Evaluation of serum neutrophil gelatinase-associated lipocalin (NGAL), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) levels, and their relations with disease type and activity in inflammatory bowel diseases

Abstract

Background/aim: Inflammatory bowel disease (IBD) mainly encompass two entities called ulcerative colitis (UC) and Crohn's disease (CD), both of which are chronic, progressive and, inflammatory conditions of the gastrointestinal tract. Various indicators and non-invasive markers have been sought and used in IBD patients to help assessing disease activity and treatment effectiveness, although none of them are proven to yield definite results in full correlation with the clinical, endoscopic, and histopathological examinations. The aim of the current study was to investigate the relationship of serum neutrophil gelatinase-associated lipocalin (NGAL), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) levels with disease type and activity and to assess their potential use in establishing diagnosis and activity status of IBD, namely UC and CD.

Materials and Methods: A total of 111 IBD patients with determined active and inactive disease periods and 70 matched controls were recruited. Serum NGAL levels of the patients and the control group were measured using commercially available ELISA kits. ADMA and SMDA levels were accomplished by high performance liquid chromatography

Results: The IBD group had significantly higher serum levels of NGAL (p= 0.001), ADMA (p= 0.0001), and SDMA (p= 0.0001) in comparison to the control group. Likewise, serum NGAL, ADMA, and SDMA levels were significantly higher in the active IBD group compared to the inactive IBD group (p= 0.0001). Active UC and active CD patients similarly had significantly higher levels of serum NGAL, ADMA, and SDMA than the respective levels in inactive UK and inactive CD patients (p= 0.0001).

Conclusion: Serum NGAL, ADMA and SMDA levels are increased in patients with IBD and serum NGAL, ADMA and SMDA concentrations are significantly higher in active IBD patients than inactive IBD patients. Our results suggest these biomarkers may serve in estimating IBD activity and severity.

1. Introduction:

The etiology and pathophysiology of inflammatory bowel disease (IBD) remain obscure. In genetically predisposed individuals, IBD develops in connection to environmental factors which result in an inappropriate immune response leading to chronic intestinal inflammation and destruction of microvascular endothelial cells in the small bowel and/or colon [1].

The clinical course of IBD is marked by alternating episodes of active disease and remission. Ulcerative colitis (UC) and Crohn's disease (CD) constitute the two major IBD forms characterized by epithelial cell destruction as well as a remarkable mucosal infiltration of inflammatory cells including granulocytes, macrophages, lymphocytes, and plasma cells [1,2].

Oxidative stress and endothelial dysfunction play a key role in IBD pathogenesis. Increased endothelial cell proliferation and activity has been blamed to cause an altered intestinal microcirculation which in turn compromises the local intestinal blood supply, eventually giving rise to the formation of local necrosis and mucosal ulcerations [3,4].

Lately, a growing body of research has focused on the use of various non-invasive activity indicators and markers in tests to assess disease activity and treatment effectiveness in IBD patients. Nevertheless, none of such testing yet achieved fully compatible results with clinical, endoscopic, and histopathological examinations. [5,6].

Asymmetric dimethylarginine (ADMA) is a marker of endothelial dysfunction [7]. ADMA is synthesized through a post-translational modification of the amino acid arginine. Its function as a competitive inhibitor in cellular intake of the enzyme nitric oxide synthase (NOS) and L-

arginine is noteworthy, as this hampers nitric oxide (NO) production playing a multifactorial role in various human diseases [7,8].

Symmetric dimethylarginine (SDMA) is a closely related stereoisomer of ADMA which is produced in almost equal amounts. Although SDMA does not inhibit NOS itself, it acts as a competitive inhibitor of cellular L-arginine uptake, thus, reducing the amount of the substrate available for NOS and eventually causes attenuation of NO production [7,8].

NO is a key molecule which is not only involved in endothelium-dependent vasodilation but also employed in regulatory functions such as proliferation of smooth muscle cells in the vascular wall, luminal cell-cell interactions, inhibition of platelet adhesion and aggregation, monocyte adhesion and inhibition, thereby contributing to the control of vascular homeostasis and maintenance of blood supply to organs [7-9]. Considering further functions it exerts on the vascular system including stimulation of angiogenesis and inhibition of superoxide radicals, NO has been called an “endogenous anti-atherogenic molecule”. ADMA and SDMA selectively inhibit the synthesis of such an important anti-atherogenic molecule thus deprive the vascular system making use of the protective effects of NO, subsequently eliciting a pathophysiological impact [7,9,10].

Higher levels of ADMA induce endothelial dysfunction which clinically manifests as vasodilation arising from impaired endothelium as well as platelet hyperaggregation and increased monocyte adhesion [9-11].

Neutrophil gelatinase-associated lipocalin (NGAL), also known as GAL, lipocalin 2, siderocalin, 24p3 or LCN2, is primarily identified in circulating neutrophils, but it may also exist in several tissues although at quite lower levels [12]. On the other hand, NGAL synthesis and release into circulation is remarkably increased by inflammatory stimuli that damage the small and large intestinal epithelial cells, respiratory cells, renal tubular cells, and hepatocyte endothelial cells [13,14].

The pathogenic bacteria have adapted several mechanisms to utilize the protein-bound iron on the host cells. Among them, the best recognized is the siderophores which are defined as low-molecular-weight ferric chelators [15]. NGAL binds to the iron-loaded siderophore to sequester the iron away from the environment thus creating a bacteriostatic effect on the microorganisms which rely on iron [15,16]. Consequently, a recent hypothesis proposes that, NGAL elicits a bacteriostatic effect by blocking the iron acquisition required by the bacteria for reproduction and other functions, and therefore attributes an essential role to NGAL as a part of the innate immune system [16].

Inflamed colon harbors an increased synthesis of NGAL and its release into the circulation which reflects neutrophil activation. On the other hand, with its bacteriostatic effects and capability to prevent neutrophil chemoattraction, NGAL is also claimed to play role in the pathogenesis of several inflammatory diseases of the colon [17, 18]. In this regard there are several former studies in the literature investigating fecal NGAL levels in various diseases of the colon and questioning its applicability as a biomarker or a diagnostic test in these disorders [5,8,17].

Although results are encouraging to support NGAL as an early marker of inflammatory conditions, studies on its use in IBD are limited in number. Furthermore, studies evaluating serum levels of NGAL as a possible biomarker in the diagnosis of IBD and determination of disease activity have controversial results [5,17,18].

In this context, the aim of the current study was to investigate serum NGAL, ADMA, and SDMA levels in IBD patients and evaluate their possible associations with disease type and activity and also to assess their potential use as a biomarker in establishing diagnosis and activity status of IBD, namely UC and CD.

2. Material and methods

2.1. Study design, setting, and participants

The present cross-sectional study was performed in Outpatient Department of Gastroenterology at Selcuk University, Medical Faculty Hospital. One hundred and eleven consecutive IBD patients admitted to the outpatient clinic from May 1, 2018 to February 28, 2020, who were eligible in terms of inclusion and exclusion criteria, were enrolled in the study. Current study was conducted in accordance with the ethics guidelines of the Helsinki Declaration. All patients provided signed informed consent prior to their participation into the study. Also, ethical approval was obtained from Selcuk University, Faculty of Medicine, Ethics Committee for Non-Interventional Clinical Research (2014/119).

2.2. Selection criteria for the patient and the control groups

Current study included 111 IBD patients, namely UC and CD patients aged 18 to 70 years, all of whom were followed-up in the Outpatient Department of Gastroenterology at Selcuk University, Medical Faculty Hospital. For all patients, UC and CD diagnoses were based on histological, endoscopic, radiological, and clinical criteria. Seventy age- and sex matched healthy individuals were also recruited in the study as the control group.

Exclusion criteria: Individuals who are pregnant or have other inflammatory, cardiovascular or cerebrovascular conditions, peripheral artery disease, renal failure, liver disease, chronic obstructive pulmonary disease, or cancer were excluded from the study.

For each of the participants in the patient group, weight, height, gender, age, status of cigarette smoking, type and duration of IBD, ongoing medical treatment, extraintestinal symptoms and complications were noted. Body mass indext (BMI) was calculated per person, by dividing body weight in kilograms to the square of height in centimeters.

Clinical activity in UC patients was determined according to the Truelove–Witts criteria and CCAI [19, 20]. UC patients were classified into 4 groups according to CCAI score; Inactive

or remitted disease (CCAI score ≤ 4), mild activity (CCAI score 5-10), moderate activity (CCAI score 11-17) and severe activity (CCAI score ≥18).

Crohn's Disease Activity Index (CDAI) was used to assess disease activity in CD patients [21]. CD patients were also classified into 3 subgroups according to CDAI score; Inactive or remitted disease (CDAI ≤ 150), mild activity (CDAI score 150-220), moderate and severe activity (CDAI score ≥ 220).

2.3. Laboratory analysis

For all laboratory analyses, peripheral venous blood samples were obtained from IBD patients and the control group early in the morning after 12 hours of fasting and collected into EDTA, sodium citrate and gel containing tubes. After allowing for 30 min, gel coated tubes were centrifuged at 3,500 rpm for 15 min. Once sera were separated, serum samples were aliquoted into two and were immediately transferred to freezer to be stored at −80°C until NGAL, ADMA, and SDMA analysis. Serum NGAL concentrations were measured using a commercially available sandwich-type enzyme-linked immunosorbent assay kit (ELISA; BioPorto Diagnostic A/S, Gentofte, Denmark) with an automated ELISA reader (Rayto RT 2100 C), ADMA and SMDA levels were accomplished by high performance liquid chromatography (HPLC) according to the protocol provided by the manufacturer.

Complete blood count (CBC) serum iron and ferritin concentrations were measured using automated analysers. C-reactive protein (CRP) levels were measured using the ARCHITEC C1600 device (Abbott Diagnostics, USA) and erythrocyte sedimentation rate (ESR) was measured using the Test-1 fully automated analyzer (Alifax, Italy).

2.4. Statistical analysis

Continuous variables were tested for normal distribution using one-sample Kolmogorov–Smirnov test. When the tested parameter was normally distributed, parametric tests were used for statistical analyses, and the results were presented as mean ± standard deviation (SD). For

normally distributed data, statistical comparisons were done using Student *t* test (comparison of two groups) and one-way analysis of variance (ANOVA) test (comparison of more than two groups). Post-hoc analyses for one-way ANOVA tests were done using Bonferroni test. When the tested parameter was not normally distributed, nonparametric tests were used, and the results were presented as median ± interquartile range. For non-normally distributed data, statistical comparisons were done using Mann–Whitney *U* test (comparison of two groups) and Kruskall–Wallis test (comparison of more than two groups). Post-hoc analyses were done using Mann–Whitney *U* test with Bonferroni correction when Kruskall–Wallis test yielded significant results.

The difference between the categorical variables was tested using Chi-square test. Correlation tests (Pearson's or Spearman's rank correlation) were done to decide as to whether there is any correlation among the variables. Multiple linear regression models were applied to search for any relationship patterns between the correlations of NGAL or ADMA and the other variables. All statistical analysis was carried out using computer software SPSS for Windows version 20 (SPSS Inc., Chicago, IL, USA). Values of $p < 0.05$ were accepted as statistically significant.

The power analysis for calculation of the sample size was done using computer software "the G*Power v.3.1.5" [22]. The required sample size was 45 participants in each group for serum NGAL (power = 0.95 at $\alpha = 0.05$), 68 participants in each group for serum ADMA (power = 0.85 at $\alpha = 0.05$) and 49 participants in each group for serum SDMA (power = 0.95 at $\alpha = 0.05$).

3. Results

A total of 111 IBD patients (54 female/57 male) and 70 age- and sex-matched healthy controls (34female/36 male) were included into this study. The median duration of disease in the patient group was 53.0 (4.00-98.0) months. Out of the IBD patients, 77 had UC (69.4%)

and 34 had CD (30.6%). Among all IBD patients, 51 were in remission (45.9%), while 60 had active disease (54.1%).

3.1. Serum NGAL, ADMA, and SDMA levels in IBD patients *versus* the control group

The IBD group and the control group had no significant difference in terms of their demographic characteristics and smoking behaviors. Mean serum creatinine concentration (0.79 ± 0.14 mg/dL; 0.08 ± 0.15 mg/dL, p= 0.6) and glomerular filtration rates (GFR) (98.98 ± 17.68 mL/min; 98.25 ± 15.80 mL/min, p= 0.7) were also similar in the IBD group and the control group.

The IBD group had significantly higher levels of leukocyte count [8.07 ± 2.44 ($x10^3$/µL); 5.91 ± 1.78 ($x10^3$/µL), p= 0.0001], ESR (18.19 ± 12.58 mm/h; 5.62 ± 3.45 mm/h, p= 0.0001) and CRP [median 11.51 (5.01-30.1) mg/L; 5.31(3.10 -10.2) mg/L, p=0.0001] than the control group. Mean serum concentrations of NGAL (148.04 ± 53.21 ng/mL; 107.50 ± 23.62 ng/mL, p= 0.0001), ADMA (0.146 ± 0.07 µmol/L; 0.110 ± 0.03 µmol/L, p= 0.0001), and SDMA (0.146 ± 0.04 µmol/L; 0.117±0.01 µmol/L, p= 0.0001) were also significantly higher in the IBD group than the control group. On contrary, serum iron (57.13 ± 31.5 mg/dL; 88.02 ± 32.17 mg/dL, p= 0.0001) and ferritin (53.13 ± 22.37 ng/mL; 99.42 ± 39.67 ng/mL , p= 0.0001) concentrations were lower in the IBD group than the control group.

3.2. Serum NGAL, ADMA and SDMA levels in active vs inactive IBD patients

Demographics, biochemical and inflammatory parameters and mean serum NGAL, ADMA, and SDMA concentrations in the control group and the active and inactive IBD patients were shown in Table 1. Demographic characteristics, smoking behaviors and GFR levels were similar in active and inactive patient groups and healthy controls ($p \geq 0.05$). On the other hand, there was a significant difference between the control group and the active and inactive IBD patients in terms of serum iron, ferritin and CRP levels, ESR and leucocyte count as well as mean serum NGAL, ADMA, and SDMA concentrations ($p < 0.05$).

In comparison to the control group, both serum iron and ferritin levels were significantly lower in the active and inactive IBD patients, whereas only serum iron level was significantly lower in the active IBD group compared to the inactive IBD group.

Inflammatory parameters (leukocyte count, CRP, ESR) and mean serum NGAL, ADMA, and SDMA concentrations were significantly higher in the active IBD group than those in the inactive IBD group. On the other hand, mean serum NGAL, ADMA, and SDMA concentrations were similar in the inactive IBD group and the control group without any statistical significance in any of these parameters (Table 1).

3.3. Serum NGAL, ADMA, and SDMA levels in patients with ulcerative colitis and Crohn's disease

Statistical comparison of UC, CD, and the control group revealed significantly higher ESR (17.50 ± 13.69; 19.76 ± 9.60; 5.62 ± 3.45, p= 0.0001), CRP [median 10.13 (5.01-32.1) mg/L; 14.58 (8.10-44.01) mg/L; 5.31 (3.25-10.10) mg/L, p= 0.0001], NGAL (143.02 ± 58.28 ng/mL; 159.41 ± 36.29 ng/mL; 107.50 ± 23.62 ng/mL, p= 0.0001), ADMA (0.127 ± 0.046 µmol/L; 0.161 ± 0.030 µmol/L; 0.110 ± 0.035 µmol/L, p= 0.0001), and SDMA (0.138 ± 0.050 µmol/L; 0.163 ± 0.039 µmol/L; 0.117 ±0.018 µmol/L, p= 0.0001) levels in UC and CD groups compared to the control group (results are given for UC, CD, and control group, respectively).

Mean serum NGAL concentration was significantly higher both in UC and CD groups compared to the control group (p= 0.0001), but there was no significant difference between the UC and CD patients in terms of serum NGAL concentration (p= 0.21).

Likewise, mean serum ADMA concentration was significantly higher both in UC and CD groups compared to the healthy controls (p= 0.003, p=0.0001, respectively). Statistical analyses also revealed that CD group had significantly higher serum ADMA concentration when compared to the UC group (p=0.0001).

Similarly, mean serum SDMA concentration was significantly higher both in UC and CD groups compared to the control group (p=0.0003, p=0.0001, respectively), which was also significantly higher in CD group compared to the UC group (p= 0.007).

ESR and CRP values were, as well, significantly higher both in UC and CD groups compared to the control group (p= 0.003, p= 0.0001, respectively), which were also higher in CD group compared to the UC group, but not to the significant extent (p= 0.8, p= 0.9, respectively).

3.4. Serum NGAL, ADMA, and SDMA levels in active vs inactive ulcerative colitis and active vs inactive Crohn's disease patients

As shown in Table 2, demographic characteristic did not significantly vary among the active UC, inactive UC, active CD, inactive CD, and the control group. On the other hand, active UC and active CD patients had significantly higher levels of ESR and CRP than the respective levels in the inactive UC, inactive CD, and the control patients (Table 2). Serum NGAL, ADMA, and SDMA levels of the active UC and active CD patients were also significantly higher than the respective levels in the inactive UC, inactive CD, and the control patients (Table 2).

3.5. Analysis of the relation of serum NGAL, ADMA, and SDMA levels with disease characteristics in IBD patients

When UC patients were divided into four subgroups according to anatomical involvement of the colon as proctitis, proctosigmoiditis, left-sided colitis, and pancolitis and then have their mean NGAL, ADMA, and SDMA levels compared by ANOVA, it was found that all three parameters were significantly different between the four groups (p= 0.0001, Table 3). Subgroup comparisons revealed that serum NGAL, ADMA, and SDMA levels did not differ significantly between the proctitis and proctosigmoiditis subgroups, whereas patients with left-sided colitis and pancolitis had significantly higher serum NGAL, ADMA and SDMA levels when compared to the patients with proctitis and proctosigmoiditis. (p= 0.0001, Table

3).

UC patients were divided into four subgroups according to disease severity using CCAI index (remission, mild UC, moderate UC, and severe UC) and serum NGAL, ADMA, and SDMA levels were compared using one way ANOVA test. It was found that, all three parameters were significantly different between the four groups ($p= 0.0001$, $p= 0.003$, and $p= 0.02$, respectively). In mildly, moderately, and severely ill UC patients, serum NGAL levels were significantly higher than those of patients in remission. Serum ADMA, and SDMA levels, on the other hand, were significantly higher only in moderately and severely ill patients when compared to the patients with mild disease or in remission. There was no significant difference between the patients with mild disease or in remission in terms of serum ADMA and SMDA concentrations (Table 3).

When UC patients were further divided into subgroups according to disease severity using Truelove-Witts index (remission, mild UC, moderate UC, and severe UC) and then have their mean serum NGAL, ADMA, and SDMA levels compared using one way ANOVA test, it was found that serum NGAL and ADMA levels were significantly different between the four groups ($p=0.004$, 0.001, respectively**)**. In mildly, moderately, and severely ill UC patients, serum NGAL and ADMA levels were significantly higher than those of patients in remission ($p < 0.05$). Moreover, serum NGAL and ADMA levels of the moderate and severe subgroups were not significantly higher than the respective values of the mild disease subgroup ($p > 0.05$, Table 3).

When CD patients were divided into subgroups according to anatomic location of involvement as colonic disease, ileocolonic disease and ileal disease and then have their mean serum NGAL, ADMA, and SDMA levels compared by one way ANOVA test, it was found that serum NGAL, ADMA and SDMA levels were not significantly different among the three groups ($p > 0.05$). Although not statistically significant, highest serum levels of NGAL and

ADMA were observed in the ileocolonic subgroup (Table 3).

CD patients were divided into three subgroups according to disease severity using CDAI index scores (remission, mild CD, moderate and severe CD) as previously described. Serum NGAL, ADMA, and SDMA levels of the groups were compared using one way ANOVA test. It was found that all three parameters were significantly different between the three groups ($p= 0.0001$ for NGAL, ADMA and SMDA). Subgroup analyses revealed that patients with mild or moderate and severe disease had significantly higher serum NGAL, ADMA, and SDMA levels when compared to the patients in remission (Table 3).

CD patients were also divided into subgroups according to disease phenotype as stricturing, penetrating, and non-stricturing, non-penetrating and into another set of subgroups by medication used, and then their mean serum NGAL, ADMA, and SDMA levels compared by one way ANOVA test which did not show any significant difference among these groups (Table 3).

3.6. Correlation Analysis

Correlations of serum NGAL and ADMA concentrations with several clinic, laboratory and demographic parameters are given in Table 4 and Table 5, respectively. Both NGAL and ADMA were found to be positively correlated with leukocyte count, ESR, and CRP levels, Truelove-Witts activity index and CDAI scores, anatomical localization of UC and status of IBD activity and they were negatively correlated with serum iron and ferritin levels. Multiple linear correlation analyses, on the other hand, revealed correlation of serum NGAL and ADMA concentrations with leukocyte count, ESR, CRP and serum iron, and ferritin levels.

4. Discussion

IBD develops as a result of chronic intestinal inflammation and the destruction of the intestinal microvascular endothelial cells, but its etiology and pathophysiology is still not fully known [1-3].

NGAL synthesis is increased in the inflamed colonic epithelium, reflecting the neutrophil activation which decreases neutrophil chemoattraction and displays bacteriostatic effects. Therefore, a crucial role has been attributed to NGAL in the inflammatory disease of the colon [17, 18].

The NO generation from L- arginine is inhibited by ADMA and SDMA. The distinctive characteristic of ADMA, a competitive inhibitor of the cellular uptake of L-arginine and NOS, is that it is accepted as a marker of endothelial dysfunction. As for SDMA, another competitive inhibitor of the cellular uptake of L-arginine, it is mostly excreted with urine. While 80% of ADMA is metabolized, 20% is excreted in urine [8, 10, 11].

As for the major findings in our study, we found that serum NGAL, ADMA, and SDMA levels were significantly higher in IBD patients compared to the healthy controls. Serum levels of NGAL, ADMA and SDMA were also found to be higher in the active IBD group compared to the inactive IBD group but no statistical differences were observed between the inactive IBD group and the control group. In subgroup analyses, serum levels of NGAL, ADMA, and SDMA were found to be similar in UC and CD patients. For both UC and CD, serum levels of NGAL, ADMA, and SDMA were significantly higher in patients with active disease compared to those with inactive disease.

In the study conducted by Nielsen et al. on 37 IBD patients, one of the first studies in this field, it was found that while serum NGAL levels were increased in IBD patients there was no sufficient difference to distinguish the disease activity [17]. In that study, the small number of patients might have been the possible cause of the different results obtained in terms of disease activity. Similar to our study, the studies recently conducted by Yeşil et al. [5], Oikinomou et al. [18], and Budzynska et al. [23] reported that serum NGAL levels were significantly higher in the IBD group compared to healthy individuals. While no significant difference in NGAL values was found between active and inactive IBD patients in the study

conducted by Yeşil et al. who compared these patient groups based on their endoscopic and clinical activity indices; significant differences were reported between active *versus* inactive IBD, UC and CD patients in the other two studies, similar to our study. In addition, while serum NGAL level was found to be higher in UC patients compared to patients with CD in the study by Yeşil et al.; no significant difference was observed between UC and CD patients in the other two studies, as well as our study. These conflicting results might have arisen from the inclusion of patients with varying degree of colonic involvement into the relevant studies, as NGAL level is considered to be lower in patients with involvement confined to ileum [5,18].

In a recent study, Thorsvik et al. [24] demonstrated that not only the serum NGAL levels were higher in IBD patients, but they were also significantly higher in the active UC and CD patients compared to inactive UC and CD patients, corroborating our study. Moreover, our study revealed correlation of the serum NGAL levels with clinical activity index, CRP, ESR, leukocyte, iron, and ferritin. While there was no correlation with the clinical and endoscopic activity indices in the study conducted by Yeşil et al. [5]; correlations were identified in the study conducted by Oikinomou et al. [18], in line with our study.

Mean NGAL levels vary in patient and control groups in various studies conducted with NGAL. It is known that people from different regions have different intestinal microbiota and even the intestinal microbiota of the people in the same region may show differences [25, 26]. This may account for the different mean NGAL values.

Another finding in our study was that serum NGAL levels are significantly increased in CD patients with colonic involvement and ileo-colonic involvement compared to ileal involvement. Similarly, UC patients with expanded colonic involvement, as in pancolitis, had higher serum levels of NGAL compared to patients with more localized disease. However, difference in NGAL levels was insignificant among the disease phenotypes (stricturing,

penetrating, non-stricturing, non-penetrating) in CD. This suggest that the disease phenotype does not have a direct effect on the serum NGAL levels. Some previous studies in the literature have also reported similar findings [5, 18].

ADMA and SDMA have been increasingly recognized as toxic non-proteinogenic amino acids in a wide range of human diseases over the past decades [8]. Emerging clinical and experimental evidence indicates that ADMA and SDMA are involved in the pathophysiology of endothelial dysfunction, atherosclerosis, oxidative stress, inflammation, and impaired immunological function [8]. The most well-known effect of ADMA and SDMA at pathological concentrations is the inhibition of NO production. [7-*10*]

In the past studies, it has been reported that the endothelial dysfunction in IBD patients is associated with the decreased NO-dependent dilation and that the diminished microvascular perfusion contributes to the impaired wound healing and persistent chronic inflammation [2, 27].

In the study conducted by Owczarek et al. [28] on 31 UC and 32 CD patients, it was reported that serum ADMA and SDMA concentrations in the IBD group were significantly increased compared to the control group; and in addition, both levels were also significantly higher in the active UC and CD groups compared to the inactive UC and CD groups. The authors also noted that these metabolites correlate with the clinical activity of CD [28[. In our study, such a correlation was observed with both UC and CD activation indices (CCAI, CDAI). Of note, our study includes a quite higher number of patients and evaluated smoking and the drugs used in treatment as well as the subgroups of these patients, the location of the disease, and its relationship with its phenotype.

In a recent study carried out by Korpacka et al. [29], no difference was found in serum ADMA and SDMA levels between the group of IBD patients and the control group. The clinicians further reported that although there was no significant difference in ADMA value

between active *vs* inactive UC and active *vs* inactive CD; SDMA was significantly lower in CD group compared to the control group and active UC group. Give the inherent proinflammatory and pro-oxidative function of SDMA, these results are quite conflicting [8].

The analytical techniques used might have been another reason of such different results obtained in the studies. So far, various analytical methods including high-performance liquid chromatography, gas chromatography-mass spectrometry (MS), liquid chromatography with MS detection, ultra-high performance liquid chromatography (UPLC)-MS/MS, and enzyme-linked immunosorbent assay (ELISA) have been used to quantifiy serum ADMA and SDMA concentrations [8, 30].

While MS-based methods are sensitive, different results are delivered through various MS systems. ELISA methods, on the other hand, tend to overestimate ADMA concentrations. For both ADMA and SDMA only moderate degree of correlation was identified between quantification by ELISA compared with that by UPLC-MS/MS [8, 30]. Standardized analytical techniques are required in order to reliably assess serum ADMA and SDMA concentrations on a routine basis in clinical practice.

Apart from the inclusion of patients with different extent of colonic involvement in the studies [5, 18], another reason why NGAL, ADMA, and SDMA results are conflicting in the differentiation of active and inactivated disease states in IBD patients could be that although exclusion criteria have been strictly applied, the conditions such as viral or bacterial infections, diverticulitis or ischemic colitis might have not been completely excluded. This may have an effect on serum NGAL, ADMA, and SDMA levels. Moreover, although validated in several studies, clinical activity indices such as CCAI and CDAI still have some limitations in detecting and representing the disease activation, their accuracy rates are not precise [31, 32].

Main limitations of our study were the small sample size (especially number of CD patients)

and the lack of endoscopic scoring component of the disease index. Moreover, taking the different mean NGAL values reported in different publications into account, we consider that it would be appropriate to assess regional mean NGAL levels with large scale studies.

Almost all studies demonstrating an association between ADMA or SDMA and clinical diseases have measured blood plasma levels of ADMA or SDMA, and not their tissue levels [7, 8]. Any changes in plasma ADMA levels are not correlated with intracellular ADMA levels in different tissues. The state of activation or inhibition of NOS is, therefore, dependent on the local intracellular L-arginine to ADMA ratio [8]. For this reason, it is important to note that systemic and tissue ADMA levels should be assessed simultaneously to elucidate the relative importance of different mechanisms regulating ADMA homeostasis.

In conclusion, serum NGAL and ADMA and SMDA levels are significantly higher in IBD patients compared to healthy controls and they are also higher in patients with active disease compared to patients with remission when IBD patients are concerned. Serum NGAL, and ADMA levels displayed correlations with several clinical activity indices in the current study. These findings imply that, these parameters may have a potential to be utilized as biomarkers in determining the disease activity in IBD patients. Having said that, future studies to be conducted with a larger number of patients and in diverse patient populations will guide us to pinpoint their utilization in the era.

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Tables:

Table 1. Analysis of demographics, and laboratory parameters in active IBD, inactive IBD and control groups

Parameters	Inactive IBD	Active IBD	Control	P*	Inactive *vs* Active IBD p
Age (years)	44.57 ± 11.88	41.25 ± 15.80	44.94 ± 7.86	0.1	0.4
Female/ Male	25/26	29/31	34/36	0.9	-
†Duration of Disease (m)	66.00(13.75-93.00)	43.00(3.00-78.00)	-	0.04	-
BMI (kg/m^2)	25.93 ± 4.22	25.97 ± 4.10	24.33 ± 3.34	0.2	-
Leukocytes ($x10^3/\mu L$)	7.07 ± 1.67[a]	8.92 ± 2.67[b]	5.91 ± 1.78	0.0001	0.0001
†CRP (mg/L)	4.93(4.19-10.49)	17.11(8.01-35.8)	4.22(3.00-5.00)	0.0001	0.0001
ESR (mm/h)	10.09 ± 6.64[a]	25.08 ± 12.36[b]	4.62 ± 3.45	0.0001	0.0001
NGAL (ng/mL)	114.29 ± 31.74	176.72 ± 51.04[b]	107.50 ± 23.62	0.0001	0.0001
ADMA (µmol/L)	0.124 ± 0.099	0.165 ± 0.038[b]	0.110 ± 0.035	0.0001	0.0001
SDMA (µmol/L)	0.124 ± 0.41	0.165 ± 0.46[b]	0.117 ± 0.18	0.0001	0.0001
GFR (mL/min)	97.20 ± 16.43	100.50 ± 18.68	98.25 ± 15.80	0.5	-
Iron (mg/dL)	70.48 ± 31.68[a]	47.00 ± 27.38[b]	88.02 ± 32.17	0.0001	0.0001
Ferritin (ng/mL)	52.92 ± 22.48[a]	53.30 ± 26.50[b]	99.42 ± 30.67	0.0001	1
Smoking					
Smoker	12	15	18	0.9	-
Non-smoker	26	31	35		
Former smoker	13	14	17		

Results were presented as mean±SD, and † median and interquartile range (Q1-Q3). m, month; BMI, body mass index; ESR, erythrocyte sedimentation rate; CRP, C- reactive protein; GFR, glomerular filtration rate. P^*, intragroup statistical p value; a= Inactive IBD *vs* Control $p< 0.05$; b= Active IBD *vs* Control $p< 0.05$

Table 2. Comparative analysis of active ulcerative colitis vs inactive ulcerative colitis and active Crohn's disease vs inactive Crohn's disease

	Active UC	Inactive UC	Active CD	Inactive CD	Control	p
Age (year)	43.09 ± 17.41	45.79 ± 11.36	38.85 ± 13.36	38.00 ± 13.24	44.94 ± 7.86	0.09
BMI	25.5 ± 3.40	26.05 ± 3.41	26.48±4.89	25.33 ± 7.54	24.33 ± 3.34	0.07
Leukocytes ($x10^3/\mu L$)	8.98 ± 2.29[abc]	7.28 ± 1.72	8.86 ± 3.15[de]	5.90 ± 0.58	5.91 ± 1.78	0.001
NGAL (ng/mL)	178.61 ± 63.91[abc]	114.87 ± 34.43	174.2 ± 27.32[de]	111.18 ± 8.23	107.50 ± 23.62	0.0001
ADMA (µmol/L)	0.157 ± 0.047[abc]	0.104 ± 0.03	0.175 ± 0.020[de]	0.118 ± 0.002	0.110 ± 0.035	0.0001
SDMA (µmol/L)	0.156 ± 0.052[abc]	0.124 ± 0.044	0.177 ± 0.036[de]	0.120 ± 0.002	0.117 ± 0.018	0.0001
†CRP(mg/L)	16.00(7.25-28.25)[abc]	5.23(3.19-10.43)	18.10(9.01-44.01)[de]	4.30(3.19-10.39)	3.00(2.00-4.00)	0.0001
ESR (mm/h	26.08 ± 15.32[abc]	10.72 ± 6.92	23.76 ± 16.89[de]	6.75 ± 3.49	5.62 ± 3.45	0.0001

Results were presented as mean±SD, and † median and interquartile range (Q1-Q3). m, month; BMI, body mass index; ESR, erythrocyte sedimentation rate; CRP, C- reactive protein; GFR, glomerular filtration rate

a= active UC *vs* inactive UC, b= active UC *vs* inactive CD, c= active UC *vs* control, d= active CD *vs* inactive CD, e= active UC *vs* control, $p< 0.05$ denotes statistical significance

Table 3. Analysis of the relation of serum NGAL, ADMA, and SDMA levels with disease characteristics in IBD patients

	N	NGAL	p	ADMA	p	SDMA	p
Ulcerative Colitis (UC)	77						
Location of UC			0.0001*		0.0001*		0.0001*
Proctitis	11	109.44 ± 44.02		0.106 ± 0.047		0.116 ± 0.023	
Proctosigmoiditis	44	122.65 ± 41.75	0.2	0.111 ± 0.034	0.7	0.123 ± 0.061	0.7
Left-sided colitis	10	205.72 ± 52.25	0.0001ab	0.186 ± 0.045	0.0001a	0.193 ± 0.057	0.0001ab
Pancolitis	12	196.22 ± 53.27	0.0001ab	0.157 ± 0.035	0.003b	0.181 ± 0.025	0.0001ab
CCAI Classification			0.001*		0.003*		0.02*
Remission	45	122.58 ± 41.51		0.114 ± 0.043		0.134 ± 0.055	
Mild	25	165.53 ± 72.18	0.01c	0.138 ± 0.046		0.137 ± 0.043	0.8
Moderate	5	194.41 ± 52.86	0.03c	0.176 ± 0.026	0.01c	0.171 ± 0.021	0.03 c
Severe	2	192.90 ± 5.89	0.02c	0.178 ± 0.002	0.01c	0.180 ± 0.028	0.02 c
Truelove-Witts Classification			0.004*		0.001*		0.14*
Remission	43	122.16 ± 40.97		0.109 ± 0.033		0.129 ± 0.044	
Mild	18	163.37 ± 79.51	0.05c	0.140 ± 0.063	0.07	0.140 ± 0.07	
Moderate	12	177.02 ± 59.13	0.01c	0.160 ± 0.038	0.03c	0.161 ± 0.037	
Severe	4	173.63 ± 22.52	0.01c	0.165 ± 0.015	0.03c	0.167 ± 0.022	
Location of CD	34		0.1*		0.07*		0.8*
Colon	5	150.50 ± 43.21		0.154 ± 0.032		0.168 ± 0.051	
Ileum	13	146.52 ± 25.89		0.149 ± 0.028		0.157 ± 0.047	
Ileum-colon	16	172.66 ± 38.81		0.174 ± 0.027		0.167 ± 0.030	
CDAI Score			0.0001*		0.0001*		0.0001*
<150	8	111.18±8.23		0.118 ± 0.002		0.120 ± 0.002	
150-219	18	168.17±20.54	0.0001d	0.168 ± 0.019d		0.158 ± 0.016	0.0001 d
>220	8	187.90±36.57	0.0001d	0.190 ± 0.014d		0.220 ± 0.031	0.0001 d
Phenotype of CD			0.22*		0.19*		0.16*
Non-stricturing, non-penetrating	21	160.03 ± 21.39		0.164 ± 0.025		172 ± 0.044	
Stricturing	6	139.42 ± 4.270		0.142 ± 0.029		0.138 ±0.020	
Penetrating	7	174.66 ± 58.38		0.170 ± 0.039		158 ± 0.031	

Treatment			0.2*		0.6		0.9*
5-ASA	66	141.78 ± 53.31		0.136 ± 0.044		0.144 ± 0.049	
5-ASA+Steroids	19	144.44 ± 52.22		0,132 ± 0,047		0.148 ± 0.060	
5-ASA+immunosuppressive	3	144.30 ± 29.02		0.126 ± 0.027		0.138 ± 0.023	
5ASA+Steroids+ immunosuppressive	12	176.72 ± 60.21		0.149 ± 0.044		0.154 ± 0.040	
Anti TNFα	11	161.51 ± 46.24		0.152 ± 0.052		0.149 ± 0.041	

CCAI, clinical colitis activity index; CDAI, Crohn's disease activity index; 5-ASA; 5-aminosalicylic acid; TNFα,Tumor necrosis factor. *= intragroup statistical (ANOVA) p value, a= p value of proctitis, and proctosigmoiditis *vs* left-sided colitis, b= p value of proctitis, and proctosigmoiditis *vs* pancolitis, c= p value of comparison against remission, d= p value of comparison against CDAI<150, p< 0.05 denotes statistical significance

Table 4. Correlation analyses for serum NGAL concentration

	Pearson's Correlation Analysis		Linear Regression Analysis	
Parameters	**r**	**p**	**β**	**p**
Age	0.043	0.56		
Duration of Disease	-0.122	0.20		
Leukocytes	0.479	0.0001	0.239	0.0001
ESR	0.583	0.0001	0.210	0.001
CRP	0.508	0.0001	0.154	0.01
Iron	-0.363	0.0001	0.009	0.8
Ferritin	-0.224	0.002	-0.020	0.6
Disease Activity Status	0.597	0.0001		
CCAI Classification	0.419	0.0001		
Truelove-Witts Classification	0.378	0.001		
CDAI Score	0.736	0.0001		
Phenotype of CD	0.095	0.5		
Location of UC	0.356	0.0001		

r, correlation coefficient; p, significance; β, standardized coefficient*;* CCAI, clinical colitis activity index; CDAI, Crohn's disease activity index; ESR, erythrocyte sedimentation rate; CRP, C- reactive protein; p value < 0.05 accepted as statistically significant.

Table 5. Correlation analyses for serum ADMA concentration

	Pearson's Correlation Analysis		Linear Regression Analysis	
Parameters	**r**	**p**	**β**	**p**
Age	0.059	0.4		
Duration of Disease	0.154	0.3		
Leukocytes	0.293	0.0001	0.140	0.04
ESR	0.317	0.0001	0.397	0.0001
CRP	0.222	0.003	0.110	0.1
Iron	-0.243	0.0001	0.060	0.9
Ferritin	-0.203	0.006	-0.42	0.5
Disease Activity Status	0.515	0.0001		
CCAI Classification	0.407	0.0001		
Truelove-Witts Classification	0.448	0.0001		
CDAI Score	0.825	0.0001		
Phenotype of CD	0.04	0.9		
Location of UC	0.502	0.0001		

r, correlation coefficient; p, significance; β, standardized coefficient; CCAI, clinical colitis activity index; CDAI, Crohn's disease activity index ; ; ESR, erythrocyte sedimentation rate; CRP, C- reactive protein; p value < 0.05 accepted as statistically significant.