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ORIGINAL ARTICLE

Relationship Between Neutrophil Gelatinase-Associated Lipocalin (NGAL) Levels and Inflammatory Bowel Disease Type and Activity

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Abstract
Background and Aim Neutrophil gelatinase associated lipocalin (NGAL) is a recently identified molecule, which is bacteriostatic, has tissue destructive effects and is pro-inflammatory with chemoattractant molecule binding properties. Our aim was to investigate the relationship between serum NGAL levels and the type and level of disease activity of IBD.
Methods A total of 92 patients [43 with Crohn's disease (CD) and 49 with ulcerative colitis (UC)], and 30 age- and sex-matched healthy controls (HC) were included in this study. Serum NGAL levels were measured using ELISA.
Results Serum NGAL levels were elevated in the IBD group [median 171, range (57–312) ng/mL] compared to the HC group [107 (45–234) ng/mL] ($p < 0.0001$) and were elevated in UC patients [188 (74–312) ng/mL] compared to CD patients [168 (57–279) ng/mL] ($p = 0.006$). When NGAL levels were further analysed based on localization of the CD and UC, the levels in ulcerative pancolitis [233 (144–312) ng/mL] were significantly higher ($p = 0.004$) than the left-sided colitis [156 (103–309) ng/mL]. Similarly, NGAL levels were significantly higher in colonic CD [207 (125–249) ng/mL] than ileal CD [114 (78–210) ng/mL], and also in ileocolonic CD [198 (57–279) ng/mL] than ileal CD ($p = 0.033$). When CD and UC groups were further categorized as active and inactive according to clinical and endoscopic activity indices, serum NGAL concentrations did not differ between inquiescent versus active stages. When a cut-off level of 129 ng/mL was used to distinguish IBD from HC, a sensitivity of 76.1 % and a specificity of 60.9 % was reached.
Conclusions The serum NGAL levels in the IBD group was significantly higher than the HC group. Serum NGAL levels were higher in more extensive colonic involvement.

Keywords Crohn's disease · Ulcerative colitis · Neutrophil gelatinase associated lipocalin · Inflammatory bowel disease

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Introduction

Inflammatory bowel disease (IBD) is composed of a group of inflammatory diseases in genetically predisposed people that have a chronic course and are characterized by episodes of remission and exacerbation. The cause and mechanism of IBD are not yet fully understood. IBD includes both ulcerative colitis (UC) and Crohn's disease (CD) [1, 2]. In these patients, many clinical activity indicators and non-invasive markers have been used to evaluate disease activity and determine treatment. However, none of these tests have produced definitive results compared to histopathological and endoscopic examinations [3, 4].

Neutrophil gelatinase-associated lipocalin (also known as NGAL, lipocalin 2, siderocalin, 24p3 or LCN2), which is mainly derived from neutrophils in circulation, is

expressed in many tissues at low levels [5]. However, its synthesis is increased in damaged intestinal and colonic epithelial cells, respiratory cells, renal tubule cells and hepatocyte endothelial cells in response to inflammatory signals [6–9]. Microorganisms require iron in order to proliferate and they meet this need mainly by synthesizing siderophores [10, 11]. Siderophores are strong iron chelators and contribute significantly to the process by which iron is captured from the surrounding medium and utilized by bacterial cells for metabolism [11]. NGAL sequesters iron in the medium by binding to iron-loaded siderophores and thus produces a bacteriostatic effect by removing iron needed by microorganisms [11, 12]. Currently, it is theorized that NGAL released from neutrophils exerts a bacteriostatic effect by blocking the uptake of iron required for bacterial proliferation and functions as one of the key molecules of the natural immune system [12].

The synthesis and secretion of NGAL is increased in colonic epithelia that are inflamed [13]. It reflects neutrophil activation and has bacteriostatic and matrix degradation properties. It also has the capability to prevent neutrophil chemoattraction [14]. Thus, NGAL has been shown to play an important role in inflammatory diseases of the colon [13–15]. Since NGAL is resistant to protease digestion and is synthesized by colonic epithelia, previous studies have investigated the measurement of fecal NGAL levels as a diagnostic test for various colonic diseases [14]. Although NGAL shows promise as an early marker of inflammatory conditions, studies investigating its use in IBD are limited. Moreover, there are insufficient studies on the use of serum levels of this molecule in determining IBD diagnosis and activity. Since NGAL is seen as an important pathogenic molecule in inflammatory diseases of the colon, the aim of this study was to investigate the utility of serum levels of NGAL in determining the diagnosis and activity of IBD.

Materials and Methods

This study was a prospectively controlled study. In total, 92 patients (43 diagnosed with CD and 49 diagnosed with UC) who had been examined and treated between January 2011 and April 2012 in the Gastroenterology Clinic of Haydarpasa Numune Education and Research Hospital were included in the study. The patients had been definitively diagnosed with IBD clinically, endoscopically, radiologically and histopathologically and had a GFR level that was higher than 90 mL/min and were between the ages of 18 and 70. Thirty healthy controls (HC), who were matched in terms of age and gender, were also included in the study. HC were selected among subjects who applied to our hospital for their routine check-up and had no known inflammatory diseases.

The patients' ages and genders, disease type and duration, bowel area affected by disease, the extent of the disease, drugs used for IBD treatment and the history of surgery for IBD were recorded. The results of routine blood tests and CRP and ESR tests, which are inflammatory markers, were retrieved from the records stored in the hospital databank.

The endoscopic examination and blood withdrawal were performed on same day. Only two authors of the manuscript performed the endoscopic examinations, but unfortunately we did not perform video records of the examinations and also did not perform the analysis of the intra- or inter-observer variability. This could be an important limitation of our study and could affect our results. Clinical activity in patients diagnosed with UC was calculated by using the Truelove–Witts criteria [16] and clinical colitis activity index (CCAI) [17]. Endoscopic activity was classified using the endoscopic Mayo score [18]. Patients with UC and a CCAI index score of ≤ 4 were classified as having inactive disease and patients whose endoscopic Mayo score was 0 or 1 were classified as being in remission. In CD cases, the Crohn's disease activity index (CDAI) was used [19]. CD patients with a CDAI score of less than 150 were considered to have inactive disease. Patients with active CD were further classified as having disease with mild activity (CDAI of 150–220), moderate activity (CDAI of 221–450) and severe activity (CDAI > 450).

Laboratory Tests

Venous blood was collected from the patient and control groups after 10–12 h of overnight fasting (to eliminate lipemia as a potentially confounding factor) into EDTA, sodium citrate and gel tubes (Becton–Dickinson, USA). After waiting for 30 min, the gel tubes were centrifuged at 3,500 rpm ($1{,}300 \times g$) for 10 min. The complete blood count (CBC), CRP and ESR were measured immediately. A CBC was measured in all patients and controls in blood samples containing EDTA. The ESR was measured in blood samples stored in the sodium citrate tube using the Westergreen method with a Sed Rate Screener 100 (SRS 100, Greiner Bio-one GmbH, Austria) instrument. The serum CRP levels were determined using the nephelometric method (Immage, Beckmann Coulter, USA).

Serum samples were divided into two portions and stored at -70 °C until the experimenters were ready to measure NGAL levels. Frozen samples were thawed and the measurements were done immediately before analysis. Repeated freezing and thawing was avoided. Serum lipocalin-2/NGAL levels were measured using the sandwich-type enzyme-linked immunosorbent assay kit (ELISA; BioVendor R&D, Czech Republic), which

is commercially available, following manufacturer's instructions.

Statistical Analysis

Non-parametric tests were used to evaluate data that was not normally distributed. In addition to descriptive statistical methods (median, interquartile range 25–75 %), the Kruskal–Wallis test was used for intergroup comparison of quantitative data, and the Mann–Whitney *U* test was used to determine which group contributed most strongly to intergroup differences. The non-parametric Spearman test was performed for correlation analyses of parameters. In order to evaluate data obtained from the study, the IBM Statistical Package for Social Sciences (SPSS) Statistics 20 program was used for statistical analyses. Sensitivity, specificity and cut-off values were calculated with receiver operating characteristics (ROC) curve analysis by using the MedCalc statistical software 12.2.1 (MedCalc, Inc., Mariakerke, Belgium) program. The results were evaluated using a 95 % confidence interval and $p < 0.05$ as the level for significance.

Results

In total, 122 subjects (92 patients and 30 HC) between the ages of 16–74 [62 females (50.8 %) and 60 males (49.2 %)] were included in the study (Table 1). Patients were divided into the IBD study groups [CD ($n = 43$), UC ($n = 49$)] and control group ($n = 30$). The IBD group and HC were found to be similar in terms of average age and gender ratios. The UC and CD sub-groups were shown to be similar to the control group in terms of age and gender distribution.

When all IBD patients were considered as a single group and compared with the control group, NGAL levels in the IBD patients group [median 171 (57–312) ng/mL] were found to be significantly higher than those of the control group [107 (45–234) ng/mL] ($p < 0.0001$). In the comparison among control [107 (45–234) ng/mL], CD [168 (57–279) ng/mL] and UC [188 (74–312) ng/mL] groups, NGAL levels were found to be statistically different ($p < 0.0001$) (Table 2).

When the NGAL levels were compared in the Crohn's and UC patient groups according to disease location, the NGAL levels were significantly different ($p = 0.006$ and $p = 0.006$, respectively). In the sub-group analysis of the CD patients, the NGAL level in the colonic type [207 (125–249) ng/mL] was significantly different than the ileal type [114 (78–210) ng/mL] ($p = 0.006$). Additionally, there was a significant difference in the NGAL levels of CD patients with ileocolonic disease [198 (57–279) ng/mL] compared to ileal disease ($p = 0.033$). In the UC subtypes, there was a significant difference in the NGAL levels between the pancolitis group [233 (144–312) ng/mL] and left colonic group [156 (103–309) ng/mL] ($p = 0.004$). No significant differences were found with the proctitis group, probably because only four patients were in this group, thus making statistical comparison difficult (Table 3).

When the UC group was compared according to the CCAI classification scores, a significant difference was not

Table 1 Demographic characteristics of the study population

Characteristic	HC	IBD	CD	UC
Patients, *n*	30	92	43	49
Gender, male/female	14/16	46/46	19/24	27/22
Median age (range), years	41.5 (24–61)	36 (16–74)	35 (19–63)	36 (16–74)
Median BMI (range), kg/m^2	25.9 (23.1–28.3)	22.4 (20.2–24.9)	21.9 (19.5–25.2)	22.55 (20.85–24.15)

HC healthy controls, *IBD* inflammatory bowel disease, *CD* Crohn's disease, *UC* ulcerative colitis, *BMI* body mass index

Table 2 Laboratory analyses of patients and controls

Measurement	HC ($n = 30$)	IBD ($n = 92$)	CD ($n = 43$)	UC ($n = 49$)
NGAL (ng/mL)	107 (74–159)	171 (127–234)	168 (105–207)	188 (142–264)
WBC (K/mm^3)	6.90 (5.60–8.70)	7.7 (6.3–9.29)	7.0 (5.9–8.75)	9.0 (7.4–10.0)
CRP (mg/L)	0.75 (0.3–0.9)	1.15 (0.32–2.72)	1.56 (0.54–3.3)	1.09 (0.29–1.92)
ESR (mm/s)	13.5 (11–16)	29 (17.0–45.8)	35 (16.5–52.5)	28 (17.5–43.0)

Data are presented as median (IQR)

NGAL neutrophil gelatinase-associated lipocalin, *WBC* white blood cells, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *IBD* inflammatory bowel disease

Table 3 Serum NGAL levels in IBD classified by potential categorical covariables

Variable	*N*	NGAL (ng/mL)	*P* value
Crohn's disease			
Disease location			
Colonic	7	207 (157–243)	0.006*
Ileal	16	114 (88–163.5)	
Ileocolonic	17	198 (149–230)	
CDAI			
<150	22	148.5 (102–220.5)	0.739
150–219	9	186 (106–199.5)	
≥220	7	192 (144–201)	
Disease behavior			
Non-stricturing, non-penetrating	21	172.5 (125–230)	0.189
Stricturing	11	139.5 (98–188)	
Penetrating	9	190.5 (102–206)	
Ulcerative colitis			
Disease location			
Proctitis	4	122 (74–302)	0.006*
Left-sided colitis	27	156 (133–240)	
Pancolitis	18	233 (161–302)	
CCAI			
<0–4	21	185.5 (137–284)	0.874
5–10	15	222.5 (144–260)	
11–17	8	131 (121–211)	
≥18	5	170 (151–276)	
Truelove–Witts index			
0	4	188 (183–302)	0.122
1	12	233 (156–294)	
2	22	144 (122–244)	
3	11	201 (151–293)	
Mayo score			
0	5	161 (130–232)	0.246
1	4	277 (168–309)	
2	23	156 (131–236)	
3	17	188 (147–302)	

NGAL neutrophil gelatinase-associated lipocalin, *IBD* inflammatory bowel disease, *CCAI* clinical colitis activity index

Data are presented as median (IQR)

* Statistically significant

found between serum NGAL levels and disease activity. A correlation was not seen between serum NGAL levels and CCAI (Spearman's r value = 0.069; $p = 0.638$) (Table 3). Additionally, no significant correlation was found between the Truelove–Witts criteria and endoscopic Mayo scores and the serum NGAL levels. In the CD group, there was no significant relationship between the degree of disease activity, represented by the CDAI clinical activity index, and the serum NGAL levels. A correlation was not seen between serum NGAL levels and CDAI and [Spearman's r values 0.142 ($p = 0.396$)] (Table 3).

No significant differences were found between the disease type in the CD group (structuring, penetrating, non-stricturing, or non-penetrating type) and the serum NGAL levels (Table 3).

When the NGAL levels in the control group [107 (45–234) ng/mL] were compared to those in the active [178.5 (78–312) ng/mL] and inactive [168 (57–309) ng/mL] IBD groups, no significant difference was found in the serum NGAL levels. In these comparisons, the disease activity classifications of the UC and CD groups were done by using CCAI and CDAI, respectively. When the CD and UC groups were classified as active and inactive according to CDAI and CCAI, respectively, there was no significant difference between the serum NGAL levels and disease activity.

In the correlation analysis between various laboratory and phenotypic parameters and serum NGAL levels in CD, UC and control groups, a weak positive relationship was seen only with thrombocyte count in CD (r 0.345, $p < 0.05$).

When the ROC curve was graphed to investigate the diagnostic value of serum NGAL for distinguishing IBD from the control group, the most suitable cut-off value with a 95 % confidence interval was ≥129 ng/mL and the sensitivity and specificity were found to be 76.1 % (66.1–84.4 %) and 60.9 % (45.4–74.9 %), respectively [AUC: 0.720 (0.638–0.793), $p < 0.005$] (Table 3).

Discussion

It seems reasonable to use inflammatory markers for the purpose of diagnosis and determination of disease activity in IBD patients, in which the main pathogenic mechanism is intestinal inflammation. The synthesis and secretion of NGAL increases in inflamed colonic epithelia, has immunoregulatory functions and reflects neutrophil activation. In this study that investigated the use of NGAL in diagnosis of IBD and evaluation of disease activity, the serum NGAL levels in the IBD patient group were found to be significantly higher than in the control group. When the UC and CD patient groups were compared to HC, there continued to be a significant difference in NGAL serum levels.

As a result of this study, which showed that serum NGAL levels in both UC and CD groups were higher than in HC, it can be inferred that serum NGAL can be helpful in the diagnosis of IBD patients. When a NGAL cut-off value of ≥129 ng/mL is used, sensitivity and specificity were found to be 76 and 61 %, respectively. Based on these values, it is thought that serum NGAL measurements, although not perfect, may be able to be used in conjunction with other methods for diagnosis.

While this study was being conducted, Oikonomou et al. [15] reported a similar study with the same methodology. In their study, IBD patients were compared with HC and patients with irritable bowel syndrome, and the serum NGAL levels in the IBD group were found to be significantly higher. According to these results, a cut-off value of 75 ng/mL was 95 % sensitive and 83 % specific for distinguishing IBD patients from HC. Moreover, the mean serum NGAL level of HC in their study was reported to be 60.80 ± 20.30 [15]. This value was markedly lower than the mean ± SD and median values (117 ± 53 and 107 ng/mL, respectively) in the control group in the present study. In another study measuring serum NGAL levels, the median value of HC was reported to be 24.1 ng/mL [14]. Surprisingly, among the three studies, there is a marked difference in the mean values of serum NGAL levels in healthy individuals. Factors contributing to these differences could be the use of different kits to measure NGAL levels, different groups performing the tests, racial factors, differences in blood collection times and differences in transport and processing of serum samples. Another factor that contributes to our different results from aforementioned studies is that two different persons performed the endoscopic examinations of the patients, and we did not record videos of the examinations so we did not perform the intra- or interobserver variability. Recently, in literature it was reported that in different regions people has different gut microbioata. Even in the same region, every person has his/her own gut microbiota which differ from others [20–23]. This fact also could have affected our different results. In the literature, it is emphasized that serum NGAL levels increase with age [24, 25]. Thus, the age of the population being tested seems also to be an important factor. Regardless of the underlying reason, marked differences among the studies lead to obvious differences among serum NGAL cut-off levels recommended for use in disease diagnosis, thus preventing the determination of one global cut-off value that can be used around the world.

In one study that investigated serum NGAL levels, it was reported that there was no difference between UC and CD patients and that serum NGAL was ineffective at reflecting disease activity [13]. Due to the fact that the number of patients in that study was low (14 CD patients, 23 UC patients and 20 HC) and that the primary objective of the study was not to investigate serum NGAL levels, it is more difficult to draw conclusions from those results. However, in the study by Oikonomou et al. [15], although there was no difference between serum NGAL levels in the UC and CD groups, the serum NGAL level was found to be helpful in distinguishing active and inactive disease in both groups. In our study, serum NGAL levels in the UC patient group were found to be significantly higher than those in the CD group. It is possible that the differences seen in our study were not identified in the earlier studies due to the low number of patients in the first study discussed above and because of the fact that there were only four patients with isolated colon involvement in the second study. Similar to our study, Oikonomou et al. [15] reported that the serum NGAL was more effective in the UC group. It is not surprising that the serum NGAL levels were found to be lower in the CD group because the colonic involvement in the CD group was more limited compared to the UC group.

Another important finding in this study is that there was a significant difference between serum NGAL levels and disease location in both the CD and UC groups. In the UC patient group, serum NGAL levels were found to be significantly higher in pancolitis than in disease restricted to the left colon. However, the fact that a difference was not found between proctitis and other locations was attributed to the low number of patients with proctitis in this study (4 patients). In CD, the serum NGAL level was found to be significantly higher in patients with colon involvement and ileocolonic involvement compared to patients with ileal involvement and only ileal involvement, respectively. These findings support the data found in previous studies and highlight that NGAL, which is a specific neutrophil marker, increases in areas of local inflammation and reflects the size of the inflammatory response. However, in the comparison done according to disease phenotype (stricturing, penetrating, non-stricturing, or non-penetrating), no significant difference was found among groups in terms of serum NGAL levels. This result indicates that disease phenotype does not significantly affect serum NGAL levels.

It was found in this study that serum NGAL levels were not effective in distinguishing inactive disease from active disease. After a detailed examination of patient data, it was found that although serum NGAL levels in both patients with active and inactive disease were higher compared to the control group, there was a wide distribution of data (Table 3). Thus, there were patients with similar NGAL values in both the active and inactive disease groups. Similar to the present study, the study by Nielsen et al. [13] found that serum NGAL levels were ineffective in distinguishing active from inactive disease. In the study by Oikonomou et al., serum NGAL levels were helpful in distinguishing active disease from inactive disease and were also useful in distinguishing disease severity when compared with indexes such as CCAI and CDAI. It has previously been frequently reported that the CDAI and CCAI indexes contain subjective criteria and neither are completely reliable [26–28]. The findings in the study by Oikonomou et al. [15] may be due to more careful selection of patient groups or may be the result of other limitations or obvious differences in serum NGAL measurements as described above. Moreover, the fact that there is no gold standard test for serum NGAL values and that none of the

scoring systems currently in use are highly reliable lead to difficulties in every study that investigates the use of cytokine or serum marker for IBD disease activity determination. The results of this study suggest that serum NGAL levels may not be the ideal marker to distinguish active from inactive disease and to determine IBD activity.

The fact that cigarette smoking has negative effects in CD and protective effects in UC indicates that there are different pathogenic mechanisms in these two subtypes of IBD. In this study, the effect of cigarette smoking on serum NGAL level was investigated and it was shown that serum NGAL levels were not affected by smoking in both the UC and CD groups. This result was confirmed by another recently published study [15].

In the literature, it has been reported that drugs used by IBD patients and the effects of these drugs on inflammation can affect serum NGAL levels [15, 29]. In this study, as has been found in past literature, the serum NGAL levels were significantly lower in patients using 5-ASA [15]. We examined this result in light of disease severity or extent of involvement in the patients solely taking 5-ASA. When all the data were combined, it was found that there is a relationship between the extent of colonic involvement and the low serum levels in patients using drugs with a low immunoregulatory effect due to mild disease. However, as was found in past studies, no utility could be found for NGAL and disease activity determination. Taken together, these data suggest that there are many factors specific to the patient and disease pathogenesis that can affect serum NGAL levels.

NGAL was found in this study to increase in IBD patients in a manner that seemed to be related to the colonic inflammatory load. However, before serum NGAL can be used for diagnostic purposes, it is necessary to define it's a diagnostic cut-off value based on a study that evaluates large patient groups. It is also important to establish a normal range for NGAL in the healthy population as well as a standard method for measuring NGAL. A consensus on the relationship of NGAL to disease activity needs to be reached, given the differing results in past literature, by future studies involving larger numbers of patients.

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Conflict of interest None.

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