



# Fecal Calprotectin: A Screening Marker for the Early Detection of Necrotizing Enterocolitis among Children in Egypt

Ayman Emil Eskander<sup>1</sup>, Rania Hosni Tomerak<sup>1</sup>, Mona Safwat<sup>1</sup>, Carolyne Ghobrial<sup>1</sup>, Mohammad Nabih Almohammady<sup>1</sup>, Walaa Abdelfattah<sup>2\*</sup>

1. Department of Pediatrics, Faculty of Medicine, Cairo University, Cairo, Egypt

2. Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt

## ABSTRACT

**Background:** Necrotizing enterocolitis (NEC) is a lethal disease affecting newborns with significant morbidity and mortality rates. Moreover, it is the most eminent gastrointestinal threat affecting premature newborns. Unfortunately, early symptoms and signs are usually vague; therefore, there is a special demand for sensitive biomarkers in this regard. This study aimed to investigate the role of fecal calprotectin in stage I NEC and identify specific cut off value at this stage to differentiate stage I NEC from other gastrointestinal disorders.

**Methods:** This cross-sectional study was conducted at New Children Hospital, Cairo University, Egypt. In total, 100 newborns were included in this study who were assigned to the patient group with stage I NEC ( $n=60$ ) and control group ( $n=40$ ) with age and gender-matched newborns. Fecal calprotectin level was assessed using an enzyme-linked immunosorbent assay in both groups. Follow up of the patient group was performed for the development of stage II or III NEC.

**Results:** The patient group obtained significantly elevated levels of fecal calprotectin, compared to the control group ( $P=0.000$ ). Within the patient group, 43 (71.66%) newborns developed stage II or III NEC, whereas 17 (28.33%) cases developed no NEC. In addition, the level of fecal calprotectin was significantly higher in the group who developed stage II or III NEC ( $P=0.001$ ). According to the receiver operating characteristic (ROC) curve, the cutoff value of 109.5  $\mu\text{g/g}$  feces showed 100% sensitivity and specificity, and the area under the ROC curve was equal to 1 in differentiating NEC from other conditions.

**Conclusion:** The study showed that fecal calprotectin can be used as a sensitive and specific marker for the early detection of necrotizing enterocolitis.

**Keywords:** Cutoff, Fecal calprotectin, Necrotizing enterocolitis, Neonates, Screening

## Introduction

Necrotizing enterocolitis (NEC) is a serious inflammatory disorder that is characterized by intestinal necrosis with sufficiently great morbidity and mortality rates in the neonatal intensive care units (NICU) (1). It affects about 1%-5% of newborns in the NICU, especially premature and low birth weight infants (2). Continuous advances have been achieved in the care of critically ill neonates; however, the complications and mortality rates of NEC have remained unchanged (3).

Abnormal inflammatory response by the newborn intestine to luminal microbes is the main theory for NEC pathogenesis (4). The immune system in neonates is vulnerable (5), which disrupts the pro-inflammatory and anti-inflammatory mediator balance; moreover, the dysbiosis in gut microbiota can contribute to the disease occurrence (6, 7, 8). The NEC has three clinical stages according to Bell's staging system (9). The classic form usually presents with feeding intolerance, abdominal distension, and blood in

\* Corresponding author: Walaa Abdelfattah, Department of Clinical and Chemical Pathology, Cairo University, Cairo, Egypt. Tel: 00201001058405; Email: walaafattah@cu.edu.eg

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the stool. Complications appear in the form of abdominal discoloration and intestinal perforation that may lead to peritonitis (10). Many preventive measures have been previously described (11,12); however, the prognosis of NEC is generally poor owing to its rapid onset and progression to death, as well as its morbidity when the infant survives (13).

The diagnosis of NEC remains challenging since the initial presentation is nonspecific with no single reliable laboratory tests or imaging tools (14). Therefore, it is mandatory to search for highly sensitive and specific biomarkers for the screening of NEC (15).

Calprotectin is one of more than 20 proteins in the so-called S-100 family that forms about 60% cytosolic protein in neutrophils. It has antimicrobial activity as an antifungal and antibacterial agent (16). It inhibits metalloproteinases and chelates with zinc and manganese ions since it is proposed to inhibit microbial proliferation (17).

During intestinal inflammation, neutrophils migrate towards the gut and are sequestered into its wall. Following that, calprotectin is released from activated neutrophils into the intestinal lumen and exhibits pro-inflammatory properties (18). Its amount in stool reflects the movement of neutrophil into the gut lumen; therefore, it is a good indicator of the severity of inflammation (19). Calprotectin resists heat, proteolysis, and degradation by bacteria in the gut. Moreover, it participates in the diagnostic workup of neonates suspected to have inflammatory bowel disease (20).

Fecal calprotectin has been extensively studied in NEC, and its concentration was found to be elevated in newborns with NEC, compared to healthy neonates. Its levels appeared to decline after the treatment initiation, which may highlight its role as a potential marker for monitoring response to therapy (21). However, it is not known yet whether high calprotectin concentrations at the beginning of bowel symptoms recognize neonates with true NEC versus other bowel disorders. Accordingly, its significance as an early screening marker remains unknown. Moreover, no definite cut-off values have been settled for screening in early bowel symptoms (22). It is hypothesized that measuring its level early in disease could help as a screening marker.

## Methods

This cross-sectional study included 100 newborns who were admitted to the NICU at New

Children Hospital, Cairo University, Egypt. After obtaining parental informed consent, stool samples were collected from the newborns over the period from April 2017 to March 2018. The study protocol was approved by the Ethics Committee of the Pediatrics Department, Cairo University, Egypt. The inclusion criteria were: 1) gestational age of fewer than 35 weeks, 2) age of more than 7 days old since fecal calprotectin is high in the first week of life, and 3) male and female gender. On the other hand, the preterm infants with a gut anomaly or those who underwent surgery were excluded from the study.

Subsequently, the newborns were assigned to the patient (n=60) and control groups (n=40). The patient group included preterm infants with feeding intolerance in stage I NEC according to the modified Bell's criteria which included systemic signs, such as apnea, bradycardia, lethargy, and temperature instability, as well as intestinal signs, such as recurrent gastric residual, abdominal distention, and occult or gross blood in the stool. Moreover, they were followed up for the development of stage II and III NEC. On the other hand, the control group included preterm infants with the same age and gender who were free of clinical criteria of NEC according to the modified Bell's staging.

The two groups were subjected to thorough history taking including prenatal, natal, and postnatal history, as well as the amount and type of feeding, and symptoms of feeding intolerance. The clinical examinations included the signs of prematurity, neonatal reflexes, vital signs, as well as abdominal and other system examinations. Furthermore, radiological investigations included chest X-ray and erect abdominal radiographs, and laboratory investigations included complete blood count, C-reactive protein (CRP) (reference range of CRP is less than 6mg/L), and fecal calprotectin measurement by Enzyme-Linked Immunosorbent Assay (ELISA).

## Data Collection

One stool sample was collected from each neonate with feeding intolerance, as well as those in the control group with no obvious gastrointestinal pathology after the first week of delivery (between 1-2 weeks old). In total, one to five grams of stool were collected from diapers and placed in a suitable clean container with no preservative. The collected samples were stored at 2-8°C for up to six days or frozen at -20 °C for up to one year. Stool samples from this study were stored at 2-8°C in a refrigerator and then

delivered to the laboratory within four days of collection. Moreover, the samples were stored frozen at -20 until the time of the assay. Frozen samples were thawed and equilibrated to room temperature before extraction and testing.

### **Calprotectin Assay**

The fecal extract was performed, and the concentration of calprotectin was measured using ELISA specific for calprotectin (EDI™ Quantitative Fecal Calprotectin ELISA kit from Epitope Diagnostics, Inc KT- 849). The collected samples were diluted in two steps with 1:40 and 1:9 before measurement. In total, 50 mg of stool was added to Extraction Buffer using an inoculation loop (39 parts of the stool volume), vortexed to dissolve stool sample, and set at room temperature vertically for 30 min for sedimentation. A clear supernatant with no particles (0.15 mL) was transferred to a clean tube with 1.2 ml Extraction Buffer and mixed well. This extracted sample was subjected to the measurement of fecal Calprotectin.

The ELISA kit was intended for the measurement of human calprotectin quantitatively in stool samples using the two-site "sandwich" technique with two selected antibodies that bind to different epitopes of human calprotectin. Optical density was obtained by reading the absorbance at 450 nm. A standard curve was drawn by adding the absorbance of all standard levels on the ordinate against their concentration. The human calprotectin values for the test samples were calculated directly from the standard curve according to their corrected absorbance.

### **Statistical Analysis**

Data were tabulated and analyzed in SPSS software (version 16.0). Nominal data were shown as frequency and percentage, and their comparison was carried out using the Chi-square test. In addition, numerical data were described as mean $\pm$ SD and compared using a t-test. In the same line, the nonparametric data were described as median and range, and numerical associations were tested using Pearson's correlation coefficient. Sensitivity, specificity, and odds ratios were utilized to test the potential risk factors. A p-value less than 0.05 was considered statistically significant.

## **Results**

This study included two groups of patients and control. There were 60 neonates in the first group who had feeding intolerance (i.e., suspected NEC

or stage I NEC). They were furtherly categorized into two subgroups according to the clinical course of the disease. Following that, 43 cases developed stage II and III NEC and were assigned into the NEC group, and 17 neonates had no NEC and remained in stage I NEC or non-NEC group. On the other hand, the control group included healthy neonates (n=40).

### **Demographic Characteristics**

The mean ages of the neonates in the patient and control groups were  $17.02\pm5.35$  and  $15.95\pm5.80$  days, respectively. Moreover, regarding gender, the majority of the patients were female in the patient (55%, n=33) and control groups (55%, n=22). The mean age of the cases in stage II and III NEC group was  $16.56\pm5.35$  days, and that of the neonates in stage I NEC group was obtained at  $18.18\pm5.35$  days.

Moreover, the number of male newborns (n=22, 51.2%) was higher than that of females in the NEC group (n=21, 48.8%). Moreover, the non-NEC group included 5 (29.4%) and 12 (70.6%) male and female neonates, respectively. No statistically significant difference was observed among the studied groups regarding age and gender (Tables 1-2).

### **Antenatal History**

The patient group obtained significantly higher fetal distress, compared to the control group ( $P=0.006$ ). There was no statistically significant difference between NEC and non-NEC groups regarding fetal distress.

### **Physical Examination**

#### **A) Vital Signs**

The patient group had significantly higher heart and respiratory rates, as well as significantly lower mean arterial blood pressure, compared to the control group ( $P=0.000$ ,  $P=0.004$ , and  $P=0.000$ , respectively). Moreover, there was a significantly decreased mean arterial blood pressure in the patient group, compared to the control group; however, no significant difference was observed between the patient and control groups regarding temperature. Furthermore, no statistically significant difference was noticed between NEC and non-NEC groups regarding heart rate, respiratory rate, or mean arterial blood pressure.

#### **B) General and Systemic Examination**

All neonates in the patient group had cyanosis, poor neonatal reflexes, or altered conscious level according to the pediatric Glasgow Coma Scale

**Table 1.** Demographic characteristics and laboratory data of the patients and controls

	Patient group	Control group	P-value
Demographic Characteristics			
Age (days)	17.02±5.35	15.95± 5.80	
Gender	Males 27 (45%) Females 33 (55%)	Males 18 (45%) Females 22 (55%)	
General and Systemic Examination			
Weight (grams)	1629.67 ± 413.75	1705.25 ± 338.49	0.320
Laboratory Investigations			
Fecal calprotectin(µg/g)	143.30 (40.50- 2510)	32.15 (25-90)	0.000

**Table 2.** Demographic characteristics and laboratory data of Stage I and Stagell and III groups

	Stage I NEC	Stage II and III NEC	P-value
Demographic Characteristics			
Age (days)	18.18±5.35	16.56± 5.35	
Gender (N)	Males 5 (29.4%) Females12 (70.6%)	Males 22 (51.2%) Females21(48.8%)	
General and Systemic Examination			
Weight (grams)	1569.41±390.05	1653.49±424.83	0.469
Laboratory Investigations			
Fecal calprotectin (µg/g)	69 (40.50 - 103)	167 (116 - 2510)	0.001

NEC: Necrotizing enterocolitis, ICU: Intensive care unit, ELISA: Enzyme-Linked Immunosorbent Assay, CRP:C-reactive protein, I/T: Immature to total neutrophils ratio, ROC: Receiving Operator Curve, AUC: Area under the ROC curve

(Patient group was stupor and obtained Glasgow coma scale score of 6-8 score, whereas the control group was conscious and alert and obtained Glasgow coma scale score of 9-15). It is worth mentioning that none of these conditions were observed in the control group. All neonates in the control group and 39 (65%) cases in the patient group had jaundice, which showed a statistically significant difference between these two groups in this regard ( $P=0.000$ ).

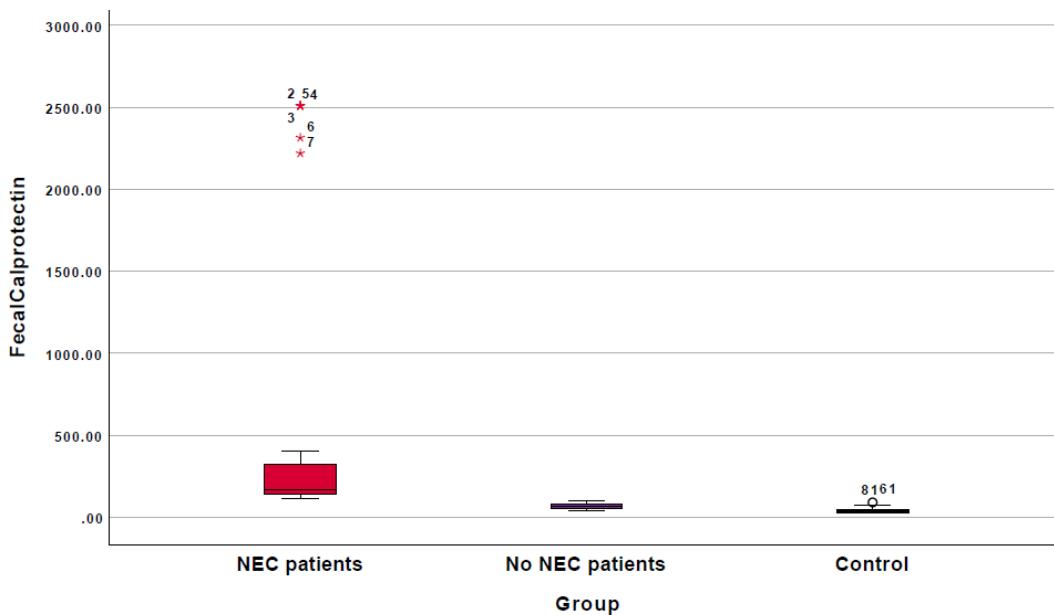
The patient group had sufficiently significant higher rates of the insertion of central venous and umbilical catheters, compared to the group ( $P=0.043$  and  $P=0.000$ , respectively). None of the studied newborns required urinary catheterization. There was no significant difference between the NEC and non-NEC groups regarding the insertion of a central venous catheter. However, umbilical catheters were inserted in 82.4% and 53.5% neonates in the non-NEC and NEC groups, respectively, which had a significantly higher incidence in the non-NEC group ( $P=0.045$ ).

The mean weights of the neonates in the patient and control groups were  $1629.67\pm13.75$  and  $1705.25\pm338.49$  gr, respectively. Moreover, the mean weights of the newborns in the NEC and non-NEC groups were estimated at  $1653.49\pm424.83$  and  $1569.41\pm390.05$  gr, respectively. There was no statistically significant difference between patient and control groups or between NEC and non-NEC groups regarding weight ( $P=0.320$  and  $P=0.469$ ).

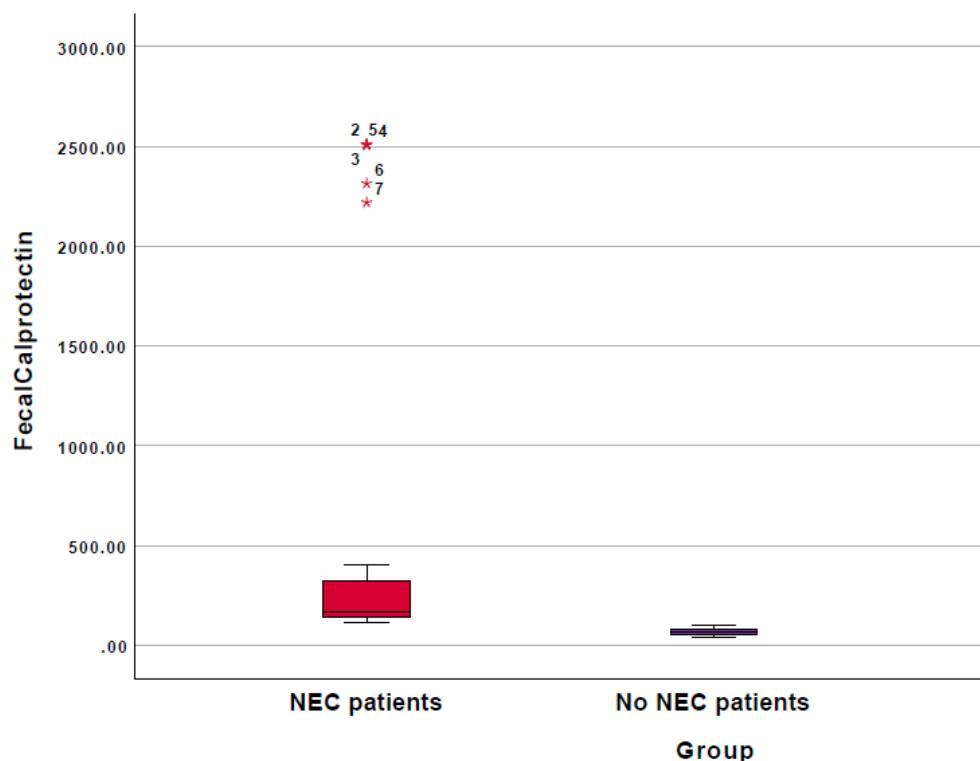
### Laboratory Investigations

The patient group obtained significantly higher rates in terms of total leucocytic count, immature to total neutrophils (I/T) ratio, CRP, potassium level, and serum creatinine (all had a P-value of 0.000), compared to the control group. However, this group had significantly lower rates regarding hemoglobin, platelet count, sodium level, and total calcium ( $P=0.013$ ,  $P=0.000$ ,  $P=0.000$ , and  $P=0.030$ , respectively). There were no statistically significant differences between NEC and non-NEC groups regarding other parameters in the laboratory results.

Regarding the fecal calprotectin, the patient group obtained a median fecal calprotectin concentration (143.30 µg/g) ranged between 40.50 µg/g and 2510 µg/g, whereas the corresponding value in the control group was estimated at 32.15 µg/g ranged between 25 µg/g and 90 µg/g. Moreover, the patient group obtained significantly elevated rate of fecal calprotectin, compared to the control group ( $P=0.000$ ) (Figure 1). In the same line, statistically higher fecal calprotectin measurements were found in the NEC group, compared to the non-NEC group ( $P=0.001$ ), and the NEC group had a median level of 167 µg/g ranged between 116 µg/g and 2510 µg/g. On the other hand, the non-NEC group obtained a median of 69 µg/g ranged between 40.50 µg/g and 103 µg/g (Figure 2). Regarding the other parameters in this study, there were no correlations between them and the fecal calprotectin level.



**Figure 1.** Comparison of NEC patients, non-NEC patients, and control group regarding fecal calprotectin



**Figure 2.** Comparison of NEC group and suspected NEC group regarding fecal calprotectin

With regard to fecal calprotectin cut off point, the fecal cutoff value equals 109.5 µg/g feces using Receiving Operator Curve (ROC). Moreover, relatively strong diagnostic capability showed 100% sensitivity and specificity for stage I NEC, and the area under the ROC curve (AUC) was

equal to 1 ( $P=0.000$ , Tables 1-2).

## Discussion

Early detection of NEC in stage I and prevention of progression into the more serious stage II and III remains a promising goal for every

neonatologist. Given the inability to predict the ultimate course of NEC in its early stage based on clinical variables alone, this study attempted to select preterm infants with feeding intolerance in the suspicious state of NEC and followed them for the development of Stage II and III. Moreover, the fecal calprotectin concentrations in the patients were measured and compared with those of the healthy neonates.

The results showed that fecal calprotectin was significantly elevated in patients with feeding intolerance, compared to the healthy neonates. Furthermore, the value of measuring fecal calprotectin was added in suspected NEC cases and its precision to predict the progression to more progressive stages. According to the results, the group in stages II and III NEC had a significantly higher fecal calprotectin level, compared to the suspected NEC group (stage I NEC).

In addition, it was revealed that a cutoff value of fecal calprotectin equal to 109.5 µg/g feces with a sensitivity and specificity of 100% in differentiation between newborns eventually developed NEC (stage II and III) and those with other diagnoses at the stage of suspicion of NEC (stage I) (AUC of ROC was 1). Therefore, these findings provide strong evidence that fecal calprotectin might be considered a promising screening marker that can be added for the investigations of preterm infants suspected to have NEC.

When preterm neonates develop feeding intolerance, abdominal distension, or other signs of intestinal distress, NEC is generally considered in the differential diagnosis. At the onset of the symptoms, it is usually difficult to know precisely whether the condition is indeed NEC, particularly when abdominal X-rays are non-diagnostic. Unfortunately, 50% of the affected neonates will develop progressive intestinal necrosis requiring urgent operation (23,24), and with intestinal involvement in polyarteritis nodosa, the mortality rate reaches 100%.

Patients who survive after developing NEC are facing serious morbidities. Despite promising trials, to date, no specific biomarker has been discovered to be useful as a stand-alone diagnostic test for the disease at its earlier stages. Fecal calprotectin has been intensively studied in NEC; however, few of these studies have focused on neonates with feeding intolerance in the early suspicious stage (stage I NEC) (25).

In our study, the mean age of developing feeding intolerance of the patient group was

obtained at  $17.02 \pm 5.357$  days, and there was no significant difference between patients and controls in terms of gender, gestational age, or maternal age. This result is in line with the findings of previous studies (26, 27). Age, gender, gestational age, and maternal age have no effect on the development of stage II and III NEC in patients with stage I NEC. This is also consistent with the results of a previously conducted study (25).

Some prenatal, natal, postnatal, and nutritional factors that may affect the progression of NEC form the suspected state. Additionally, intrauterine fetal distress showed a significantly higher incidence in the group of feeding intolerance which may be then considered a risk factor. However, it had no effects on the progression from suspected state to established NEC, compared to a study performed by Boo et al., in 2000 (28). They revealed no significant difference between the group of feeding intolerance and the tolerated oral feeding group regarding perinatal asphyxia.

Prenatal factors had no effects on the development of stage II and III NEC from the suspected state. This finding was in line with the results of previously conducted studies (25,27, 29). Cardiopulmonary status of neonates has been studied as an important risk factor for NEC development since the cardiorespiratory baseline changes may even precede abdominal signs (30).

In our study, the feeding intolerance group had significantly higher heart and respiratory rates, as well as a significantly lower mean arterial blood pressure, compared to the control group. However, these cardiopulmonary changes had no effects on the development of stage II and III NEC, compared to the results of a previously conducted study by Samuels et al. in 2017 (8).

Several studies have focused on inserted devices and considered them as risk factors for NEC development (31-33). In our study, the patient group showed a significantly higher rate of insertion of both the central and umbilical venous catheters, compared to the control group. In this regard, the findings in the present study showed that inserted devices could predispose to the development of NEC, which was not in line with the findings obtained by Boo et al., in 2000. They stated that stage I had higher rates of umbilical catheter insertion than stages II and III, whereas no effects were observed on disease progression using a central venous catheter.

Several studies have declared the importance of measuring fecal calprotectin in the diagnosis of NEC and its utility as a noninvasive prognostic marker for the progression of the disease (34). The results of the current study were compared to the findings of a previously performed study on concentrations of calprotectin. In a study carried out by Campeotto et al. in 2007 (26), a median fecal calprotectin level was obtained at 226 µg/g (16 mg/g-4775 µg/g). Moreover, in a study performed by Moussa et al. in 2016 (27), fecal calprotectin was higher in the study group with a mean value of  $334.33 \pm 236.61$  µg/g., compared to the control group.

The results of this study confirmed the findings of a previously performed study in which Reisinger et al. compared stage I with stages II and III NEC in 2012 (25). They found significant differences between NEC and non-NEC groups regarding fecal calprotectin. A cutoff value lower than that obtained in a previously conducted study was observed in this study; however, its sensitivity was greater. According to a study conducted by Reisinger et al. in 2012, a fecal calprotectin cutoff value of 286.3 µg/g feces was obtained with a sensitivity and specificity of 81% and 79%, respectively. Moreover, the AUC of the ROC curve was estimated at 0.82.

Calprotectin measurement could be affected by many factors, such as gestational and postnatal age (35). The current study revealed no significant correlation between fecal calprotectin level and gestational or postnatal age. Furthermore, the results were consistent with the findings of a study conducted by Campeotto et al. in 2007 (26), whereas they were not in line with those obtained from a study performed by Josefsson et al. in 2007 (36). They observed a positive relationship between fecal calprotectin level and postnatal age; however, Rougé et al. in 2010 (37) found a negative relationship between fecal calprotectin and gestational age. Fecal calprotectin level may be affected by the volume of enteral feeding, which is regarded as a discrepancy point among several studies (36, 37). There was no significant correlation between fecal calprotectin level and volume of enteral feeding in this study, which was in line with the findings in a study conducted by Campeotto et al. in 2007.

This study may have relevant applications since fecal calprotectin can be considered a promising screening marker that can be added for the investigation of preterm infants suspected to have NEC. It should be assessed after the first

week of life in these preterm newborns. This can help in early diagnosis, treatment, and application of preventive measures, which can improve the outcome of NEC and prevent deterioration to more serious disease with many complications. The majority of the published studies provided reference values for fecal calprotectin in healthy full-term and preterm neonates; however, few of them provided the corresponding values in NEC neonates. One of the limitations of this study was a small sample size. Therefore, further studies are recommended to be conducted on a larger scale and are required to evaluate the cutoff value due to the paucity of similar studies and the presence of differences in cutoff points among them.

## Conclusion

It is concluded that fecal calprotectin level was significantly higher in feeding intolerance neonates, compared to healthy controls, especially newborns who developed stages II and III NEC. A cut off value of fecal calprotectin in stage I NEC equal to 109.5 µg/g feces showed 100% sensitivity and specificity, as well as area under ROC curve that was equal to 1. These values are valuable to predict the NEC in preterm newborns who are at the stage of suspicion (stage I) for progression into stages II and III.

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## Conflicts of interest

The authors declare that they have no conflict of interest regarding the publication of this paper.

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