RESEARCH ARTICLE

The relationship between neutrophil/lymphocyte and platelet/lymphocyte ratios with oxidative stress in active Crohn's disease patients

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Abstract

Background: This study investigated the relationship between the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio **(**PLR) values with oxidative stress in active Crohn's disease (CD) patients. We investigated whether these parameters were useful for follow-up assessments of CD activity.

Methods: Forty-nine patients with a confirmed diagnosis of CD (24 active and 25 inactive) and 38 control subjects were enrolled in the study. We measured serum activity of superoxide dismutase (SOD) using an enzyme-linked immunosorbent assay (ELISA) and malondialdehyde (MDA) levels using a spectrophotometric method. Neutrophil, lymphocyte and platelet counts were recorded, and the NLR and PLR values were calculated from these parameters.

Results: Patients with active CD exhibited significantly higher serum levels of MDA (p =0.007), NLR (p =0.034), and PLR (p =0.026) than inactive CD patients. Receiver operating characteristic (ROC) curve analysis demonstrated that the optimum cut-off values of MDA, NLR, and PLR based on the differences between active and inactive patients were 0.14 μmol/L, 2.58, and 192.26, respectively. The NLR value increased in active patients with elevated MDA levels as a dependent variable (B: 0.422, p =0.029).

Conclusions: We suggest the use of MDA, PLR, and NLR values as a noninvasive test to evaluate disease activity in CD patients. NLR values may also reflect the presence of oxidative stress, and this value may be efficient and useful in determining CD activity. Hippokratia 2016, 20(4): 268-273

Keywords: Malondialdehyde, superoxide dismutase; neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, oxidative stress, Crohn's disease

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Introduction

Crohn's disease (CD) is characterized by a chronic uncontrolled inflammation that affects the gastrointestinal tract and exhibits periods of relapse and remission[1,2]. Various methods are used to evaluate CD activity, such as investigations based on endoscopic, clinical symptoms and laboratory results (e.g., C-reactive protein, fecal calprotectin, and lactoferrin). Evaluations of inflammation are crucial to follow-up of the disease and to prevent complications[3]. Endoscopic procedures are invasive and not widely available methods to establish a definitive diagnosis in determination of disease activity. Therefore, reliable and noninvasive markers to determine disease activity are required.

Activated and massively infiltrating leukocytes produce large quantities of reactive oxygen species (ROS). ROS are destructive and may contribute significantly to the pathogenesis of CD[4]. Oxidative stress plays a role as a potential etiologic and triggering factor in CD[5,6]. Oxidative stress is a condition in which the antioxidant mechanisms, such as superoxide dismutase (SOD), cannot neutralize the increase in ROS levels in the cell[2,7]. Oxidative stress[8,9] or inflammatory biomarkers, such as the neutrophil-to-lymphocyte ratio (NLR)[10,11] or platelet-to-lymphocyte ratio (PLR)[12], are used as markers in the assessment of disease activity in inflammatory bowel disease (IBD). Malondialdehyde (MDA) is a lipid peroxidation (LPO) product that reflects the damage of reactive oxygen species to lipid membranes, and MDA is a biomarker of oxidative stress[7].

Our study investigated the relationship between the oxidative stress markers MDA and SOD, which play a

role in the etiopathogenesis of IBD, and NLR and PLR to assess inflammatory parameters and other routinely used inflammatory biomarkers, such as the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP). Our study evaluated the usability of these parameters as markers of disease activity in CD.

Materials and Methods

Patient and control groups

Forty-nine patients with CD and 38 healthy volunteers agreed to participate in this study. The ethics committee of the Medical Faculty of Kocaeli University [decision: KOU KAEK.2013/77] approved this cross-sectional study, which was performed between July 2013 and December 2013. Written informed consent was obtained from each participant. CD patients attended the Gastroenterology unit of our hospital for their follow-up visits. The diagnosis of CD was based on the standard clinical, radiological, endoscopic, and histological criteria at the initial diagnosis of their disease. The severity of disease activity was evaluated in each patient according to the CD Activity Index (CDAI). The CDAI is composed of a symptom-based scoring system, including the frequency of liquid stools, the severity of abdominal pain, general well-being, the presence of extra-intestinal manifestations, abdominal mass, current use of antidiarrheal treatment, hematocrit level, and body weight. Clinically inactive disease was defined as an estimated CDAI score of 150 or lower, and patients with CDAI scores higher than 150 were considered to have clinically active disease[13]. According to disease activity, the 49 CD patients were divided into two groups: 24 patients with active CD (ACDP) and 25 patients with inactive CD (ICDP). Most patients continued using their current medical treatments for CD during the study. CD patients who were smokers, diabetics, pregnant women, patients suffering from another chronic inflammatory disease or malignancy, patients with any evidence of an acute viral or bacterial infection and patients who were consuming antioxidant supplements within the preceding four weeks were excluded from the study. Healthy controls included volunteers with no history of CD. Thirty-eight age and sex matched healthy subjects over 18 years of age were recruited as controls from the hospital employees, who underwent their annual routine health screening. The healthy controls were volunteer non-smokers, without any prior medical diagnosis, who had not consumed any supplemental antioxidants or other drugs within the preceding four weeks. Disease duration, extra-intestinal manifestations, disease location and behavior, and drug intake were recorded.

Analytical Procedures

Venous blood samples from all participants were drawn from the antecubital vein after an average fasting period of ten hours before noon and collected into anticoagulant-free tubes. Serum was obtained via centrifugation (3,000 rpm for 15 min) within 30 min and immediately frozen and stored at -40°C until analysis was performed for MDA and SOD.

Serum MDA levels were measured spectrophotometrically using the thiobarbituric acid reactive substances (TBARS) method[9]. Serum SOD activity was analyzed using enzyme-linked immunosorbent assay (ELISA) kits and an Alisei Quality System Seac Radin Company analyzer (Cayman Chemical, Ann Arbor, MI, USA).

Protocols were based on published methods or the manufacturer's instructions. Complete blood count (CBC), CRP and ESR results were obtained from the laboratory database, which was requested during routine follow-ups. CBC, ESR, and CRP analyses were performed in the hematology and biochemistry laboratories of the hospital. CBC was analyzed using laser impedance technology in combination with flow-cytometric laser optical analysis and a Cell-Dyn 3700 analyzer (Abbott Diagnostics, Wiesbaden, Germany). The neutrophil, platelet, and lymphocyte counts were recorded from CBC parameters in laboratory tests, and the NLR and PLR values were calculated from these parameters. ESR was estimated using the Westergren method and an Alifax Test-1 THL Automated Analyzer (Alifax, Italy). CRP was estimated using an immunoturbidimetric method in human serum and the Architect c16000 autoanalyzer (Abbott Diagnostic, USA).

Statistical Analysis

Values are expressed as percentages (n), means ± standard deviation or medians (with 25^{th}-75^{th} percentiles). The Kolmogorov-Smirnov test was used to test the normality of data distribution. Variables that did not conform to a normal distribution were logarithmically transformed (base-10) to reduce the skewness of the data.

Comparisons of continuous variables between groups were performed using Student's t-test, one-way analysis of variance and Tukey's and Tamhane's T2 posthoc tests. Comparisons of categorical variables between groups were performed using the Yates' chi-square test. The relationship between parameters was defined as the dependent variable in ACDP, and NLR and PLR were evaluated using linear regression.

Receiver operating characteristic (ROC) curves were constructed for the MDA, NLR, PLR, ESR, and CRP variables, and the areas under the ROC curve values were calculated with 95 % confidence intervals (CIs) for comparison.

Optimal cut-off values for use in the diagnosis were calculated with 95 % CIs for sensitivity, specificity, and positive and negative predictive values. Analyses were performed using IBM SPSS Statistics software (IBM SPSS, IBM, Armonk, NY, USA) version 20.0, and $p < 0.05$ was considered significantly significant. The power of the study was calculated as 97 % (α: 0.05, β: 0.20).

Results

Table 1 shows the demographic features of the participants, and Table 2 presents the clinical characteristics of ACDP and ICDP groups. Table 3 shows the comparison of

Table 1: Demographic characteristics of the 49 patients with Crohn's disease and the 38 healthy controls who were enrolled in the study.

	CD (n =49)	Controls (n =38)	p values
Age (years)	39.00 (31.00 - 48.00)	34.00 (29.75 - 39.25)	0.441
Female / Male	23 (46.9 %) / 26 (53.1 %)	20 (52.6 %) / 18 (47.4 %)	0.887
Duration of disease (months)	30.00 (3.50 - 70.00)	-	

Values are expressed as numbers (% or range in brackets) or medians (25^{th} - 75^{th} percentiles in brackets), CD: Crohn's disease.

Table 2: Clinical characteristics of the 49 patients with Crohn's disease (CD) who were divided into two groups: 24 patients with active CD (ACDP) and 25 patients with inactive CD (ICDP).

	ACDP (n =24)	ICDP (n =25)
Location of disease		
Ileitis	10 (45.5 %)	15 (60 %)
Colitis	4 (18.1 %)	2 (8 %)
Ileocolitis	8 (36.4 %)	8 (32 %)
Extraintestinal manifestations		
No	22 (91.7 %)	21 (84 %)
Arthritis	2 (8.3 %)	1 (4 %)
Ankylosing spondylitis	-	1 (4 %)
Sacroileitis	-	2 (8 %)
Behavior of disease		
Non-stricturing non-penetrating	9 (37.5 %)	16 (64 %)
Stricturing	9 (37.5 %)	5 (20 %)
Penetrating	6 (25 %)	4 (16 %)
Medications*		
Azathioprine	14 (58.3 %)	13 (52 %)
5-Aminosalicylic acid	10 (41.7 %)	4 (16 %)
Corticosteroids	13 (54.2 %)	10 (40 %)
TNF-α blocker	2 (8.3 %)	7 (28 %)
No medications	5 (20.8 %)	-

Values are expressed as numbers (% or range in brackets), ACDP: patients with active Crohn's disease, ICDP: patients with inactive Crohn's disease, *: Some patients were taking more than one medication at the same time.

Table 3: Comparison of oxidative stress and inflammation markers in the 24 patients with active Crohn's disease (ACDP), the 25 patients with inactive Crohn's disease (ICDP) and the 38 healthy controls.

	Active CD (n =24)	Inactive CD (n =25)	Controls (n =38)	p values of ACDP vs ICDP	p values of ACDP vs Controls	p values of ICDP vs Controls
MDA (μmol/L)	0.17 (0.13-0.23)	0.13 (0.11-0.17)	0.12 (0.08-0.14)	**0.007****	**<0.001***	0.412
SOD (U/ml)	67.18 ± 6.78	65.71 ± 7.34	67.58 ± 8.10	0.808	0.980	0.653
NLR	3.55 (2.14-5.04)	2.41 (1.89-2.62)	1.65 (1.41-2.01)	**0.034****	**<0.001***	0.056
PLR	223. 92 (164.32-329.04)	161.19 (130.39-204.24)	107.60 (92.91-132.63)	**0.026****	**<0.001***	**<0.001***
ESR (mm/h)	29.00 (17.00-54.00)	15.00 (10.00-21.00)	4.00 (2.00-10.50)	**0.049****	**<0.001***	**<0.001***
CRP (mg/dl)	1.31 (0.57-9.00)	0.61 (0.19-0.98)	0.28 (0.10-0.65)	**0.004****	**<0.001***	0.523

Values are expressed as the means ± standard deviation or medians (25^{th}-75^{th} percentiles in brackets), ACDP: patients with active Crohn's disease, ICDP: patients with inactive Crohn's disease, **: The mean difference is significant at the 0.05 level, *: The mean difference is significant at the <0.001 level.

MDA, SOD, NLR, PLR, ESR, and CRP values of all participants. Serum MDA levels, which is an indicator of oxidative stress, were significantly higher in ACDP vs ICDP, p =0.007; ACDP vs healthy controls, p <0.001; ICDP vs healthy controls, p =0.412, respectively. However, serum SOD activity was not significantly different between groups (ACDP vs ICDP, p =0.808; ACDP vs healthy controls, p =0.980; ICDP vs healthy controls, p =0.653, respectively).

Our study demonstrated that NLR and PLR values were significantly higher in ACDP compared to ICDP (p =0.034 and p =0.026, respectively) and healthy controls (p <0.001). ESR and CRP levels were significantly higher in ACDP compared to the healthy controls (p <0.001). ESR (p =0.049) and CRP (p =0.004) levels were significantly higher in ACDP than ICDP. NLR (p =0.056), PLR, and ESR levels were also significantly higher in ICDP

Table 4: Receiver operating characteristic (ROC) analyses of markers to differentiate patients with active Crohn's disease (ACDP) from patients with inactive Crohn's disease (ICDP).

Variables and cut-off values	SEN (95 % CI)	SPE (95 % CI)	PPR (95 % CI)	NPR (95 % CI)	AUC (95 % CI)	AUC p values
MDA (>0.1404 µmol/L)	66.67 (44.7-84.4)	72.00 (50.6-87.9)	69.6 (46.5-87.1)	69.2 (48.2-85.7)	0.714 (0.567-0.834)	0.004
NLR (>2.58)	69.57 (47.1-86.8)	76.00 (54.9-90.6)	72.7 (49.2-89.6)	73.1 (52.2-88.4)	0.703 (0.553-0.826)	0.013
PLR (>192.26)	60.00 (36.1-80.9)	76.00 (54.9-90.6)	66.7 (41.0-86.7)	70.4 (49.8-86.2)	0.690 (0.535-0.819)	0.022
ESR (>16 mm/hr)	78.26 (56.3-92.5)	68 (46.5-85.1)	69.2 (48.2-85.7)	77.3 (54.6-92.2)	0.740 (0.593-0.856)	0.002
CRP (>1.18 mg/dl)	56.52 (34.5-76.8)	88.00 (68.8-97.5)	81.2 (53.3-96.2)	68.7 (50.0-83.9)	0.752 (0.606-0.865)	<0.001

ROC: Receiver operator characteristics curve, CI: confidence interval, SEN: sensitivity, SPE: specificity, PPR: positive predictive rate, NPR: negative predictive rate, AUC: area under the curve, ACDP: patients with active Crohn's disease, ICDP: patients with inactive Crohn's disease, MDA: malondialdehyde, NLR: neutrophil-to-lymphocyte ratio, PLR: platelet-to-lymphocyte ratio, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

Table 5: Linear regression analyses results as dependent variables of neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR) in patients with active Crohn's disease (ACDP).

	NLR		PLR	
	B (95 % CI)	p	B (95 % CI)	p
MDA	0.422(0.048-0.796)	0.029**	0.065(-0.372-0.503)	0.760
ESR	0.174(-0.044-0.393)	0.112	0.082(-0.173-0.338)	0.511
CRP	-0.044(-0.205-0.116)	0.573	-0.055(-0.243-0.132)	0.546

CI: confidence interval, NLR: neutrophil-to-lymphocyte ratio, PLR: platelet-to-lymphocyte ratio, ACDP: patients with active Crohn's disease,**: The mean difference is significant at the 0.05 level.

compared to the healthy controls (p <0.001). However, there was no significant difference between ICDP and healthy controls for MDA and CRP levels (p =0.412 and p =0.523, respectively).

ROC curve analysis revealed that the optimum cut-off values of MDA, NLR, PLR, ESR, and CRP determined CD activity (differentiation of ACDP from ICDP) as 0.14 µmol/L, 2.58, 192.26, 16 mm/hr, and 1.18 mg/dl, respectively. Table 4 shows the cut-off values, sensitivity, specificity, positive predictive value, negative predictive value, and area under the curve (AUC) and AUC p values of all variables with 95 % CIs. The sensitivities of MDA, NLR, PLR, ESR, and CRP at the cut-off values to define ACDP were 66.67, 69.57, 60, 78.26, and 56.2, respectively, and the specificities were 72, 76, 76, 68, and 88, respectively (Table 4).

Figure 1 shows the comparison of ROC curves of MDA, NLR, PLR, ESR, and CRP variables for ACDP vs ICDP. There was no significant difference between paired comparisons of the AUC values of parameters (AUC values) (CRP vs ESR, MDA, NLR, PLR, p =0.530, 0.824, 0.496, and 0.869, respectively; ESR vs MDA, NLR, PLR, p =0.488, 0.247, and 0.531, respectively; MDA vs NLR, PLR, p =0.775, and 0.953, respectively; NLR vs PLR p =0.694). The results of our linear regression analysis revealed that NLR changed in an MDA-dependent manner (B: 0.422, p: 0.029) (Table 5).

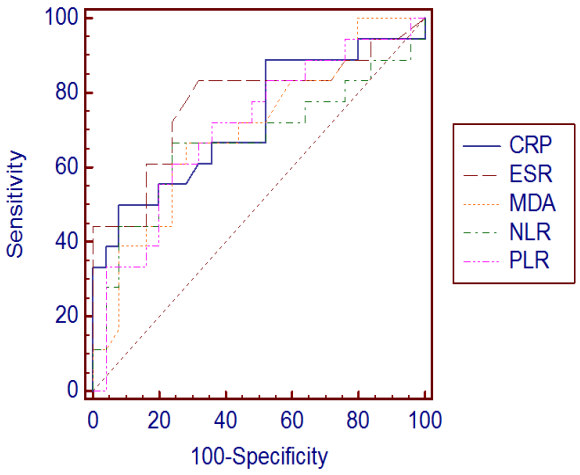

Figure 1: Comparison of the receiver operating characteristic (ROC) curves of malondialdehyde (MDA), between neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) variables. There were no significant pair-wise differences in the area under the curve (AUC) values (CRP vs ESR, MDA, NLR, and PLR, p =0.530, 0.824, 0.496, and 0.869, respectively; ESR vs MDA, NLR, and PLR, p =0.488, 0.247, 0.531 respectively; MDA vs NLR, and PLR p =0.775, and 0.953, respectively; NLR vs PLR, p =0.694).
MDA: malondialdehyde, NLR: neutrophil-to-lymphocyte ratio, PLR: platelet-to-lymphocyte ratio, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein.

Discussion

Our study demonstrated significantly higher values of NLR, PLR, and MDA with ACDP. NLR values also changed in an MDA-dependent manner. ESR was the

most sensitive test (78 %), with an optimum cut-off value of 16 mm/hr, but it exhibited the lowest specificity (68 %) in determining CD activity. However, CRP exhibited the highest specificity (88 %), with an optimum cut-off value of 1.18 mg/dl and the lowest sensitivity (56 %). NLR was the second-most sensitive test (69 %) with a cut-off value of 2.58. NLR and PLR were the second-most sensitive tests with the same specificity value (76 %), with an optimum cut-off value of 192.26. However, the sensitivity of PLR (60 %) was lower compared to NLR in our study. Similar results of NLR were demonstrated in IBD[10,11].

Neutrophils, lymphocytes, and platelets play important roles in the pathogenesis of mucosal tissue injury in CD[14-16]. Neutrophils play an essential function in the maintenance of intestinal homeostasis. Neutrophils also contribute to the inflammatory response via facilitating the recruitment of other immune cells and mucosal healing via the release of mediators that are necessary for inflammation resolution. Neutrophils are the most abundant white blood cells in the circulation, constitute approximately 50-70 % of all circulating leukocytes, and produce massive amounts of reactive oxygen species to destroy pathogens[14].

MDA was the third-ranking marker for sensitivity (66.7 %) and specificity (72 %) values with a cut-off value of 0.14 µmol/L. Comparisons of the ROC curves of the investigated parameters demonstrated that serum values of NLR and PLR and serum levels of MDA could be as important as the serum levels of other routinely used inflammatory parameters, such as CRP and ESR, for disease follow-up. Many studies have demonstrated the association of chronic intestinal inflammation with increased oxidative stress[7,9,17,18]. Many studies investigated the use of LPO products, such as MDA, for determining disease activity in IBD due to the higher levels of oxidative stress, but this parameter was not meaningful[8,9,19].

The antioxidant enzyme SOD was not important for CD activity in our study, which is consistent with Achitei et al[9]. However, Karp et al observed decreased SOD activity[20]. Antioxidant activity decreases in CD patients, and one of the drugs used in CD patients, 5-aminosalicylic acid (5-ASA), exerts an antioxidant effect[21]. Most of our patients, especially most of the ACDP group, were using 5-ASA, which may explain the absence of a significant difference in SOD levels between the groups as a result of the antioxidant activity of this treatment, which is consistent with Alzoghaibi et al[21].

Patients' use of various medications during the study and in long term, is one limitation of the current study. It might be possible to create subgroups comprised of patients using the same medication, to evaluate drug effects in the current study and provide an opportunity to observe differences in these subgroups attributed to these drugs. However, the number of subgroups was insufficient to evaluate the same drug in our cohort. Another limitation is the number of healthy controls. Finding healthy non-smoking men to enroll in the control group was difficult.

It might have been more suitable to perform such a study in newly diagnosed and treatment-naïve patients to exclude possible drug effect. However, we enrolled patients with ongoing treatment and in various stages of the disease. Our results suggest that these inflammatory parameters may be useful in the determination of CD activity as these parameters provide the attending physician with data regarding the increased oxidative stress in the patient (a reflection of LPO) and the ability to use a cheap and easy-to-use test in the routine follow-up of CD patients. Therefore, these features increase the clinical value of NLR. NLR may also be widely studied in routine laboratories because this measurement is clinically useful, cheaper and uses a simpler calculation than CBC. NLR may also reflect the present oxidative stress, which is more efficient and valuable compared to the other parameters investigated. We suggest that further studies should be performed with the inclusion of additional oxidant and antioxidant parameters.

Conflict of interest

Authors declare no potential conflicts of interest.

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