



Assessing the activity of Inflammatory Bowel Disease using the serum marker: Neutrophil Gelatinase Associated Lipocalin (NGAL)

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Cover Page Footnote

We would like to thank all staff members, colleagues and employees in IBD clinic Mansoura specialized medical Hospital & laboratories of the Clinical pathology department of Mansoura Faculty of medicine.

ORIGINAL STUDY

Assessing the Activity of Inflammatory Bowel Disease Using the Serum Marker: Neutrophil Gelatinase Associated Lipocalin

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Abstract

Background: IBD, or inflammatory bowel disease, is an active and remitting illness. One of the most overexpressed genes in the colonic mucosa of patients with ulcerative colitis (UC) and Crohn's disease (CD) is lipocalin 2, the coding gene of neutrophil gelatinase associated lipocalin (NGAL). We investigated NGAL's applicability in assessing IBD activity.

Methods: This study was a single center case control type. Thirty of the 60 IBD patients who participated in the study were in remission, and 30 were in active disease. A 28 healthy controls were included. Enrolled were patients with CD or UC. The control group and all patients underwent ELISA testing for serum NGAL, erythrocyte sedimentation rate, C-reactive protein, and complete blood count. Patients received ileo-colonoscopy along with fecal calprotectin. The CDAI (Crohn's disease activity index) for CD and the MAYO score for UC were used to assess the IBD activity.

Results: The mean \pm SD ng/ml (38.01 ± 9.52) of NGAL was significantly higher in patients with active IBD than in those in remission (21.14 ± 4.22). It revealed a highly significant correlation with IBD's endoscopic and clinical activity. Serum NGAL exhibits 100 % sensitivity and 100 % specificity in its ability to differentiate between healthy controls and patients with active IBD, with an area under the curve 95 % confidence interval = 1.00 (0.94–1.0) at the cutoff of 18.53, *P* less than 0.0001.

Conclusion: Patients with active IBD can be easily distinguished from healthy controls using serum NGAL. Moreover, it can distinguish between IBD active and remittent patients. In comparison to other markers like CRP, ESR, or fecal calprotectin, NGAL performs better statistical predictor in terms of IBD activity.

Keywords: Activity and remission, Fecal calprotectin, Inflammatory bowel disease markers, Neutrophil gelatinase-associated lipocalin

1. Background

The autoimmune condition of inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), is characterized by recurrent episodes of chronic inflammation of the intestine. An aberrant and persistent immune response to gut microbes is thought to be

the cause of IBD, which is triggered by an individual's genetic susceptibility. Therefore, it entails a complicated interplay between immune responses and genetic, environmental, or microbial factors (Zhang and Li, 2014).

To assess the patients' disease activity and the effectiveness of their treatment, a variety of non-invasive markers and clinical activity indicators

Acronyms: CD, Crohn's disease; UC, Ulcerative colitis; IBD, Inflammatory bowel disease; Hb, Hemoglobin; MCV, Mean corpuscular volume; NGAL, Neutrophil gelatinase-associated lipocalin; CDAI, Crohn's disease activity index; WBCs, White blood cells; CRP, C reactive protein; FC, Fecal calprotectin.

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have been used. However, when compared with endoscopic and histopathological analyses, none of these tests have yielded conclusive findings (Vilela *et al.*, 2012; Vermeire *et al.*, 2006).

Different tissues express low amounts of neutrophil gelatinase associated lipocalin (NGAL) or lipocalin 2 (LCN 2), which is mostly released by circulating neutrophils (Xu *et al.*, 1994). Because of its capacity to absorb and reduce siderophores released by specific bacteria, it has bacteriostatic effects on infections, which prevent growth of bacteria (Devarajan, 2010).

When compared with healthy individuals, among the genes that are overexpressed in the colonic mucosa of UC and CD patients is LCN 2, the NGAL coding gene. Since NGAL is small (25 kDa), it can be secreted and is relatively stable, making it useful for assessing disease activity and evaluating its impact on the pathophysiology of IBD (Østvik *et al.*, 2013).

Fecal NGAL levels have been measured and used as a diagnostic marker for a number of colonic diseases in numerous previous studies (Devarajan, 2010; Østvik *et al.*, 2013; Nielson *et al.*, 1999). The use of this molecule's serum levels to diagnose and monitor the progression of IBD, however, has not received enough attention from researchers. For this reason, in our study, we looked into the suitability of LCN 2 serum levels in determining IBD activity.

2. Methods

Over the course of a year, from 2021 to 2022, a single center case control study was carried out at the IBD clinic of the specialized medical hospital and the Clinical Pathology department of Mansoura Faculty of Medicine. It was conducted on a convenient sample of 60 IBD patients, of whom 30 were in remission and the remaining 50 % were active. There were 28 healthy control patients. Enrolled were IBD patients with either CD or UC.

This study included patients with a confirmed diagnosis of IBD (UC or CD) based on European Crohn's and Colitis Organization (ECCO) guidelines 2019, who were older than 18 years, either active or in remission, may not be receiving therapy at the time of diagnosis or be receiving it now, and who fell into class I–III of the American Society of Anaesthesiologists.

Patients unable or unwilling to give consent to a colonoscopy or flexible sigmoidoscopy, who had total colectomy, or malignant conditions such as colorectal cancer, or had surgically localised sources of rectum bleeding (piles, fissures, sinuses, etc.) were all excluded. Additionally, patients with chronic renal failure or liver cell failure were

excluded because LCN is expressed at low levels in hepatocytes, endothelial cells, and kidney tubular cells.

Using the following formula, the sample size was determined based on the correlation coefficient. 2013 (Hulley *et al.*, 2013); Hulley *et al.* (0.05) is the desired level of significance. NGAL level would be substantially more common in the IBD group than in the control group, according to our hypothesis. A 90 % power to detect a difference in the group proportions of 0.3372 would be achieved with group sample sizes of 60 IBD patients and 28 control patients. The Z test with pooled variance is a two-sided test statistic that is utilized. The test's significance (α) level was set at 0.050. With this design, the actual significance (α) level attained is 0.037. 1.9600 was the standard normal deviate for α ($Z\alpha$). 1.2816 was the standard normal deviate for β ($Z\beta$).

The history-taking process, comprehensive clinical examination, laboratory testing and other procedures were applied to all patients and the control group. The Clinical Pathology Department of the Mansoura Faculty of Medicine conducted laboratory tests in its laboratories. Complete blood counts (CBCs) using automated hematology analyzers, the Cell-dyn 1700 and Cell-dyn emerald hematology analyzers, hemoglobin (Hb) (gm/dl), red blood cell and white blood cell counts (RBC and WBC) (103/cmm), platelet counts (103/cmm), the Erythrocyte Sedimentation Rate (ESR) monitored by VES-Matic 20, C-reactive protein (CRP) measured by Cobas C311, and serum NGAL assessed by ELISA were among the tests performed.

Serum NGAL assessment by ELISA: 3 ml of venous blood were extracted from the patients and centrifuged for 15 min at 3000 rpm. Serum samples were stored in (-40°C) till the analysis was done. Human LCN-2 (Neutrophil Gelatinase Associated Lipocalin) ELISA Kit (Wuhan Fine Biotech Co., Wuhan, China), Catalogue No. EH0012, was used to measure NGAL levels in subject sera in accordance with manufacturer's instructions (Detection Method: Sandwich ELISA, Double Antibody). Using the supplied dilution buffer, samples were diluted ($\times 10$) so that the diluted target protein concentration fell within the kit's ideal detection range (0.313–20 ng/ml). NGAL concentrations were obtained via Infinite F50 ELISA Reader (TECAN, Männedorf, Switzerland).

In addition to sigmoidoscopy or ileo-colonoscopy using a Pentax PK 100 video scope, patients were exposed to faecal calprotectin through stool samples. In addition to the colonic biopsy's histological inspection, which was done after it was embedded in paraffin and fixed in 10 % formalin solution for

further analysis. Following hematoxylin and eosin staining. IBD activity was measured for UC using the MAYO score (Schroeder *et al.*, 1987) and CD using the CDAI (Best, 2006).

After being informed about the study's procedures, the patients or their family members who were taking part in it provided written informed consent. Every laboratory and endoscopic procedure used in this study was carried out in compliance with all applicable Mansoura University rules and regulations. We obtained informed consent from each participant and/or their legal guardian(s). This study has been approved by the Institution Research Board (IRB) of Mansoura Medical College (MS.20.10.14) in Egypt.

Version 25.0 of the IBM SPSS software program was used to establish data analysis. Utilizing percentages and numbers, the qualitative data was described. The median (lowest and maximum) and interquartile range (ICR) were used to characterize quantitative data that were not normally distributed after the Kolmogorov–Smirnov test was used to confirm normality. Each test comprised two tails. Nonparametric tests: more than two independent study groups were compared using the Kruskal Wallis test, and two groups were compared using the Mann–Whitney *U* test. The correlation between continuous, non-normally distributed data was determined using the Spearman correlation coefficient. A *P* value, or probability value, was deemed statistically significant if it was less than 0.05. The receiver operating characteristics (ROC) curve was used to determine the cutoff.

3. Results

The results of binary logistic regression, which was used to determine the impact of serum NGAL greater than 26.7, fecal calprotectin greater than 154, WBC count greater than 6.6, and MCV less than or equal to 70 on the probability that individuals with IBD will experience active disease, are displayed in this table. There was a statistically significant correlation between all 4 variables and active IBD. Participants with serum NGAL greater than 26.7, fecal calprotectin greater than 154, WBCs count greater than 6.6, and MCV less than or equal to 70 have 126, 36, 5.7, and 8.1-times higher odds to exhibit active disease.

4. Discussion

Neutrophils are the main source of the multi-functional protein called NGAL. It is not only secreted in urine or stool, but it can also be found in

serum. Inflammatory, malignant, and infectious diseases all exhibit high expression of it, including IBD. NGAL is overexpressed in the epithelium of the colon during periods of inflammation. In 2018, it was reported that NGAL is expressed in both the colon and small intestine entero-endocrine cells (Stallhofer *et al.*, 2015).

In the current study, we examined serum NGAL levels in IBD patients with active or remission form of the disease and contrasted them with those of healthy controls. Serum level of NGAL was in active IBD patients (mean \pm SD ng/ml) equal to (38.01 \pm 9.52) which was significantly higher than in patients with remission (21.14 \pm 4.22), with a *P* less than 0.0001 difference (see Tables 1 and 2).

Table 3 indicates that there was a highly significant correlation ($r = 0.8$, $P < 0.0001$) between NGAL and the clinical and endoscopic activity of IBD (Mayo score). With an area under the curve (AUC) of 95 % Confidence interval (CI), serum NGAL exhibits 100 % sensitivity and 100 % specificity in differentiating between patients with active IBD and healthy controls. In Figs. 1 and 2, at the cutoff of 18.53, CI (0.94–1.0) = 1.00, *P* less than 0.0001. The AUC of NGAL, which can differentiate between patients with active IBD and those in remission, was 0.97 with 95 % CI (0.89–0.99), *P* less than 0.0001, and at the cutoff of 26.95, it had a sensitivity of 93.3 % and a specificity of 93.3 %.

Additionally, it was discovered that, at the cutoff of 14.91, the AUC of NGAL for differentiating between remittent IBD patients and healthy controls was 0.95, by a 95 % C.I (0.86–0.99), with sensitivity 93.33 %, and specificity 89.29 % ($P < 0.0001$).

NGAL serum levels in individuals with active IBD were found to be significantly higher (median [IQR], 36.85 [21.19–73.64] ng/mL; $P = 0.01$) compared with healthy controls (24.25 [17.75–35.12] ng/mL). As a result, there was a greater chance that NGAL would accurately reflect the activity of the UC disease with AUC = 0.75, specificity = 0.63, sensitivity = 0.83, and $P = 0.0002$ (Stallhofer *et al.*, 2015). Oikonomou and colleagues (Oikonomou *et al.*, 2012) reported that serum NGAL has a specificity of 68, 84, and 79 %, and a sensitivity of 100, 96, and 95 %, respectively, for differentiating between patients with IBS, healthy controls, and inactive IBD. These results were consistent with the findings of numerous other studies. In 2017, Budzynska *et al.* (2017) discovered nearly identical outcomes. The same results for both serum and fecal NGAL were also noticed (Thorsvik *et al.*, 2018; Abdulganieva *et al.*, 2022).

A distinct investigation, however, did not discover a distinction in serum NGAL concentrations between the quiescent and active phases. Level of

Table 1. Comparisons of the three study groups.

Features	A Group (control) N = 28	B Group (remission) N = 30	C Group (active) N = 30	P-value
Age (y)				*0.888
18–30	11 (39.3 %)	8 (26.7 %)	10 (33.3 %)	
31–40	10 (35.7 %)	11 (36.7 %)	12 (40 %)	
41–50	6 (21.4 %)	8 (26.7 %)	5 (16.7 %)	
>50	1 (3.6 %)	3 (10 %)	3 (10 %)	
Sex				#0.382
Male	19 (67.9 %)	15 (50 %)	17 (56.7 %)	
Female	9 (32.1 %)	15 (50 %)	13 (43.3 %)	
Occupation				#0.002
Working	24 (85.7 %) a	14 (46.7 %) b	14 (46.7 %) b	
Unemployed	4 (14.3 %) a	16 (53.3 %) b	16 (53.3 %) b	
Residence				#<0.001
Rural	3 (10.7 %) a	17 (56.7 %) b	18 (60 %) b	
Urban	25 (89.3 %) a	13 (43.3 %) b	12 (40 %) b	
Current smoking	4 (14.3 %)	4 (13.3 %)	4 (13.3 %)	*1.000
Comorbidities	4 (14.3 %)	3 (10 %)	3 (10 %)	*0.838
Serum NGAL (ng/ml)	13.39 ± 2.04 a	21.14 ± 4.22b	38.01 ± 9.52c	§<0.001

Notes: N (%) of categorical data are compared using either the *Fisher's exact test or the #Chi-square test. Multiple z-tests for occupation and residence are presented as letters (similar = insignificant, different = significant difference). Quantitative data (Serum NGAL) is mean ± SD and are compared by §Welsh ANOVA. Games-Howell post-hoc tests are presented as letters (similar = insignificant, different = significant difference).

Table 2. Comparing various factors between 30 inflammatory bowel disease cases that are active and 30 inflammatory bowel disease cases that are in remission.

Variable Mean ± SD	Active inflammatory bowel disease cases (30 cases)	Remittent inflammatory bowel disease cases (30 cases)
WBCs 10 ³ /cmm	8.32 (±2.45)	7.04 (±2.39)
Hb gm/dl	10.97 (±1.71)	11.623 (±1.49)
MCV (fl)	73.63 (±9.14)	78.62 (±7.59)
Platelets 10 ³ /cmm	281.97 (±82.26)	308.67 (±102.8)
CRP	18.62 (±25.27)	7.733 (±8.67)
ESR	18.93 (±10.75)	16.43 (±7.2)
FC (mg/kg)	266 (±99.6)	122.47 (±43.8)

The terms hemoglobin (Hb), c reactive protein (CRP), erythrocyte sedimentation rate (ESR), mean corpuscular volume (MCV), white blood cells (WBCs), and fecal calprotectin (FC) are related.

serum NGAL (129 ng/ml) was found to be the cut off level with 76.1 % sensitivity and 60.9 % specificity in distinguishing IBD from healthy controls (Yeş et al., 2013).

70 % of the patients in our study were underage of 40 years. 42 % of them were females, and 58 % were males. 54 (90 %) patients had UC; of these, 28 (51.9 %) had a remission MAYO score of 0–1, 26 (48.1 %) had an activity by MAYO score of 2–3 (Table 1).

Table 3. Correlation between various significant factors and neutrophil gelatinase associated lipocalin in the cases under study.

Parameter	Correlation coefficient	P-value
Mayo score*	0.8 (Spearman)	<0.001
ESR	0.298 (Pearson)	0.02
CRP	0.377 (Pearson)	0.003
FC	0.699 (Pearson)	<0.001

Test of significance is Pearson's correlation and *Spearman's correlation.

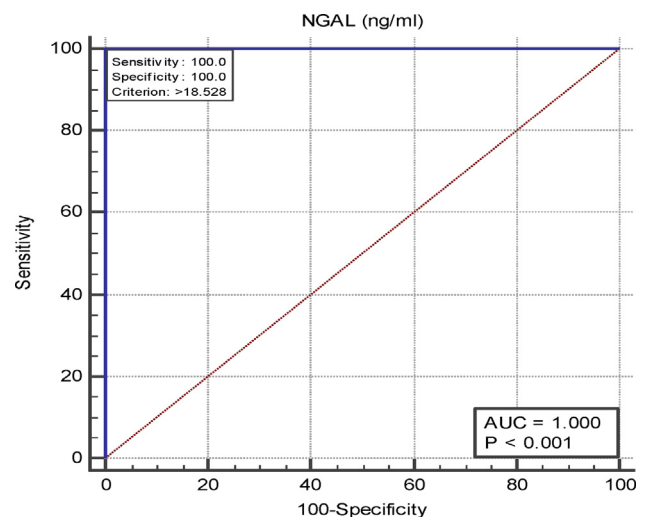


Fig. 1. Cutoff value by receiver operating characteristics curve of serum neutrophil gelatinase associated lipocalin in differentiating active versus control.

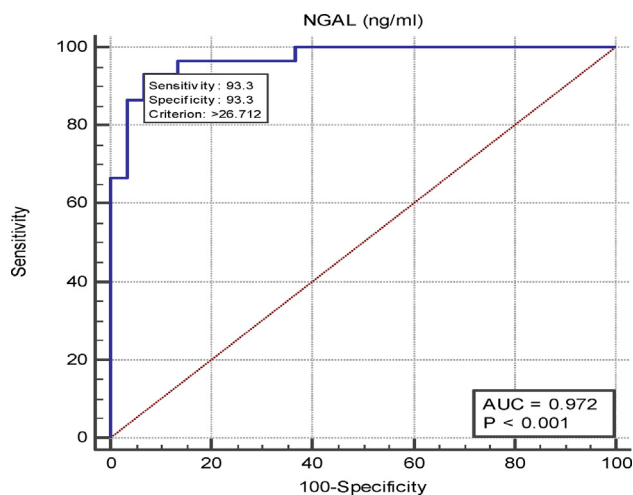


Fig. 2. Cutoff value by receiver operating characteristics curve of serum neutrophil gelatinase associated lipocalin in differentiating active versus remittent inflammatory bowel disease.

Seven patients had proctitis, 19 had proctosigmoiditis, and 34 (56.7 %) had pan-colitis, according to the extent of UC. Six (10 %) patients had a CD diagnosis; two had a CDAI less than 150 remission and the other four had an activity CDAI greater than 150. All CD patients had inflammatory type of CD. There was a history of appendectomy for three of them.

Moreover, 53.3 % of the patients had extra-intestinal manifestations, such as thromboembolic,

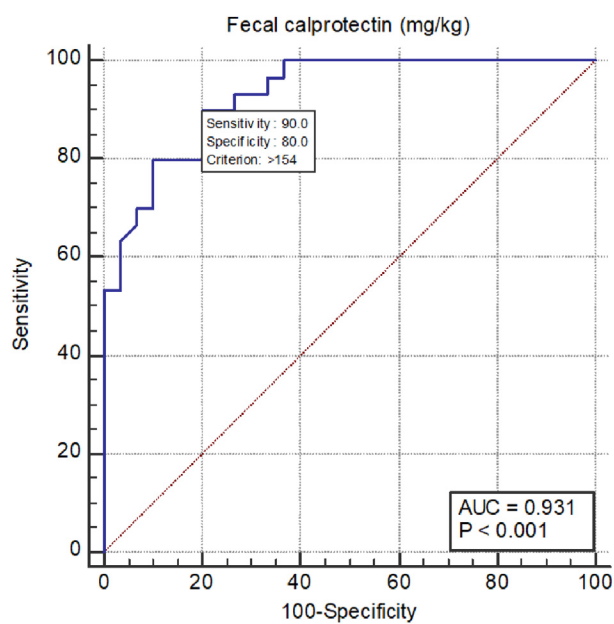


Fig. 3. Cutoff value of fecal calprotectin by receiver operating characteristics curve in differentiating active versus remittent inflammatory bowel disease.

musculoskeletal, or skin or eye manifestations, all of which were managed with medication alone. Every one of our patients was undergoing traditional medical treatment. Azathioprine (AZA) and frequent steroid use were taken by 63.3 % of the population, whereas 36.7 % were taking 5 amino salicylic acid (5 ASA) (pentasa) and local steroids.

With respect to fecal calprotectin's capacity to discriminate between IBD remittent cases and active cases as in Fig. 3; at the cutoff of 154 mg/kg, the findings demonstrated an AUC of 0.93 CI (0.83–0.98), with 90.0 % sensitivity and 80.0 % specificity ($P < 0.0001$). Additionally, there was a highly significant positive correlation found between serum NGAL and fecal calprotectin ($r = 0.69$, $P < 0.0001$). Compared with fecal calprotectin, NGAL shows a significantly greater predictability of IBD activity ($P < 0.0001$), see Table 4.

This is consistent with the results of Zollner *et al.*, (2021) (Zollner *et al.*, 2021), who discovered a strong correlation between the two parameters to predict clinical and endoscopic disease activity, as well as comparable sensitivity and specificity. Further, a multicentric cross-sectional study with about 400 patients found that NGAL and fecal calprotectin levels are beneficial additions for evaluating disease activity in asymptomatic UC patients (Magro *et al.*, 2017).

Additionally, Table 3 of our study demonstrated a mildly positive correlation ($r = 0.38$; $P = 0.003$) between CRP and NGAL. However, there was only a 0.56 AUC of CRP, with a 95 % CI (0.43–0.69), $P = 0.42$, separating active IBD patients from those in remission. In contrast, the ESR had a sensitivity of 33.3 % and a specificity of 83.3 %, with a 0.55 CI (0.41–0.67), $P = 0.54$. Therefore, when NGAL is compared with other markers such as CRP or ESR, it shows better statistical performance for IBD activity. These findings are consistent with De Bruyn *et al.*'s (de Bruyn *et al.*, 2015) findings that NGAL was superior to CRP and could be used alone or in conjunction with CRP to differentiate between mucosal healing (de Bruyn *et al.*, 2015).

Only a small number of studies (after revision of literature) involving Egyptian patients with UC-IBD have demonstrated the utility of serum NGAL as a marker for evaluating patients' disease activity and response to different lines of therapy (Nooh *et al.*, 2018; Fiefel *et al.*, 2020).

In conclusion, because serum NGAL is less expensive than faecal calprotectin and has a high sensitivity and specificity for active cases, it can be recommended for the assessment of IBD activity. It may also serve as a sensitive prognostic marker with highly significant prediction of disease activity.

Table 4. Predictors of the probability of having active inflammatory bowel disease (univariate analysis).

Predictor	P-value	Crude odds ratio	95 % Confidence interval
Serum NGAL (ng/ml)	<0.001	r (1)	r (1)
≤26.7		126	19.5–814
>26.7	<0.001	r (1)	r (1)
≤154		36	8.1–160
>154	0.004	r (1)	r (1)
WBCs count		5.7	1.7–18.9
≤6.6	0.011	r (1)	r (1)
>6.6		8.1	1.6–40.7
MCV (fl)	0.011	r (1)	r (1)
>70		8.1	1.6–40.7
≤70			

Notes: Confidence interval, CI; Crude odds ratio, or COR; Reference category, r(1).

Binary logistic regression is used to test significance.

Declarations

Ethics approval and consent to participate Mansoura University's Faculty of Medicine's Ethical Committee gave its approval for the study and the patients' participation. MS.20.10.14 is the code number.

Ethics of Humanity was given the all-clear by the Mansoura University Faculty of Medicine's Ethics Committee.

Agreement to publish: The manuscript has been approved for submission and publication in this journal by all authors. Availability of data and material: Data were gathered from the medical records that were registered in accordance with the Helsinki Declaration of 1975, as amended in 2008, and the institutional ethics committee.

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Confirmation: The manuscript has been approved for submission to this journal by all authors. This manuscript's content has never been released or submitted for publication before.

Conflicts of interest

Nothing to announce.

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