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Clostridioides (Clostridium) difficile 감염에서 대변 Neutrophil Gelatinase-Associated Lipocalin과 Calprotectin의 생물학적 표지자로서의 임상적 유용성

Clinical Utility of Fecal Neutrophil Gelatinase-Associated Lipocalin and Calprotectin as Biomarkers of *Clostridioides (Clostridium) difficile* Infection

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Background: Current methods for diagnosing *Clostridioides difficile* infections (CDIs) fail to provide information on their severity. Fecal neutrophil gelatinase-associated lipocalin (NGAL) and calprotectin are candidate biomarkers for evaluating the severity of intestinal inflammation. We assessed fecal NGAL and calprotectin levels in patients with CDI and compared these values between subgroups of patients. We also evaluated their utility in predicting CDI clinical outcomes.
Methods: A total of 147 leftover fecal samples were obtained; 97 samples were from patients with CDI and 50 were from routine healthcare checkups. Fecal calprotectin and NGAL levels were measured using a Quantitative Fecal NGAL ELISA Kit and Quantitative Fecal Calprotectin ELISA Kit (Epitope Diagnostics, USA).
Results: Significant differences in fecal NGAL and calprotectin levels were observed between CDI patients and healthy controls ($P<0.0001$ for both). Significant differences in fecal NGAL and calprotectin levels were also seen between patients with high and low *tcdB* gene load ($P=0.005$ and 0.006, respectively). Fecal calprotectin levels were lower in patients with leukopenia ($P=0.002$), and high calprotectin levels were associated with severe CDI and treatment failure ($P=0.021$ and 0.033, respectively).
Conclusions: Fecal NGAL and calprotectin levels were higher in patients with CDI than in healthy controls and correlated with high *tcdB* gene loads. Leukopenia patients with CDI had significantly lower levels of calprotectin and the assessment should be regarded with caution. High fecal calprotectin levels were also associated with severe CDI and treatment failure. This warrants future studies with more patients and in-depth analyses.

Key Words: *Clostridioides difficile*, Fecal neutrophil gelatinase-associated lipocalin, Calprotectin

INTRODUCTION

Clostridioides difficile, previously known as *Clostridium difficile*, is a anaerobic gram-positive spore-forming bacterium that is prevalent in soil [1]. Active *C. difficile* infections (CDIs) are a leading cause of antibiotic-associated nosocomial diarrhea and colitis in hospitals in the industrialized world [2]. Current diagnostic methods [3, 4] for CDI recommend a combination of a toxin enzyme immunoassay (EIA), nucleic acid amplification test, and glutamate dehydrogenase assay, but these tests do not provide direct information on intestinal inflammation status [5-7]. As fecal biomarkers are more reflective of intestinal inflammation status than serum-based biomarkers, there is a need for easily measurable fecal bio-

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markers that reflect intestinal inflammation status [8]. Among fecal biomarkers, fecal neutrophil gelatinase-associated lipocalin (NGAL), calprotectin, and lactoferrin are the most researched biomarkers related to intestinal inflammation; although they are not yet considered gold standards for CDI diagnosis and monitoring, research is ongoing [9-11]. The absence of evidence-backed biomarkers for CDI has led to the dependence on clinical symptoms and other non-specific laboratory tests for determining disease severity and treatment progress. As CDI tends to occur in multimorbid patients, dependence on non-specific indicators is highly problematic and the results may be potentially confusing. In this study, we demonstrated that fecal NGAL and calprotectin levels were higher in patients with CDI than in healthy controls. We also evaluated the utility of these biomarkers for determining disease severity, as measured by existing international clinical guidelines for CDI, and for predicting the clinical outcomes of CDI.

MATERIALS AND METHODS

1. Study population and sample preparation

A total of 147 leftover fecal samples, consisting of 97 samples from patients with CDI and 50 samples from routine healthcare checkups, were obtained between November 2017 and March 2018. This study protocol was approved by the Institutional Review Board of our institution before the enrolment of the first patient (KUH1200089). Fecal NGAL and calprotectin levels were measured in residual fecal samples that would otherwise have been discarded, and no further tests were performed. Therefore, we were given an exemption for written informed consent from the enrolled patients. CDI diagnosis was established by testing symptomatic patients (diarrhea) with a *C. difficile* glutamate dehydrogenase assay and real-time polymerase chain reaction (PCR) assay for *tcdB* toxin genes using the Xpert *C. difficile* Assay (Cepheid, Sunnyvale, CA, USA). The demographic and clinical characteristics of the study population are shown in Table 1. The medical records of the *tcdB*-positive patients were reviewed and the patients were divided into the subgroups described in Table 2. A cycle threshold (C_t) value of 26.3 was used as the cut-off for high or low toxin *tcdB* gene load [4]. Leukopenia was defined as a white blood cell (WBC) count of less than $<4\times10^9$/L, while treatment failure was defined as the persistence of symptoms after treatment or any change in the medication from oral metronidazole to oral vancomycin. CDI severity was assessed as prescribed by the Infectious Diseases Society of America/Society for Healthcare Epidemiology of America (IDSA/SHEA) [3] and European Society of Clinical Microbiology and Infectious Diseases (ESCMID) [12]. The clinical criteria of CDI

Table 1. Demographic and clinical characteristics of the study population

	Controls (N=50)
Females (%)	29 (56.9)
Age (yr), mean ± SD	63.8 ± 10.9
	Patients (N=97)
Females (%)	40 (41.2)
Age (yr), mean ± SD	64.6 ± 17.4
Severe CDI according to IDSA/SHEA guidelines (%)	21 (21.7)
Severe CDI according to ESCMID guidelines (%)	77 (79.4)
Leukopenia (%)	17 (17.5)
30-day all-cause mortality (%)	10 (10.3)
Treatment failure (%)	17 (17.5)
History of antibiotic usage within 3 days (%)	62 (63.9)

Abbreviations: IDSA/SHEA, Infectious Diseases Society of America/Society for Healthcare Epidemiology of America; ESCMID, European Society of Clinical Microbiology and Infectious Diseases; CDI, *Clostridioides difficile* infection.

Table 2. Criteria for subgroups of patients with CDI

Leukopenia (WBC count $<4\times10^9$/L)
Low/high *tcdB* gene load (C_t value ≤ 26.3)
Antibiotic usage within 3 days
IDSA/SHEA and ESCMID severity
Low/high fecal NGAL and calprotectin levels*

*Defined as being lower or higher than median values (6.51 and 88.82 μg/g for NGAL and calprotectin, respectively).
Abbreviations: WBC, white blood cell; IDSA/SHEA, Infectious Diseases Society of America/Society for Healthcare Epidemiology of America; ESCMID, European Society of Clinical Microbiology and Infectious Diseases; NGAL, neutrophil gelatinase-associated lipocalin; CDI, *Clostridioides difficile* infection.

Table 3. Clinical criteria of CDI severity

	IDSA/SHEA clinical definition	ESCMID clinical definition*
Non-severe	Leukocytosis (WBC count $<15\times10^9$/L) and serum creatinine level <1.5 mg/dL	-
Severe	Leukocytosis (WBC count $\geq 15\times10^9$/L) or serum creatinine level ≥ 1.5 mg/dL	Age ≥ 65 years or Leukocytosis (WBC count $\geq 15\times10^9$/L) or serum creatinine level ≥ 1.5 mg/dL or decreased blood albumin (<3.0 g/dL) or other comorbidity
Fulminant	Hypotension or shock, ileus, megacolon	-

*The ESCMID clinical definition does not include a non-severe or fulminant category.
Abbreviations: WBC, white blood cell; IDSA/SHEA, Infectious Diseases Society of America/Society for Healthcare Epidemiology of America; ESCMID, European Society of Clinical Microbiology and Infectious Diseases; CDI, *Clostridioides difficile* infection.

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severity as defined by these two societies are shown in Table 3.

2. Measurement of fecal calprotectin and NGAL levels

Fecal samples were prepared using a Fecal Calprotectin Sample Collection Kit (Epitope Diagnostics, San Diego, CA, USA). The collected fecal matter was added to 10 mL of sample extraction buffer and frozen at –70°C before being used in the ELISA assay. Fecal calprotectin and NGAL concentrations were measured using a Quantitative Fecal Calprotectin ELISA Kit and Quantitative Fecal NGAL ELISA Kit (Epitope Diagnostics, San Diego, CA, USA), respectively [13, 14].

3. Statistical analysis

Data are expressed as the median and interquartile range. Groups were compared using the Mann-Whitney U and Kruskal-Wallis tests. Receiver-operating characteristic (ROC) curves and area under the curve (AUC) were used to determine cut-off levels. Microsoft Excel (version 14.0.7113.5005, Microsoft, Seattle, WA, USA) and MedCalc Software (version 16.8.4, MedCalc Software bvba, Ostend, Belgium) were used for statistical analyses. A *P* value less than 0.05 was considered statistically significant.

RESULTS

1. Fecal biomarker levels in patients and controls

We compared the levels of both fecal biomarkers in patients with CDI and healthy controls. The concentrations of the fecal biomarkers were higher in patients than in healthy controls ($P<0.0001$, Fig. 1). We then performed ROC curve analysis to determine the optimum cut-off value for differentiating between healthy controls and patients with CDI (Fig. 2). The optimal cutoff and AUC values were 4.25 μg/g and 0.767, respectively, for NGAL, and 7.66 μg/g and 0.889, respectively, for calprotectin. Pearson's correlation analysis of the two fecal biomarkers also confirmed the high correlation between the two biomarkers ($r=0.608$, $P<0.0001$).

2. Fecal biomarker levels in patient subgroups

We compared fecal NGAL and calprotectin levels in subgroups of patients with CDI (Table 4). Fecal NGAL and calprotectin levels were significantly different in the high and low *tcdB* gene load groups ($P=0.005$ and 0.006, respectively). As both fecal biomarkers are found in neutrophils, we then compared their levels in non-leukopenia and leukopenia patients. While no significant difference was observed in NGAL levels ($P=0.553$), the difference in calprotectin levels was statistically significant ($P=0.004$). No statistically significant differences in fecal biomarker levels were associated with antibiotic usage within 3 days of diagnosis or for IDSA/SHEA clinical severity grade ($P=0.137$ and 0.430, respectively). For ESCMID clinical severity, the differences in fecal NGAL levels were not statistically significant, but the differences in fecal calprotectin levels were significantly different ($P=0.16$ and 0.021 respectively).

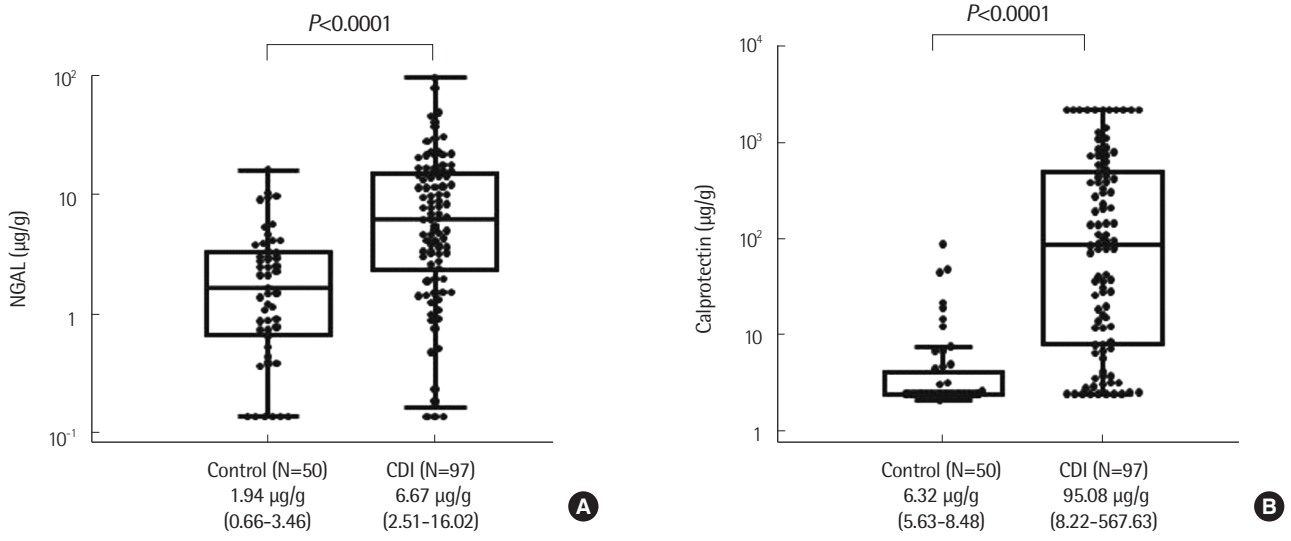
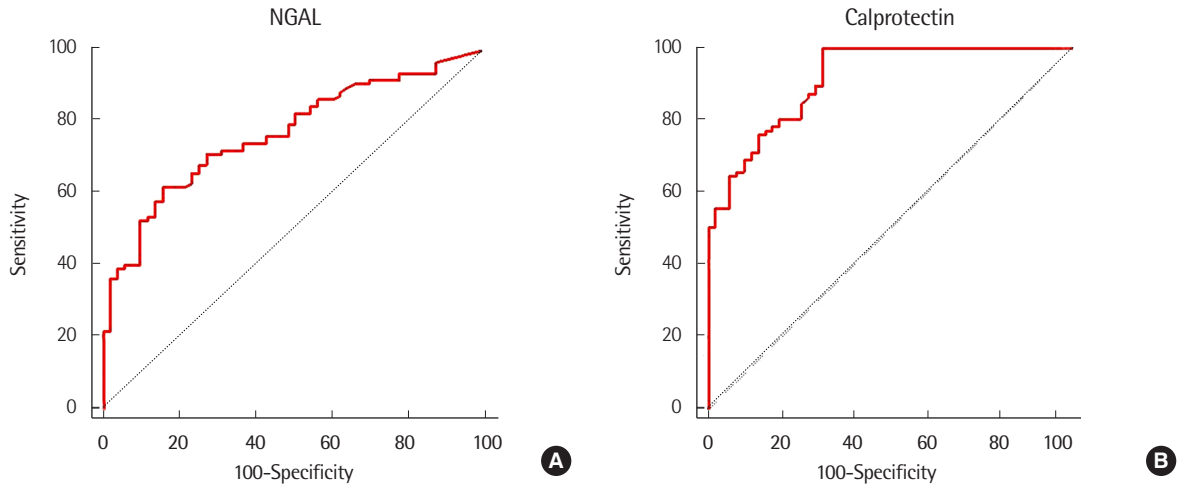


Fig. 1. Comparison of fecal (A) NGAL and (B) calprotectin levels in patients with CDI and healthy controls. All data are median and interquartile ranges.



	NGAL	Calprotectin
Optimal cutoff (μg/g)	4.25	7.66
AUC (95% CI)	0.767 (0.691-0.843)	0.889 (0.825-0.933)
P	<0.0001	<0.0001

Fig. 2. ROC curves for fecal (A) NGAL and (B) calprotectin levels in the healthy control group and patients with CDI.

Table 4. Evaluation of fecal NGAL and calprotectin levels in subgroups of patients with CDI

		NGAL (μg/g)	*P*	Calprotectin (μg/g)	*P*
Leukopenia	Yes (N=18)	3.80 (3.27-10.16)*	0.553	12.43 (2.89-41.26)*	0.004†
	No (N=79)	7.19 (2.12-16.03)*		139.82 (13.95-677.76)*	
Antibiotic use within 3 days	Yes (N=62)	9.06 (3.51-18.21)*	0.098	77.78 (4.24-419.67)*	0.253
	No (N=35)	5.16 (1.56-14.83)*		141.83 (14.22-674.51)*	
tcdB gene load	High (N=39)	10.27 (4.01-21.01)*	0.005†	274.05 (41.17-1,019.61)*	0.006†
	Low (N=58)	4.60 (1.46-11.94)*		35.91 (6.86-304.96)*	
IDSA/SHEA severity	Severe (N=23)	10.27 (3.34-22.47)*	0.137	141.83 (3.74-1,339.82)*	0.430
	Non-severe (N=74)	5.40 (2.43-14.46)*		82.48 (8.67-419.67)*	
ESCMID severity	Severe (N=77)	7.19 (3.30-16.36)*	0.160	112.05 (13.53-624.66)*	0.021†
	Non-severe (N=20)	3.39 (1.25-15.38)*		11.95 (4.30-224.64)*	

*All data are median and interquartile ranges; †$P<0.05$ indicates statistical significance.
Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; IDSA/SHEA, Infectious Diseases Society of America/Society for Healthcare Epidemiology of America; ESCMID, European Society of Clinical Microbiology and Infectious Diseases; CDI, *Clostridioides difficile* infection.

Table 5. Fecal NGAL and calprotectin levels by clinical outcome

		NGAL (μg/g)	*P*	Calprotectin (μg/g)	*P*
30-day all-cause mortality	Yes (N=10)	7.72 (4.45-23.76)*	0.26	296.95 (40.47-1,094.96)*	0.159
	No (N=87)	6.44 (2.12-15.5)*		79.46 (7.46-442.77)*	
Treatment failure‡	Yes (N=6)	8.53 (4.19-19.08)*	0.13	382.19 (63.97-1,503.52)*	0.033†
	No (N=80)	5.46 (1.98-15.38)*		77.78 (7.08-426.49)*	

*All data are median and interquartile ranges; †$P<0.05$ indicates statistical significance; ‡Treatment failure was defined as the persistence of symptoms after treatment or as a change in the medication.
Abbreviation: NGAL, neutrophil gelatinase-associated lipocalin.

3. Comparison of fecal biomarkers for the prediction of clinical outcomes

We analyzed the correlation between fecal NGAL and calprotectin levels and the clinical outcomes among the patient groups (Table 5). There was no statistically significant difference in 30-day all-cause mortality associated with either fecal NGAL or cal-

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protectin (P=0.260 and 0.159, respectively). Excluding the 11 patients that failed to show up for follow-up, a significant association with treatment failure observed for fecal calprotectin (P=0.033) but not for fecal NGAL (P=0.13).

DISCUSSION

This is the first study to explore the relationship between fecal NGAL and calprotectin levels in patients with CDI. As both fecal biomarkers are known to be upregulated in the intestine in inflammatory conditions, the increase in the levels of both fecal biomarkers in the intestine and the feces is reasonable [5-8, 15]. As both fecal NGAL and calprotectin are primarily found in neutrophils, we hypothesized that their levels in leukopenia patients differ [16, 17]. In comparison with non-leukopenia patients, CDI patients with leukopenia had lower fecal calprotectin levels. Thus, fecal calprotectin levels may be an unreliable CDI marker in leukopenia patients. We also calculated the best cut-off value to distinguish between patients with and without CDI for both fecal biomarkers. However, fecal calprotectin levels are also known to increase in response to *Campylobacter* infections and inflammatory diseases of the intestine [5, 15]. Hence, these biomarkers may be able to be used to estimate disease severity or monitor treatment progress. Towards this end, we analyzed the association between fecal biomarker levels and disease severity using existing clinical severity guidelines [3, 12], and found that fecal calprotectin level, but not NGAL level, could be used to stratify patients according to the ESCMID guidelines. Our finding indicates that fecal calprotectin may serve as a more useful biomarker than fecal NGAL. However, neither fecal biomarker could be used stratify patients according to the IDSA/SHEA guidelines. This failure to successfully stratify patients according to the IDSA/SHEA guidelines may be related to the definition of severity criteria and the characteristics of the patient population involved in this study [3]. Unlike the ESCMID guidelines, the IDSA/SHEA guidelines do not include age as a criterion and have a third category for fulminant severity. In our study group, only two patients had fulminant CDI, thereby rendering any statistical analysis futile owing to the low numbers.

Fecal NGAL and calprotectin are both non-specific markers; although their use as diagnostic markers may be limited, these molecules are still useful for predicting disease severity and outcomes [6, 8, 15, 18, 19]. The finding that *C. difficile tcdB* PCR C_t values were lower in patients with high biomarker levels may be indicative of a link between disease severity and fecal NGAL and calprotectin levels. However, some previous studies have shown that the EIA toxin is a more accurate predictor of patient mortality than *tcdB* PCR [9, 20]. A statistically significant relationship was observed between higher calprotectin, but not fecal NGAL, levels and treatment failure. When only non-leukopenia patients were included in the analysis, there was also a statistical association between higher fecal calprotectin levels and higher 30-day all-cause mortality. These results should be confirmed in future studies, as this was a single-center study on a low number of patients [20, 21]. As both fecal biomarkers are primarily secreted by neutrophils [16, 17], it is logical to theorize that their levels cannot increase in leukopenia patients. Therefore, fecal calprotectin should not be used as a biomarker for clinical outcomes in leukopenia patients. Furthermore, while hypervirulent ribotypes have been reported in Korea [22], their prevalence is lower than that in countries outside of East Asia, leading to lower mortality [23, 24].

The present study has a few limitations. It is a retrospective study and we could not monitor the changes in fecal NGAL and calprotectin levels [25], as the patients underwent CDI treatment. We also did not measure serum NGAL and calprotectin levels. We could have potentially employed the fecal toxin EIA to differentiate between actual CDI and colonization [9, 21, 26]. As we used leftover samples, we could not confirm whether the healthy controls were free of *C. difficile*. Future studies should enroll patients with non-CDI diarrhea as a control group to clarify the relationship between NGAL and calprotectin levels and CDI. In addition, when classifying patients based on CDI severity, the relative lack of patients with severe CDI limited the statistical power of our analysis. While the Xpert *C. difficile* assay presumptively identifies ribotype 027, with none identified among our samples, the fact that we did not perform genotyping for *C. difficile* ribotypes is another limitation of this study. Peretz et al. [27] noted that fecal calprotectin levels were higher in patients with ribotype 027; future studies should aim to distinguish between ribotypes. Finally, considering the poor state of fecal samples from patients experiencing diarrhea, it is possible that the fecal NGAL and calprotectin levels detected by ELISA are highly variable. Another factor related to fecal samples is that the initial sample was diluted in 10 mL of sample extraction buffer; this volume was larger than the

8-mL reagent used in the nucleic acid amplification test assay [13, 14, 28].

In conclusion, this is the first study to evaluate the utility of both fecal NGAL and calprotectin levels in determining disease severity and treatment status of CDI. We demonstrate that fecal NGAL and calprotectin levels were higher in patients with CDI than in healthy controls and highlight their significant relationship with *tcdB* gene load. High fecal calprotectin levels were significantly associated with disease severity and treatment failure. Fecal calprotectin levels were significantly lower in leukopenia patients, suggesting the need for the cautious interpretation of fecal calprotectin levels in leukopenia patients. Further studies with a larger group of patients are warranted.

요 약

배경: 현재 *C. difficile* 감염(CDI)에 쓰이는 진단법은 그 중증도에 대한 정보를 제공하지 못한다. 장내 염증 중증도에 대한 표지자 중 대변 내 neurophil gelatinase-associated lipocalin (NGAL)과 calprotectin이 있다. 본 연구에서는 CDI 환자에서 대변 NGAL과 calprotectin 농도를 측정하였고 여러 요인으로 분류하여 비교하였다. 또한 이 표지자들의 임상적 지표로서의 유용성을 평가하고자 하였다.

방법: CDI 환자의 잔여 대변 97검체 및 건강 검진 환자의 잔여 대변 50검체로 147개의 대변 검체를 수집하였다. 대변 내 NGAL과 calprotectin 농도는 Quantitative Fecal Calprotectin ELISA Kit 및 Quantitative Fecal NGAL ELISA Kit (Epitope Diagnostics, USA)를 사용하여 측정하였다.

결과: 대변 NGAL과 calprotectin 농도 모두 CDI 환자군과 대조군 간 유의한 차이가 있었다($P<0.0001$). CDI 환자 군에서 대변 NGAL과 calprotectin 모두 *tcdB* 유전자 농도에 따라 유의한 차이가 있었다($P=0.005$, 0.006). Calprotectin 농도는 백혈구감소증 환자에서 유의하게 낮았으며($P=0.002$), 중증도와 치료실패와 유의한 연관성이 있었다($P=0.021$, 0.033).

결론: 대변 NGAL과 calprotectin 농도는 CDI 환자에서 건강인에 비해 유의하게 높고 *tcdB* gene load와도 유의한 상관 관계가 있었다. 백혈구감소증 환자에서 calprotectin 농도가 유의하게 낮으므로 주의가 필요하다. 대변 내 calprotectin 농도는 중증도 및 치료실패와 연관이 있었고 더 많은 환자를 대상으로 추가적인 분석이 필요하다고 생각한다.

Conflicts of Interest

None declared.

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