRP and MDA in differentiating active from inactive CD (according to CDAI). The AUC for

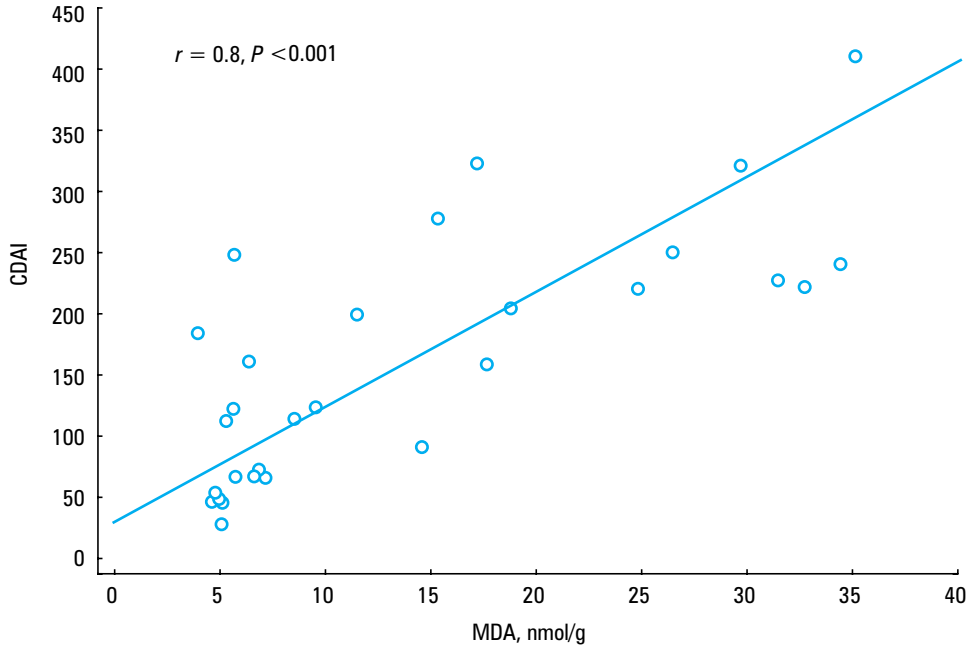

FIGURE 2 Spearman correlation of malondialdehyde (MDA) levels in the saliva of patients with Crohn disease and the activity of the disease according to the Crohn's Disease Activity Index (CDAI)

TABLE 4 Coefficients of correlation between the analyzed serum and saliva biomarkers and C-reactive protein level and catalase activity in patients with Crohn disease

Parameter		CRP	CAT
MDA	Serum	$r = 0.6$ $P < 0.001$	$r = -0.6$ $P < 0.001$
	Saliva	$r = 0.7$ $P < 0.001$	$r = -0.4$ $P = 0.001$
FRAP	Serum	$r = -0.5$ $P < 0.001$	$r = 0.5$ $P < 0.001$
	Saliva	$r = -0.4$ $P = 0.01$	$r = 0.3$ $P = 0.11$
GSH	Serum	$r = -0.5$ $P < 0.001$	$r = 0.8$ $P = 0.001$
	Serum	$r = -0.6$ $P < 0.001$	$r = 0.4$ $P = 0.003$

Abbreviations: see TABLES 1 and 2

CRP was 0.85 (95% CI, 0.76–0.94) with a cut-off point of >4.25, Youden index of 0.62, sensitivity of 0.62, and specificity of 1.00. For MDA, the AUC was 0.95 (95% CI, 0.90–1.00), the cut-off point was >3.82, sensitivity was 0.93, and specificity was 0.87. The AUC for FRAP was 0.94 (95% CI, 0.89–0.99) and for GSH, 0.86 (95% CI, 0.78–0.95).

DISCUSSION CD is a chronic inflammatory condition involving a disrupted oxidant–antioxidant balance that leads to the development of complications.[3,11,15,21,25,42] CD is associated with increased oxidative stress, which is accompanied by increased production of ROS; however, the data on the levels of antioxidants in CD are inconclusive.[10,43]

In this study, we examined the disease activity in relation to inflammation and oxidant–antioxidant indicators as a potential tool in early diagnosis of CD patients. This is the first report of the differences in indices of lipid peroxidation and antioxidant levels in serum and saliva of patients with active and inactive CD. Comparing the results of other studies, we noted an upward trend for MDA levels, depending on the severity of CD. In addition, the increased MDA levels correlate with visible symptoms of inflammation and can be a good indicator for early diagnosis. Additionally, this parameter correlated positively with CDAI and CRP, and negatively with FRAP, GSH, and CAT.

Oxidative stress is involved in the pathogenesis of numerous diseases and is associated with an increase in lipid peroxidation, as indicated by higher concentrations of its products, such as MDA.[11,16,18,21,44,45] These compounds participate in apoptosis signaling by influencing the link between Bcl-2 and Bax, and in mitochondrial regulation and the regulation by $CD8^+$ lymphocytes through nuclear factor-κB (NF-κB) activity.[5,6,14,20,46]

The major intracellular antioxidant enzymes are superoxide dismutase (SOD), GSH, and CAT.[12,15,47] The biological roles of GSH include protecting against oxidative stress, in addition to many other activities.[6,48,49] A shortage of GSH or disturbance in its homeostasis contributes to the pathogenesis of several diseases. CD is associated with decreased intestinal mucosal levels of GSH and SOD, which supports the role for oxidative stress in CD.[3,21] Activities of low-molecular-weight antioxidants, proteins, and enzymatic systems contribute to the total plasma antioxidant capacity.[3,13,43,47,48] CD patients have low levels of numerous antioxidants (eg, vitamins A, C, and E, SOD, GSH, and CAT) in blood and the intestinal mucosa.[13,15,21,25,42,43]

The effects of oxidative stress on CD are intensified by lipid peroxidation.[3,18,45] The role of MDA in CD remains unclear. MDA participates

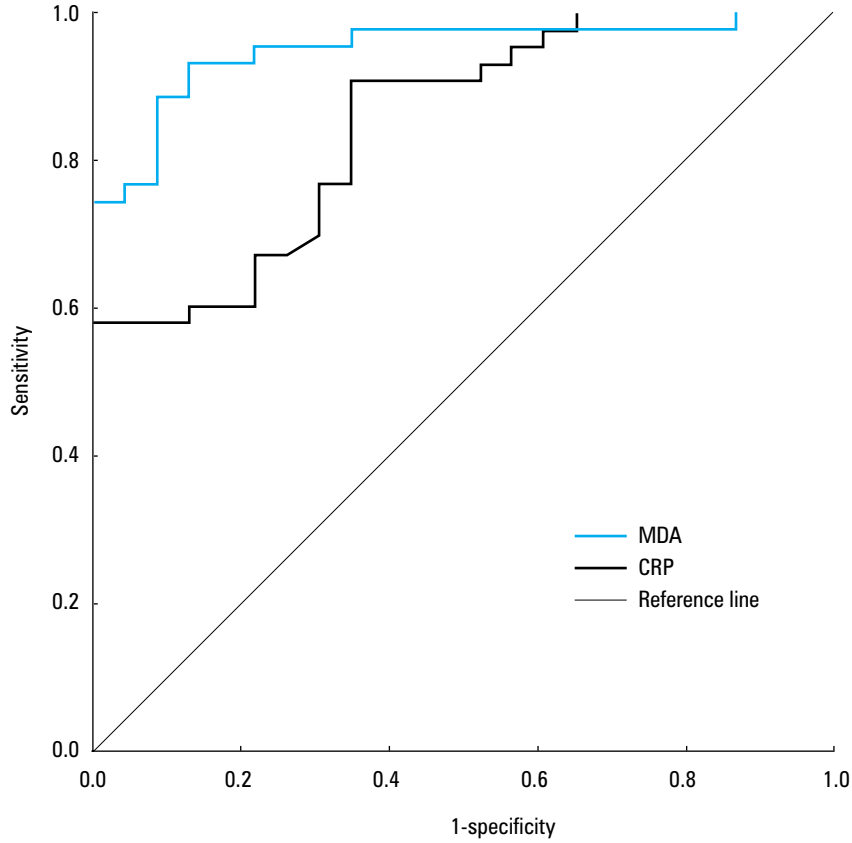

FIGURE 3 Receiver operating characteristic curve analysis comparing serum C-reactive protein (CRP) and malondialdehyde (MDA) levels. Diagonal segments are produced by ties.

in apoptosis signaling and in p85-dependent regulation through NF-κB/p65.[14,15,46] Oxidative stress suppresses signal transfer and transduction mechanisms, which results in enterocyte dysfunction and apoptosis, and functional impairment.[12,14,15,17,18] A better understanding of the role of oxidative stress in CD may facilitate the development of antioxidant therapies.[15,49]

Kruidenier et al[50] confirmed that oxidative stress may occur in CD when there is an imbalance in the levels of ROS and antioxidants. Jahanshahi et al[51] showed that TBARS levels were significantly increased and antioxidant activity was lower in the saliva of CD patients than in controls. These results were in line with a report by Alzoghaibi et al[52] that plasma MDA levels were higher in CD patients than in controls. Boehm et al[10] evaluated the diagnostic utility of MDA/TBARS as markers of CD and concluded that the combination of CRP and MDA/TBARS may facilitate the diagnosis of CD.

Our analysis of the ROC curves revealed the diagnostic utility of CRP and MDA in differentiating patients with active and nonactive CD. CRP currently constitutes the best biochemical marker of CD; however, it is far more useful in the monitoring of the course of the disease than for its diagnosis. Cases are known where an endoscopic examination revealed inflammation but CRP levels were within the reference range.[34]

It was reported that hydrogen peroxide itself acts as an immunomodulator capable of attracting neutrophils, intensifying white blood cell rolling, lymphocyte-T activation, and angiogenesis,[34,53] thus worsening the ongoing inflammation. Moreover, hydrogen peroxide is a key precursor of hydroxyl radicals, ROS, particularly efficient in initiation of lipid peroxidation if it is insufficiently neutralized, which is indicated by decreased activity of CAT determined in our study in white blood cells. As we demonstrated in our previous study, red blood cells, and in this study, white blood cells—as the key cells providing antioxidant defense mechanisms (eg, antioxidant enzymes)—are threatened in patients with CD due to, for example, enzyme damage or exhaustion of their processing capacity in relation to the elevated amount of the substrate. This leads to persistent imbalance and development of oxidative stress. Then, elimination of hydrogen peroxide may be inhibited. In this study, the activity of CAT, an enzyme participating in neutralization of hydrogen peroxide was reduced, while the activity of SOD was significantly reduced in active disease, as reported in our earlier study.[29] This may result in the formation of lipid hydroperoxides, which not only disturb the integrity of cell membranes, but also may exacerbate the existing inflammation through attracting neutrophils and stimulation of proinflammatory cytokines.[10,53] Thus, we believe that, in this case, oxidative stress indices in combination with the indices of antioxidant defense better reflect the degree of the actual changes in the diagnosis of CD than CRP alone. This applies particularly to changes which may not be visible, especially in inactive disease.

In this study, antioxidant activity, measured using FRAP and GSH levels, was significantly reduced in CD. FRAP levels were lower in the serum of active CD patients than inactive CD patients and controls, which suggests antioxidant

depletion. However, saliva FRAP levels did not differ significantly between active and inactive CD, although they were decreased in both groups compared with controls. FRAP levels were significantly increased in saliva compared with the serum of the active and inactive CD patients and controls. Increased total antioxidant activity in saliva may be a compensatory response to oxidative stress in inflammation. Serum and saliva GSH levels were significantly lower in patients with active CD compared with inactive CD and controls. Therefore, antioxidant activity is reduced in active CD compared with clinical remission.

In our study, antioxidant activity was decreased in patients with active CD and increased in those in remission, and this is consistent with other reports.[13,26,47] It is important to consider all factors that influence oxidative stress markers, including duration of CD, clinical status, medications, sample type, and time of sample collection.

Saliva has the potential to be used for noninvasive diagnosis of oral and systemic diseases.[30,32,51,53] Characterization of salivary biomarkers associated with CD and related to its severity could have a major impact on the diagnosis and monitoring of CD.[27,51] Jahanshahi et al[51] reported that the saliva of CD patients had higher levels of nitric oxide and lipid peroxidation markers, as well as decreased antioxidant activity. Rezaie et al[28] reported that several antioxidants as well as lipid peroxidation, nitric oxide, tumor growth factor β, total antioxidant capacity, and albumin and uric acid levels in saliva were decreased in patients with active CD.[28]

In this study, we observed that differences in MDA, FRAP, and GSH levels in saliva were similar to those in serum; therefore, we suggest that saliva can be used to assay lipid peroxidation and antioxidant activity in CD patients. We used 2 biological fluids to evaluate the level of changes, from the local character through systemic response or preceding symptoms in the oral cavity.

The frequency of the oral cavity lesions in CD patients has been reported from 2% to 37%.[27] These changes may overlap or precede the intestinal symptoms of CD.[54] The oral cavity symptoms can be classified as specific lesions, when a macroscopic examination indicates changes similar to those observed on endoscopy in the intestine and nonspecific changes.[54] In most CD patients, these lesions are asymptomatic, yet in some patients, discomfort may occur.[52] In this study, we also examined if noninvasive screening tests for CD (saliva test) are possible not only for gastroenterologists but also for dentists, who often deal with patients without evident clinical symptoms of CD.

This study has several limitations. First, other inflammatory, oxidative stress, and antioxidant parameters were not studied. Second, all patients with CD were treated chronically with azathioprine, and we cannot exclude an effect of this medication on the results. Third, evaluating a larger number of patients would increase the statistical power of the tests and thus the value of the research.

Conclusions The increased lipid peroxidation product (MDA) and decreased FRAP and GSH levels in the serum and saliva of patients with active CD, and to a lesser extent those with inactive CD, confirm that CD patients are under oxidative stress. Positive correlation of MDA with the clinical activity and inflammation, and comparison of the ROC curves for MDA and CRP suggest that MDA could be a valuable indicator in early diagnosis of CD.

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CONTRIBUTION STATEMENT KS and WK conceived the concept of the study and contributed to the study design. KS, DC, RD-R, DO, and JP-P were involved in data collection. TM and DO were involved in blood and saliva sample analysis. All authors edited and approved the final version of the manuscript.

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