The value of serum neutrophil gelatinase associated lipocalin levels in predicting the inflammatory bowel disease patients with axial involvement.

Serum nötrofil jelatinaz ilişkili lipokalin düzeylerinin aksiyal tutulumu olan inflamatuvar barsak hastalığını predikte etmedeki değeri

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Background and Aims: *We hypothesized that serum neutrophil gelatinase-associated lipocalin levels in inflammatory bowel disease patients with axial involvement would be higher than serum neutrophil gelatinase-associated lipocalin levels in both an inflammatory bowel disease patients group without axial involvement and in healthy controls. We therefore attempted to demonstrate the value of using neutrophil gelatinase-associated lipocalin levels to predict inflammatory bowel disease patients with axial involvement.* ***Material and Methods:*** *The study was performed in a total of 123 cases between April 2011 and May 2012, and included a control group (n=40), an inflammatory bowel disease group (n=64), and an inflammatory bowel disease+axial involvement group (n=19). We excluded patients with peripheral joint involvement because of the different pathogenesis from axial involvement. We could not separate the axial arthropathy group due to a limited number of cases.* ***Results:*** *Paired comparisons were performed to determine the difference. There was a statistically significant difference in the neutrophil gelatinase-associated lipocalin levels among the groups (p<0.01). The neutrophil gelatinase-associated lipocalin levels of the inflammatory bowel disease+axial involvement group was determined to be significantly higher than the inflammatory bowel disease group and the control group (p=0.001; p=0.004), respectively. neutrophil gelatinase-associated lipocalin levels in the inflammatory bowel disease group were determined to be significantly higher than the control group (p=0.001).* ***Conclusions:*** *We recommend the identification of a diagnostic threshold value of neutrophil gelatinase-associated lipocalin as an inflammatory marker in patients with inflammatory bowel disease and inflammatory bowel disease and axial joint involvement since neutrophil gelatinase-associated lipocalin levels increase in these groups and seems to be related to the severity of colonic inflammation.*

Key words: *Neutrophil gelatinase-associated lipocalin, inflammatory bowel disease, axial joint, axial involvement*

Giriş ve Amaç: *Biz, aksiyal tutulumu olan inflamatuvar barsak hastalarının serum nötrofil jelatinaz ilişkili lipokalin düzeylerinin, aksiyal tutulumu olmayan inflamatuvar barsak hastaları ve sağlıklı kontrollerin serum nötrofil jelatinaz ilişkili lipokalin düzeylerinden daha yüksek olacağı hipotezini ileri sürdük. Böylelikle, nötrofil jelatinaz ilişkili lipokalin değerinin aksiyal tutulumu olan inflamatuvar barsak hastalığını predikte etmedeki değerini ortaya koymaya çalıştık.* ***Gereç ve Yöntem:*** *Çalışma, Nisan 2011 ile Mayıs 2012 tarihlerinde, inflamatuvar barsak hastalığı grubu (n=64), inflamatuvar barsak hastalığı+aksiyal tutulum grubu (n=19) ile kontrol grubunu (n=40) içeren toplam 123 olgu üzerinde gerçekleştirilmiştir. Aksiyal tutulumdan farklı patojeneze sahip olması nedeniyle periferik eklem tutulumlu hastaları çalışma dışında bıraktık. Kısıtlı hasta sayısı yüzünden aksiyal artropati grubunu ayıramadık.* ***Bulgular:*** *Gruplara göre olguların nötrofil jelatinaz ilişkili lipokalin düzeyleri arasında istatistiksel olarak anlamlı bir farklılık bulunmaktadır (p<0,01). Farklılığın hangi gruptan kaynaklandığını saptamak amacıyla yapılan ikili karşılaştırmalar sonucunda; inflamatuvar barsak hastalığı+aksiyal tutulumu grubunun nötrofil jelatinaz ilişkili lipokalin düzeyinin, kontrol grubu ve inflamatuvar barsak hastalığı grubundan anlamlı şekilde daha yüksek olduğu saptanmıştır (p=0,001, p=0,004). İnflamatuvar barsak hastalığı grubunun nötrofil jelatinaz ilişkili lipokalin düzeyi de kontrol grubundan anlamlı şekilde daha yüksek saptanmıştır (p=0,001).* ***Tartışma:*** *İnflamasyon göstergesi olarak inflamatuvar barsak hastalığı grubunda ve aksiyal tutulumlu inflamatuvar barsak hastalığı grubunda düzeyi yükselen ve kolonik inflamasyon şiddeti ile ilişkili gibi görünen nötrofil jelatinaz ilişkili lipokalinin tanısal eşik değerinin değerlendirilmesini öneriyoruz.*

Anahtar kelimeler: *Nötrofil jelatinaz ilişkili lipokalin, inflamatuvar barsak hastalığı, aksiyal eklem, aksiyal tutulum*

INTRODUCTION

Arthritis poses a severe problem in daily practice due to its effect on patients' quality of life in addition to being the most common extraintestinal manifestation of inflammatory bowel disease (IBD) (1). Joint involvement in IBD is classified as axial arthropathy, peripheral arthritis, and inflammatory back pain - including ankylosing spon-

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dylitis (AS) and sacroiliitis (1). Axial arthropathy generally begins before intestinal involvement and there is no correlation between the severity of the involvement and the clinical course of IBD; even surgical treatment for IBD does not change the course of axial involvement (2). It has been shown in studies that routine laboratory tests like complete blood count (CBC), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) levels, used in the diagnosis of IBD, are not ideal markers for the diagnosis of axial joint involvement (2-4).

Despite the emergence in the use of radiological imaging due to increased sensitivity of magnetic resonance imaging (MRI), pathognomonic findings which would confirm the suspicion of joint involvement in IBD patients cannot always be determined (5,6). Therefore, a diagnosis of exclusion, together with clinical suspicion, still comprises the primary approach to a diagnosis (7).

Neutrophil gelatinase-associated lipocalin (also called as NGAL, lipocalin 2, siderocalin, 24p3 or LCN2) was first isolated from a supernatant of activated human neutrophils in 1994 (8). The main source of circulating NGAL is in neutrophils (8-10). Although it is expressed at low levels in most tissues, its expression in damaged intestinal and colonic epithelial cells, respiratory cells, renal tubular cells and hepatocyte endothelial cells increases as a response to inflammatory signals (11-15). NGAL is covalently linked to type 4 collagenase (gelatinase B, matrix metalloproteinase-9) in neutrophils. In consequence of its binding to matrix metalloproteinase-9 (MMP-9), it leads to prolongation of the effects of MMP-9 on collagen degradation by inhibiting inactivation of this enzyme (16,17). In addition to this function, it exerts bacteriostatic action by blocking ferritin necessary for bacterial growth and serves as one of key molecules of the natural immune system (18-20). When these different inflammatory conditions are evaluated together, it is clear that the synthesis and expression of NGAL does not arise specific to a unique colonic inflammatory disease and that NGAL expression can be induced irrespective of the cause of inflammation. In this study, we hypothesized that serum NGAL levels in IBD patients with joint involvement would be higher than the serum NGAL levels in both an IBD patient group without joint involvement and in healthy controls. We tried to demonstrate the value of NGAL in predicting IBD patients with joint involvement.

MATERIALS and METHODS

Between April 2011 and May 2012, we evaluated a total of 123 patients, including a control group (n=40), an IBD group (n=64), and an IBD + axial involvement (sacroiliitis + AS + inflammatory back pain) group (n=19). The ages of the patients ranged from 16 to 74 years, with a mean age of 39.1±11.5. We excluded patients with peripheral joint involvement due to the different pathogenesis of these patients from those with axial involvement. We could not separate the axial arthropathy group due to the small number of cases.

The study was approved by the local Ethics Committee of Haydarpaşa Numune Training and Research Hospital, and informed consent was obtained from all of the patients.

Clinical evaluation

Physical examination and evaluation of patients were performed regarding IBD, complaints related to sacroiliac joint involvement, peripheral joint involvement, inflammatory back pain, and clinical examination findings. HLA-B27 testing was requested from patients that had an insufficient diagnosis. The European Spondyloarthropathy Study Group Criteria was used to make the diagnosis through clinical evaluation.

Laboratory tests

Venous blood samples from the IBD patients and the control group were drawn in EDTA tubes, sodium citrate tubes and gel-containing tubes (Becton Dickinson, USA) in the morning following a 10-12 hour overnight fast (to eliminate possible interference of lipemia). Gel-containing tubes were centrifuged at 3500 RPM (1300 g) for 10 minutes after waiting for 30 minutes. CBC, ESR and CRP levels were studied without a waiting period. CBC tests were performed from EDTA blood samples on all patients and the control group and ESR rates were measured from sodium citrate blood samples, using the Westergren method and a Sed Rate Screener 100 (SRS 100, Greiner Bio-one GmbH, Austria) device. CRP measurements were performed from serum samples, using a nephelometric method (Immage, Beckman Coulter, USA).

To perform measurements of NGAL levels at a later time, serum samples were stored at -70ºC by dividing it into two portions. Frozen samples were thawed immediately before the analysis and studied. Repeated freezing and thawing procedures were avoided.

Studying serum lipocalin-2/NGAL levels

Serum lipocalin-2/NGAL levels were measured using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA; BioVendor R&D, Czech Republic) kit. As previously described, double pipetting was performed in the wells coated with polyclonal antibody specific to anti-human lipocalin-2, using standards, cont-

rols and diluted serum samples. Following incubation and washing procedures, biotinylated second polyclonal anti-human antibody was added to the well. After incubation, unbound antibody was removed by a washing procedure. A chromogen substrate was then added to the wells. Severity of yellow color occurring due to enzymatic activity after a third incubation was measured on an ELISA reader set at a wavelength of 450 nm (reference wavelength, 630 nm). Results were multiplied by the dilution factor and expressed in terms of mg/dL (21).

Radiological evaluation

Evaluation of sacroiliitis was proven by direct X-Rays of the pelvis and sacroiliac MRI.

Statistical evaluation

Evaluation of study data was obtained using NCSS (Number Cruncher Statistical System) 2007 & statistical analysis by PASS (Power Analysis and Sample Size) 2008 Statistical Software program (Utah, USA). Comparisons of quantitative data and descriptive statistical methods (mean, standard deviation, median, frequency and ratio) were performed using one-way ANOVA test for the intergroup comparisons of parameters with normal distribution. Tukey HDS test was used for the determination of the group causing differences. The Kruskal Wallis test was used for the intergroup comparisons of parameters without normal distribution and Mann Whitney U test was used for the determination of the group causing differences. Pearson's Chi-Square test was used for the comparison of qualitative data. The results were evaluated with a 95% confidence interval and at a statistically significance level, p<0.05.

RESULTS

Differences in age, gender and duration of disease were not statistically significant among groups (p>0.05) (Table 1). There was a statistically significant difference among

Table 1. Distribution of general characteristics according to the groups.

		Control (n=40)	IBD (n=64)	IBD+Joint Involvement (n=19)	p
		Mean±SD	Mean±SD	Mean±SD	
Age		42,2±10,6	37,4±11,3	38,3±13,2	[a]***0,109***
BMI		26,05±4,94	22,36±3,44	23,86±4,33	[a]***0,001****
Disease Year (Median)		-	5,6±4,3 (4,5)	7,3±5,1 (7)	[b]***0,181***
		%	%	%	***p***
Gender	Female	23 (57,5)	32 (50)	12 (63,5)	[c]***0,537***
	Male	17 (42,5)	32 (50)	7 (36,8)	

[c]0,537[a]One-Way Anova [b]Mann-Whitney U test [c]Pearson's Chi-Square test

IBD; Inflammatory bowel disease. BMI; Body mass index.

Table 2. Evaluation of hemoglobin, NGAL, erythrocyte sedimentation rate, CRP and white blood cell (WBC) levels according to the groups

	Groups			p
	Control (n=40)	IBD (n=64)	IBD+Joint Involvement (n=19)	
	Mean±SD	Mean±SD	Mean±SD	
Hgb (mg/dL)	14,01±1,12	11,44±2,57	11,84±2,16	[a]***0,001****
NGAL (ng/mL) (Median)	129,88±51,297 (122)	172,17±66,57 (168)	226,0±63,02 (234)	[d]***0,001****
ESR (mm/h) (Median)	14,02±4,63 (14)	34,03±19,50 (31)	34,47±25,06 (25)	[d]***0,001****
CRP (mg/dL) (Median)	0,84±1,36 (0,7)	2,37±3,06 (1,4)	1,89±2,58 (1)	[d]***0,006****
WBC (10^{-3}/mL) (Median)	7,08±1,81 (6,8)	8,73±4,13 (8)	7,13±2,17 (7,1)	[d]***0,014****

[a]One-Way Anova [d]Kruskal Wallis test **p<0,01 *p<0,05

Hgb; hemoglobin. NGAL; neutrophil gelatinase associated lipocalin. ESR; erythrocyte sedimentation rate. CRP; C-reactive protein. WBC; White blood cell.

groups in body mass index (BMI) (p<0.01), hemoglobin (Hgb) (p<0.01), ESR (p<0.01), CRP (p<0.01), and WBC levels (p<0.01), (Table 2). Using the Post-Hoc Tukey HSD test to determine where the differences among the groups arose, it was determined that Hgb levels were significantly higher in the control group than IBD group and IBD + joint involvement (sacroiliitis) group; ESR, CRP and WBC levels were significantly higher in IBD group and IBD + joint involvement (sacroiliitis) group than those in the control group (p=0.001; p=0.001). There was no significant difference between the IBD group and IBD + joint involvement group regarding Hgb, WBC, CRP and ESR values (p>0.05) (Table 2).

There was a statistically significant difference among the NGAL levels of cases according to groups (p<0.01). In consequence of paired comparisons performed to determine where the difference arose among the groups, it was determined that the NGAL levels of the IBD + axial involvement group was significantly higher than the IBD group and the control group, respectively (p=0.001; p=0.004). The mean NGAL level of the IBD group was determined to be significantly higher than the control group (p=0.001) (Table 2).

DISCUSSION

We observed that parameters like CRP, ESR, WBC and Hgb, frequently used by us in this study for the diagnosis of IBD and the determination of activity, were inadequate in differentiating IBD patients with axial involvement and in predicting axial involvement. However, we determined that the serum level of NGAL, whose role in IBD pathogenesis has been proven in recent studies, was significant in predicting IBD patients with axial involvement.

In previous studies, the cause of the close relationship between intestinal inflammation and joint involvement in IBD patients was investigated (21-23). While Faustini et al. tried to decipher this relationship by way of interleukins like IL17 and IL23 (22), De Vos M. emphasized genetic polymorphism, including CARD 15 (caspase recruitment domain-containing protein 15) (21), adding IL-23R polymorphism to the genetic etiology in his 2009 study (23). Although radiological imaging has moved to the forefront due to an increased use of magnetic resonance imaging (MRI) in the diagnosis of IBD with joint involvement in recent years, contradictory results and lack of pathognomonic findings created the need of new markers.

Recent studies show that NGAL expression is elevated in conditions like acute and chronic renal failure, cardiovascular disease, transplantation, sepsis and pancreatitis, and that the level of this protein could be beneficial for diagnosis (24,25). The expression and blood levels of NGAL are increased in non-infectious inflammatory disorders (26,27). Therefore the effect of NGAL in determining inflammation and its severity was worthy of investigation, especially its use in acute renal failure since it is a subject of investigation in many studies (27,28). NGAL was found to be promising, equivalent to cardiac troponin in acute renal failure; it was also shown that increased blood NGAL levels, due to rapid and early secretion in the presence of a cellular stress response, could be beneficial for diagnosis (27). Further, NGAL was introduced as an early marker of renal failure after it was shown that NGAL serum levels increased 2 hours after renal ischemia occurred, compared to 24-48 hours for an increase to be seen in creatinine levels, the traditional marker (28).

NGAL is promising as an early marker in inflammatory conditions but studies regarding its use in IBD are limited (12,29). Use of inflammatory markers for diagnosis and determination of activity in IBD, where the main pathogenetic mechanism is intestinal inflammation, appears to be reasonable. In this study, we investigated the use of NGAL to predict joint involvement in IBD patients, in which its expression and secretion increased in the inflamed colonic epithelium, reflecting neutrophil activation and having an immunoregulatory function. Our results, a first report in the literature, showed that serum NGAL levels in IBD + joint involvement patients were significantly higher than NGAL levels in IBD patients without joint involvement as well as those seen in healthy controls.

Additionally, we determined that NGAL levels of IBD patients were significantly higher than serum NGAL levels of the control group. These findings show parallels with the findings of a study performed by Nielsen et al. (12), which investigated NGAL expression by using in situ hybridization and immunohistochemical methods in different inflammatory diseases of the colon (IBD, diverticulitis, appendicitis), and in the setting of inflammatory reaction around a tumor created by the body against colon tumors (12). In consequence of investigation performed in fewer pathological samples, the authors determined that NGAL was expressed slightly, rarely and weakly in normal colonic epithelium; mRNA expression was increased in the inflammatory diseases (shown with in situ hybridization); and, accordingly, mature protein was increased (12). Although the presence of NGAL in neutrophils was shown immunohistochemically, no demonstration of in-

creased expression using in-situ hybridization tests suggested that mature neutrophils did not express NGAL (15). This data shows that NGAL is deposited in specific granules by expressing in immature bone marrow neutrophils and are secreted from mature neutrophils with stimulation. Although NGAL expression was not shown in tumor cells, the authors showed increased NGAL expression in the setting of inflammatory reaction around the tumor (2). When these different inflammatory diseases and the findings of our study are evaluated together, it is understood that synthesis and expression of NGAL does not arise specific to a unique colonic inflammatory disease and that NGAL expression can be induced irrespective of the cause of inflammation.

In this study, we have shown that a significant difference exists between serum NGAL levels of IBD patients with and without axial involvement. This suggests that NGAL could play an important role in the pathogenesis of the axial involvement, and therefore could be useful in the diagnosis.

In studies in the literature, increase in serum NGAL levels correlates with an increase in age (29,30). Therefore, the age of the study population seems to be an important factor and provides a challenge in determining a single threshold value that can be used globally. In our study, the absence of a difference among the patient groups is noteworthy and one of the major positive aspects of the study; age and gender were statistically similar between the groups.

It may be beneficial to consider NGAL, which is secreted primarily from activated neutrophils and expressed in colonic epithelium, in the presence of inflammation and other factors that affect serum NGAL levels. Additionally, the close interaction of NGAL with iron metabolism and the renal excretion of NGAL should be considered as factors that can affect serum NGAL levels (31,32). Further, exertion of a defensive effect by NGAL, by binding inflammatory lipophilic mediators and its effects on immune system in this way, and many cytokines affecting inflammatory processes and their effects on each other, should be considered in studies performed with mediators participating in inflammatory pathways (33). In our study, although there was a significant difference between the study and the control groups, there were no statistically significant difference between IBD and IBD with axial involvement in Hgb levels.

In conclusion, for the first time in the literature, our study results demonstrate the role of serum NGAL levels in predicting axial involvement in IBD patients, showing that its level increases in IBD patients and IBD patients with axial involvement. We recommend the development of a diagnostic threshold value of NGAL as an inflammatory marker since it also appears to be related to the severity of colonic inflammation. It should be evaluated in a larger cohort of patients before introduction as a diagnostic tool of axial joint involvement. Additionally, the determination of normal ranges in the healthy population, development of standards for a study method, and the introduction to routine practice using results of other studies are also needed.

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