ORIGINAL ARTICLE

A New Biomarker in the Differential Diagnosis of Acute Appendicitis: Zonulin

Akut Apandisit Ayırıcı Tanısında Yeni Bir Biyobelirteç: Zonulin

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ABSTRACT

Objective: Acute appendicitis is the most frequently encountered emergency abdominal surgery worldwide, and the clinical diagnosis is based on the history, physical examination, laboratory evaluation and imaging. Zonulin is a measurable protein that reflects intestinal permeability. This study aimed to evaluate the utility of serum zonulin levels in the diagnosis of acute appendicitis.
Material and Method: This prospective, observational study included patients aged ≥18 years who presented to the emergency medicine department of a city hospital with clinical and examination findings and were pathologically confirmed with appendicitis after the operation. A control group was formed of healthy subjects aged >18 years. Serum zonulin levels were compared between the two groups.
Results: Evaluation was made of a total of 123 voluntary subjects who met the study inclusion criteria, as 64 (52%) in the patient group and 59 (48%) in the control group. The serum zonulin levels were determined to be significantly low in the appendicitis patient group at 15.2 ± 10.3 ng/mL (p<0.001). According to the pathology reports of acute appendicitis, no significant difference was observed in the serum zonulin levels between acute phlegmonous appendicitis and acute perforated appendicitis.
Conclusion: Serum zonulin levels were determined to be significantly reduced in appendicitis patients. This suggests that serum zonulin could be a potential negative biomarker in acute appendicitis pathogenesis.
Keywords: Acute appendicitis, Zonulin, Biomarker

ÖZET

Amaç: Akut apandisit, dünya genelinde en sık karşılaşılan acil karın cerrahisidir. Klinik tanı; hasta öyküsü, fizik muayene, laboratuvar değerlendirmesi ve görüntüleme yöntemlerine dayanır. Zonulin, bağırsak geçirgenliğini yansıtan ölçülebilir bir proteindir. Bu çalışmanın amacı, akut apandisit tanısında serum zonulin düzeylerinin tanısal değerini değerlendirmektir.
Gereç ve Yöntem: Bu prospektif gözlemsel çalışmaya, bir şehir hastanesi acil tıp kliniğine başvuran, klinik ve muayene bulguları apandisit ile uyumlu olan ve cerrahi sonrası patolojik olarak apandisit tanısı doğrulanan 18 yaş ve üzeri hastalar dahil edildi. Kontrol grubunu 18 yaş ve üzeri sağlıklı gönüllüler oluşturdu. Serum zonulin düzeyleri iki grup arasında karşılaştırıldı.
Bulgular: Çalışma kriterlerini karşılayan toplam 123 gönüllü değerlendirildi; bunların 64'ü (%52) apandisit hastası, 59'u (%48) sağlıklı kontrol grubundaydı. Serum zonulin düzeyleri apandisit grubunda anlamlı derecede düşük bulundu (15,2 ± 10,3 ng/mL, p < 0,001). Patoloji sınıflandırmasına göre, akut flegmonöz ve akut perfore apandisit grupları arasında serum zonulin düzeyleri açısından anlamlı fark saptanmadı.
Sonuç: Akut apandisit hastalarında serum zonulin düzeyleri anlamlı şekilde düşük bulunmuştur. Bu bulgular, serum zonulin düzeylerinin akut apandisit patogenezinde potansiyel bir negatif biyobelirteç olabileceğini göstermektedir.
Anahtar Kelimeler: Akut apandisit, Zonulin, Biyobelirteç

INTRODUCTION

Appendicitis is currently one of the most frequently encountered causes of acute abdominal pain, but correct and rapid diagnosis remains a difficult process. Acute appendicitis is the most common abdominal surgical emergency with an incidence of 96.5-100 cases per 100,000 adults (1). Early diagnosis of appendicitis cases has a significant impact on the health status of patients and therefore, the development of correct diagnosis methods is of critical importance in clinical practice.

Zonulin plays a key role in the pathogenesis of autoimmune diseases, especially celiac disease. Proteomic analysis of human serum has identified pre-haptoglobin (HP)-2 of zonulin and has shown that HP genetic alleles have an effect on zonulin synthesis (2). It has also been shown that the interaction of zonulin and enterotoxin can prevent microorganism colonisation by increasing intestinal permeability (3). An increase in zonulin levels is observed especially in obesity, ageing, various autoimmune diseases, asthma, and inflammatory bowel diseases. Zonulin inhibitors have been investigated as potential therapeutic agents to inhibit the development of autoimmune diseases such as type 1 diabetes in particular. The effects of zonulin on intestinal permeability occur through regulation of intercellular

tight junctions. This regulation is provided by the effect of intercellular signal mechanisms through epidermal growth factor receptors and protein kinase C of zonulin (4). Zonulin is one of the few physiological mediators of paracellular intestinal permeability and is linked to the development of various chronic inflammatory disorders. Inappropriate regulation of zonulin causes functional loss of the epithelial barrier, thereby causing inflammatory bowel diseases (5). If the mechanism is considered in reverse, any inflammation forming in the intestines can be a reason for a change in the serum zonulin levels. Based on the importance of early diagnosis of appendicitis patients, the diagnostic value of biomarkers, especially serum zonulin levels used in the evaluation of intestinal pathologies, has become an area of increasing interest.

This study aimed to examine the potential benefit of the serum zonulin levels in the diagnosis of acute appendicitis.

MATERIALS AND METHODS

Study Design and Setting

Approval for this prospective, observational study was granted by the Local Ethics Committee (decision no:150, dated: 28 August 2023). The patient data were collected between 01.09.2023 and 01.03.2024. An average of 250,000 patients are admitted to the Emergency Department (ED) of our hospital annually, and of these, approximately 70% are green zone patients. The ED has 3 different areas coded as green, yellow, and red, and patients for these areas are classified according to their clinical condition. The green zone is the area in which patients are evaluated for minor trauma such as low back pain, upper respiratory tract infection, or sprained ankle. Approximately 200 acute appendicitis operations are performed per year in our hospital. This study included patients who were diagnosed with acute appendicitis confirmed pathologically and underwent surgery in our hospital within the defined 6-month period, and met the study inclusion criteria.

Participants

The sample size of the study was determined using an a priori power analysis performed with G*Power 3.1. Because the study involved a comparison between two independent groups, the "Means: Difference between two independent means (two groups)" module was used. Based on effect sizes reported in similar previous studies, an effect size (d) of 0.50, an alpha level of 0.05, and a statistical power of 0.80 were adopted. Under these parameters, the minimum required sample size was calculated as 64 participants per group, for a total of 128 participants. Starting from 01.09.2023, a total of 78 patients were evaluated who presented at the ED and were diagnosed with appendicitis as a result of physical examination, imaging methods, and general surgery consultation. The study materials were obtained from the patient group immediately after diagnosis. A total of 14 patients were excluded from the study; 8 who did not meet the study inclusion criteria and 6 with a postoperative pathology report not compatible with appendicitis. Thus, the patient group was formed of 64 patients. For the formation of the control group, blood samples were taken from 68 patients. The blood biological material was lost in 5 cases and it was learned that 4 patients were smokers, so the study control group was formed of 59 healthy subjects. The serum zonulin levels of the patients diagnosed with appendicitis were compared with those of the control group. During the study period, 64 patients with appendicitis (study group) and 59 healthy individuals (control

group) meeting the inclusion criteria were enrolled, resulting in a total sample size of 123 participants. A post hoc power analysis performed with these group sizes ($n_1 = 64$, $n_2 = 59$) and effect size d = 0.50 showed a statistical power of ≈78.5%, indicating that the study retained an acceptable level of power despite the slightly lower total sample size.

Study Inclusion Criteria

1- Patients aged >18 years who presented to the emergency medicine department of a city hospital and had a postoperative pathological diagnosis of appendicitis,

2- No diagnosis of DM, malignancy, obesity, IBD (inflammatory bowel disease), HIV (+), sepsis, gout, ankylosing spondylitis, celiac disease,

3- Written informed consent to participate in the study provided by the patient or if the patient was unconscious, by their legal representative.

Study Exclusion Criteria

1- Unwilling to participate in the study,

2- Patients diagnosed with DM, malignancy, obesity, IBD, HIV (+), sepsis, gout, ankylosing spondylitis, celiac disease,

3- Pregnancy, or age <18 years

Data collection and zonulin measurements

The demographic data (age, gender) of patients were recorded together with vital parameters such as blood pressure, pulse, temperature, and oxygen saturation, comorbidities, consciousness status, ultrasonography findings and abdominal tomography images. Appendicitis was recorded as "present" or "absent" on the radiological images and extra data were not used. For the patients with suspected appendicitis, the results of routine biochemical tests (CRP, urea, creatinine, alanine transaminase (ALT), aspartate transaminase (AST), sodium, potassium) and hematological tests (hemoglobin level, leukocyte count) were added to the data.

Following the data collection, all the samples were transported to the laboratory where the analyses were to be conducted under cold-chain conditions as recommended by the kit manufacturers, and the serum samples were stored at room temperature until assay. The laboratory personnel were blinded to patient information. All the measurements were performed by two researchers blinded to the clinical data. The serum zonulin levels were examined using the ELISA method with the 96 Human Zonulin ELISA Kit and the 96 Human Glial Fibrils Acidic Protein ELISA Kit (Bioassay Technology Laboratory – BT LAB, Shanghai, China) and the BioTek® ELx800DA™ Microplate Reader (VT, USA) and DIALAB® DIAWasher ELX5O/8 (Vienna, Austria) devices. The results obtained were recorded on case report forms. The levels were compared between the patient and control groups.

Statistical Analysis

All data were evaluated using SPSS v.22.0 software (SPSS, Inc., Chicago, IL, USA). Descriptive statistics were applied to all the study data, stated as mean ± standard deviation values for measured variables and as median values for age. Conformity of variables to the normal distribution was assessed with the Shapiro-Wilk test. A value of $p<0.05$ was considered statistically significant. The serum zonulin levels were compared between the control group and the patient group as a whole and according to the type of acute appendicitis.

RESULTS

Evaluation was made of a total of 123 subjects, comprising 80 (65%) males and 43 (35%) females. The patient group diagnosed with appendicitis included 64 (52%) patients,

Table 1: Comparisons of the quantitative parameters of the groups.

	Groups		Total	p*
	Patient group	**Control group**		
Age (years)	35.61 ± 14.01	31.95 ± 10.5	33.85 ± 12.54	0.261
	33 (18 - 73)	29 (19 - 74)	30 (18 - 74)	
Male	42 (65.6)	38 (64.4)	80 (65)	1.000*
Female	22 (34.4)	21 (35.6)	43 (35)	
Serum Zonulin level	15.2 ± 10.3	34.05 ± 22.07	24.24 ± 19.38	<0.001
	11.28 (2.66 - 41.76)	27.34 (2.23 - 68.48)	13.59 (2.23 - 68.48)	
Hemoglobin	138.59 ± 16.68		138.59 ± 16.68	
	140 (102 - 175)		140 (102 - 175)	
Leukocytes	13723.28 ± 3871.11		13723.28 ± 3871.11	
	13925 (4080 - 23510)		13925 (4080 - 23510)	
CRP	41.6 ± 49.06		41.6 ± 49.06	
	26.25 (0.8 - 229)		26.25 (0.8 - 229)	
Urea	25.86 ± 9.06		25.86 ± 9.06	
	24.5 (13 - 71)		24.5 (13 - 71)	
Creatinine	0.86 ± 0.2		0.86 ± 0.2	
	0.84 (0.45 - 1.43)		0.84 (0.45 - 1.43)	
ALT	20.33 ± 15.26		20.33 ± 15.26	
	17 (7 - 116)		17 (7 - 116)	
AST	22.3 ± 16.09		22.3 ± 16.09	
	18 (12 - 128)		18 (12 - 128)	
Sodium	138.89 ± 2.93		138.89 ± 2.93	
	139 (127 - 145)		139 (127 - 145)	
Potassium	4.41 ± 0.34		4.41 ± 0.34	
	4.32 (3.8 - 5.24)		4.32 (3.8 - 5.24)	

Mann Whitney U test; mean ± standard deviation; median (minimum-maximum)

Table 2: Comparisons of the serum zonulin levels according to the pathological diagnosis results.

Pathological Diagnosis	Serum Zonulin Levels		p*
	Mean ± SD	**Median (Min-Max)**	
Acute Appendicitis	13.64 ± 8,86	10.38 (2.66 - 41.76)	
Appendicitis Phlegmonosa	17.48 ± 12,73	11.77 (7.63 - 41.65)	0.318
Perforated Appendicitis	17.5 ± 8,88	11.5 (10.92 - 30.07)	
Total	15.2 ± 10,3	11.28 (2.66 - 41.76)	

Kruskal Wallis H test

Table 3: Relationships of the serum zonulin levels with age, CRP and leukocyte levels.

	Serum Zonulin Levels	
	r	**p**
Age	-0.013	0.882
CRP	0.030	0.812
Leukocyte	-0.059	0.645

r: Spearman's rho correlation coefficient

comprising 65.6% males and 34.4% females with a mean age of 35.61 ± 14.01 years. The control group included 59 (48%) healthy subjects, comprising 64.4% males and 35.6% females with a mean age of 31.95 ± 10.5 years. No statistically significant difference was determined between the groups in respect of gender or age (p=1.000, p=0.261, respectively).

The serum zonulin levels were determined to be 15.2 ± 10.3 ng/mL in the patient group and 34.05 ± 22.07 ng/mL in the control group, and the difference was found to be statistically significant (p<0.001) (Table 1).

No statistically significant difference was determined in the mean serum zonulin levels according to the postoperative pathology result (p=0.318). The serum zonulin levels were determined to be mean 13.64 ± 8.86 ng/mL in the acute appendicitis group, 17.48 ± 12.73 ng/mL in the acute phlegmonous appendicitis group, and 17.5 ± 8.88 ng/mL in the acute perforated appendicitis group (Table 2). No statistically significant difference was observed in the serum zonulin levels according to the demographic data of age or according to CRP and leukocyte levels (Table 3).

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Table 4: Results of the ROC analysis of the serum zonulin level in the differentiation of patients.
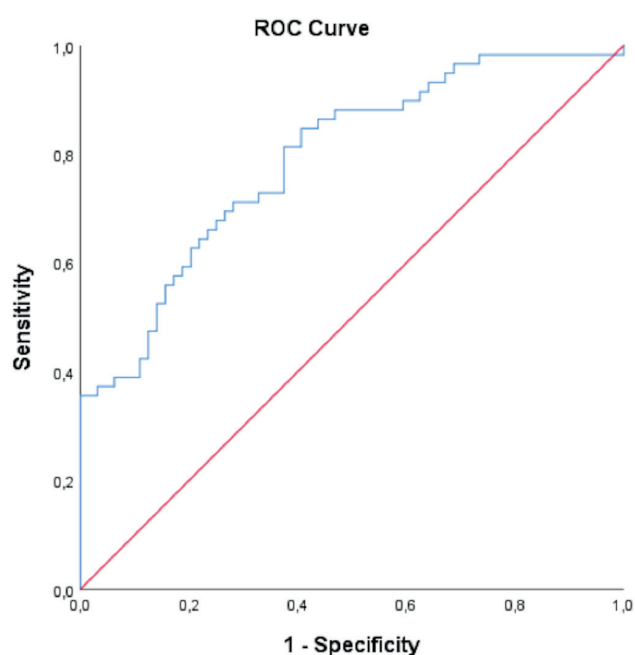
Cut off	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC (%95 CI)	p
≤11.94	84.75	59.38	65.79	80.85	0.791 (0.713 - 0.87)	<0.001

PPV: Positive Predictive Value, NPV: Negative Predictive Value, AUC: Area Under the Curve

Table 5: Comparisons of the serum zonulin levels according to gender.

	Serum Zonulin Levels		p*
	Mean ± SD	Median (Min-Max)	
Male	25.89 ± 20.33	13.24 (2.23 - 68.48)	
Female	21.17 ± 17.29	13.74 (5.33 - 68.19)	0.261
Total	24.24 ± 19.38	13.59 (2.23 - 68.48)	

*Mann Whitney U test

Figure 1: ROC curve of the serum zonulin level.

appendicitis patients, the AUC obtained of 0.791 was statistically significant (p<0.001). Following the ROC analysis, a cutoff value of ≤11.94 ng/mL was determined to have 84.75% sensitivity, 59.38% specificity, PPV 65.79%, and NPV 80.85% (Table 4) (Figure 1).

No statistically significant difference was determined in the mean serum zonulin levels according to gender, as 25.89 ± 20.33 ng/mL for males and 21.17 ± 17.29 ng/mL for females (p=0.261) (Table 5).

DISCUSSION

The results of this study demonstrated that the serum zonulin levels of patients with acute appendicitis were significantly lower than those of the healthy control group. No significant difference was determined in the serum zonulin levels of different types of appendicitis in the pathology results. Zonulin, which is the only measurable blood protein that reflects intestinal permeability, is a protein involved in the regulation of both epithelial and endothelial barrier functions, and its role in healthy and sick individuals is the subject of active research (6). In acute appendicitis, first edema develops in the intestinal wall, then necrosis. As a result of this edema, intestinal wall permeability increases, and therefore, this study was planned with the thought that serum zonulin could be high in the acute period. Contrary to expectations the serum zonulin levels were found to be low. There are studies in the literature showing that the serum zonulin levels are decreased in acute pancreatitis just as in acute appendicitis. Although the serum zonulin levels have been determined to be low in patients with acute pancreatitis and ileus, as in the current study, the mechanism has not been fully clarified (7, 8).

In 2000, Fasano reported that zonulin irregularity could contribute to a deterioration in intestinal barrier functions, food allergies, gastrointestinal system infections, and systemic autoimmune diseases, and that bacteria defined as inflammatory bowel diseases are a strong trigger of zonulin expression (9). In a study by Karin Malickova in 2017, serum zonulin levels were found to be increased in patients with Crohn's disease. However, in the same study, there was not determined to be significant increase in zonulin level in ulcerative colitis patient but an increase was observed in individuals who smoked (10).

Qi Y. et al. determined a positive correlation between age and serum zonulin levels (11). In another study by Thomas et al., it was shown that increased zonulin expression as a result of the interaction between gliadin and intestinal epithelium in celiac disease increased intestinal permeability. It was determined that in this situation gliadin undergoes paracellular translocation, interacts with submucosal macrophages, and creates a proinflammatory cytokine environment (12). In the study by Fasano, it was found that exposure to gliadin, which is an antigenic stimulus, directly led to an increase in zonulin levels in celiac patients, and this caused an increase in the intracellular penetration of antigens, including gliadin, in the intestinal submucosa (5). Wang et al. compared patients with liver cirrhosis or chronic hepatitis B with a healthy control group and reported that serum zonulin levels were significantly high in patients with hepatocellular carcinoma. This suggested that zonulin pathway disorders emerge secondary to deteriorations in the intestinal barrier (13).

Although serum zonulin biomarker has been frequently associated with gastrointestinal permeability, no study conducted on appendicitis patients could be found in the literature. Therefore, this is the first such study in literature. The aim of this study was to evaluate the utility of the serum zonulin levels as an early biomarker at the stage of diagnosis of appendicitis patients, and the results showed that the serum zonulin levels of acute appendicitis patients were significantly lower than those of the healthy control group. This suggests that serum zonulin could be a potential negative biomarker in the pathogenesis of acute appendicitis. However, this low level cannot be clearly explained. In a study by Avcıoğlu U. and Eruzun H., the serum zonulin levels were found to be low in the acute period of pancreatitis patients, but there was no significant difference in complicated pancreatitis (7). This low level of serum zonulin could not be linked to any cause, and similar negative results were obtained in the current study. A previous study conducted in our clinic included ileus patients and the serum zonulin levels in the acute period were found to be significantly low (8). On the basis of these studies, it can be said that although conditions such as diarrhea are seen

in some acute appendicitis patients, there may be decreased intestinal movements due to the development of peritonitis. Moreover, this decrease may have been determined because acute appendicitis developing in the appendix affects only a small segment of the intestine (14). However, the reason for this decrease cannot be fully explained.

The patients included in this study were all those in the specified period who underwent surgery for a diagnosis of appendicitis, which was then confirmed by the postoperative pathology result. In the patients with perforated appendicitis according to the pathology result, changes were expected in the serum zonulin values, as in the above-mentioned study of acute pancreatitis patients and in other studies in the literature (7). However, the current study results showed no statistically significant difference in the mean serum zonulin values between acute appendicitis, acute phlegmonous appendicitis, and acute perforated appendicitis. In the light of these results, it was thought that the serum zonulin levels may not determine complications in patients with acute appendicitis. However, this thought could be disproved in future studies with more equally matched numbers of cases with and without perforated acute appendicitis.

Limitations

There were some limitations to this study to be considered. As this was a single-centre study and patients were evaluated in a specific time period, the results cannot be generalised. The study included 64 patients and a control group of 59, but although the control group was smaller in number, the results were not statistically affected. There is a need for further, randomised, controlled, multicentre studies with greater numbers of participants. The serum zonulin levels were not measured postoperatively so new studies are required to investigate the effect of the operation and whether or not the serum zonulin levels increase after the surgery. These limitations can be of guidance for future studies.

CONCLUSION

The results of this study demonstrated that the serum zonulin levels of acute appendicitis patients were low, and that zonulin was a biomarker supporting the diagnosis. Therefore, it can be considered that the measurement of serum zonulin levels in patients presenting at ED with suspected acute appendicitis will help physicians at the stage of diagnosis.

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