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# Fecal Calprotectin Level in Neonates with Necrotizing Enterocolitis

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## ABSTRACT

***Background:*** Necrotizing enterocolitis (NEC) is a disease with high mortality. It is more present in premature infants and can also happen in term and late preterm neonates. It may affect any segment of the small intestine or colon. However, most commonly influences the terminal ileum and proximal ascending colon. This disease might damage the entire bowel, which can be irreversible. Intestinal mucosal defects cause the migration of large numbers of inflammatory cells into the gut lumen. Extensive mucosal affection results in increased calprotectin levels.

This study aimed to investigate the role of fecal calprotectin as a non-invasive marker in the diagnosis of NEC for the better management of infants with NEC.

***Methods:*** This case-control cross-sectional study was performed in two groups. Group 1 was the case group consisting of the neonates admitted at Suez Canal University Hospital, Neonatal Intensive Care Unit with a clinical diagnosis of NEC. All cases were evaluated by Bell's staging criteria. Group 2 included control subjects. All the studied subjects had complete medical history, full physical examination, and laboratory investigations, including complete blood count, stool analysis, and C-reactive protein. Radiological examination entailed chest X-ray and erect abdomen X-ray, abdominal ultrasonography, and the measurement of stool calprotectin.

***Results:*** Fecal calprotectin level showed a positive strong correlation with NEC stages and this was statistically significant. Regarding the sequels of NEC, our study showed a positive correlation between NEC stage and fecal calprotectin level with r of 0.911 and P-value of < 0.001. The mean level of calprotectin in stage Ia was 226.9 µg/g with the maximum in patients affected with stage IIb (875 µg/g).

***Conclusion:*** According to the findings of this study, fecal calprotectin can be used as a marker in the diagnosis of NEC and has a strong positive correlation with the severity of NEC.

***Keywords:*** Calprotectin, Necrotizing enterocolitis, Neonate

## Introduction

Necrotizing enterocolitis (NEC) is defined as an inflammatory disease with multifactorial causes and is characterized by injury to the intestinal tract with defects ranging from minimal mucosal damage to full-thickness necrosis and even intestinal perforation (1).

The disease more affects infants who weigh less than 1.5 kg and has a high rate of mortality. Although it is more prominent in premature infants, it can also occur in term and late preterm infants. The NEC can influence any part of the intestine. Nonetheless, the most common areas are the terminal ileum and proximal ascending colon (2).

The early symptoms of this disease may be subtle and may include feeding intolerance, abdominal tenderness, abdominal distention, delayed gastric emptying, ileus, decreased bowel sounds, abdominal wall erythema, and hematochezia (2). Nonspecific signs encompass apnea, lethargy, decreased peripheral perfusion, shock, cardiovascular collapse, bleeding diathesis (3), hyponatremia, metabolic acidosis, decreased platelet and leucocytes count or leukocytosis, prolonged prothrombin time, activated partial thromboplastin time, and hypofibrinogenemia (4).

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While the main etiology of NEC remains unidentified, researchers found that it is multifactorial, (e.g., ischemia and reperfusion injury) and is aggravated by the activation of inflammatory intracellular cascades (5). Moreover, some of the triggering mechanisms include the inflammation of the intestinal mucosa, release of mediators and intraluminal bile acids, and down-regulation of growth factors leading to intestinal damage (6).

Although NEC occurs at any gestational age, it is mainly a disease of prematurity. However, it can occur in full-term newborns (7) and in critical cases with refractory hypotension and small for gestational age (8-11). It should be noted that genetic element may have a role in NEC (12) and infants with the definite genotypes of various cytokines have been linked to a higher incidence of NEC (13).

Calprotectin is an abundant calcium-binding protein in neutrophilic granulocytes, comprising up to 60% of the total cytosolic protein content of neutrophils (14). Intestinal mucosal defects or the elevated permeability of the intestinal barrier permit the migration of huge numbers of granulocytes into the intestinal lumen. In severe mucosal injury, the calprotectin levels may reach ten times above the upper reference limit of 30 mg/L (15).

Calprotectin is a member of S100 proteins (16). S100A8 comprises about 20% of the neutrophil cytoplasm and is found in the nucleus of some cells (17). With this background in mind, our objectives were to evaluate fecal calprotectin as a non-invasive marker in neonates with NEC and to assess the correlation between the level of fecal calprotectin and the clinical staging of NEC.

## Methods

This case-control cross-sectional study aimed to investigate the role of fecal calprotectin as a non-invasive marker in the diagnosis of NEC. The study was conducted on two groups. Group 1 was the group of patients and consisted of the neonates admitted at Neonatal Intensive Care Unit, Suez Canal University Hospital with clinical signs diagnosed as NEC. These cases were evaluated by Bell's staging criteria. Group 2 included healthy control subjects. The exclusion criteria entailed neonates who had sepsis and neonates with gastrointestinal anomalies, such as anorectal malformation (18).

The sample type was a random sample of both males and females. The sample size was calculated according to the following equation:

$$n = 2\left[\frac{(Z_{\alpha/2} + Z_{\beta}) * \sigma}{\mu_1 - \mu_2}\right]^2$$

In which n represents the sample size required in each group, σ refers to the estimation of the standard deviation in the study group, $Z_{\alpha/2}$ of 1.96 denotes the critical value that divides the central 95% of the Z distribution from the 5% in the tail, $Z_{\beta}$ is 0.84 (i.e., the critical value separating the lower 20% of the Z distribution from the upper 80%), $\mu_1$ signifies the mean in the group 1 (18), and $\mu_2$ refers to the mean in group 2 (18). As a result, the required sample size was obtained as 29 participants in the case group and 29 matched subjects in the control group, following adding 10% drop out (19).

The staging of NEC was assessed according to the Bell system (20). All the studied subjects had complete medical history, physical examination, and laboratory investigations, including complete blood count, stool analysis, and C-reactive protein. In addition, they underwent radiological examinations, namely chest X-ray, erect abdomen X-ray, and abdominal ultrasonography. The measurement of stool calprotectin was carried out after stool collection.

In order to prepare the samples, fecal samples (50-100 mg) were collected with a disposable, breakable inoculation loop (10 µl, sterile, firm loop, T572-B, Technical Service Consultants, Lancaster, UK) and were placed into a 14-ml disposable screw cap tube (Greiner tube, Greiner GmbH, Labortechnik, Frickenhausen, Germany).

The weight of the feces was measured and the loop handle was broken off leaving the loop and 4-6 cm of the handle inside the tube. The extraction solution containing urea and citrate (Nycomed-Pharma AS) was added in a weight: volume ratio of 1:50. Following 30 s agitation on a mixer (WhirliMixer™, Fisons Scientific Equipment, UK) and homogenization for 20 min at 1400 rpm on a shaker (Ika-Vibrax-VXR, IKA Werke, Janke and Kunkel GmbH, KG, Germany), 1 ml of the homogenate was transferred to an Eppendorf tube and was centrifuged for 20 min at 10,000 g.

Next, the supernatant (0.5 ml) was collected and analyzed immediately or was frozen at −20° C for later analysis (14) by original PhiCal enzyme-linked immunosorbent assay (ELISA) kit (Nycomed Pharma AS). The stools were thawed and 5 g of

feces were suspended in 10 ml of the extraction solution (1:3 dilutions) and homogenized at room temperature for 1 min at 20,000 rpm using a rod mixer (Ultra Turrax, IKA Werke, Janke and Kunkel GmbH). The homogenate was centrifuged and the supernatants were collected for analysis.

Statistical analysis was performed by the Statistical Package for Social Sciences (SPSS) software version 16. The correlations between different parameters were evaluated using Spearman rank correlation. For all tests *P*-value < 0.05 was considered significant. All the data are expressed as mean±SD. Ethical approval was taken from the Ethical Committee, Faculty of Medicine, Suez Canal University. Written consents were taken from all the parents of the participants before including them in the study and they had the right to refuse without effect on their provided services.

## Results

Fifty-eight neonates were enrolled in the study as 29 with NEC and 29 matched controls for comparison. According to Table1, both studied groups were matched for postnatal age, gestational age, and weight and no statistically significant difference was found. Table 2 shows that the two study groups were significantly different in terms of feeding. As demonstrated in Table 3, the most frequent stage among neonates with NEC was 1A (51.7%).

The relationship between fecal calprotectin level and NEC stage is summarized in Table 4. Fecal calprotectin level showed a positive strong correlation with NEC stage and this was statistically significant. The scattered plot curve (Figure 1) shows that there is a positive correlation between the level of fecal calprotectin and the clinical staging of NEC. Table 5 shows that fecal calprotectin level was much higher among NEC cases, compared to the controls with a statistically significant difference.

According to Table 6, fecal calprotectin level was affected by NEC stage. Receiver Operating Characteristics (ROC) curve and area under the curve (AUC) were calculated to detect the best cutoff value of fecal calprotectin for the diagnosis of NEC. The sensitivity and specificity of this value were obtained from the curve (Table 6). Figure 2 indicates the sensitivity, specificity, and area under the curve for the best cutoff value of fecal calprotectin for the diagnosis of NEC. This table shows that AUC = 1 and P-value < 0.01 (statistically significant). According to Figure 2, the best cutoff value, sensitivity, and specificity were calculated as 153 units, 96.6%, and 100%, respectively.

**Table 1.** Demographic characteristics of the study subjects (N=58)

| Characteristic | NEC group | Control group | P-value |
|---|---|---|---|
| Postnatal age (day) | | | |
| Mean±SD | 5.1±0.77 | 5.6±0.98 | 0.063 (NS) |
| Gestational age (week) | | | |
| Mean±SD | 33.8±1.31 | 34.1±1.19 | 0.495 (NS) |
| Weight (Kg) | | | |
| Mean±SD | 1.49±0.18 | 1.51±0.15 | 0.929 (NS) |

NS: no statistically significant difference

**Table 2.** Type of feeding among NEC versus the control group

| Type of feeding | NEC group N (%) | Control group N (%) | P-value |
|---|---|---|---|
| Artificial feeding | 9 (31 %) | 4 (13.8 %) | |
| Expressed breast milk | 0 (0 %) | 12 (41.4 %) | < 0.001* |
| Mixed feeding | 20 (69 %) | 13 (44.8 %) | |

* Statistically significant at P < 0.05

**Table 3.** Distribution of NEC stages among affected cases (N=29)

| NEC stage | N | % |
|---|---|---|
| 1A | 15 | 51.7 % |
| 1B | 5 | 17.2 % |
| 2A | 7 | 24.1 % |
| 2B | 2 | 7 % |
| Total | 29 | 100 % |

**Table 4.** Mean fecal calprotectin level among each stage of NEC in the studied patients

| NEC stage | Fecal calprotectin level (ug/g) (mean±SD) | P-value |
|---|---|---|
| 1A | 226.9±67.1 | |
| 1B | 588±59.8 | < 0.001 |
| 2A | 776±57.7 | |
| 2B | 875±35.4 | |

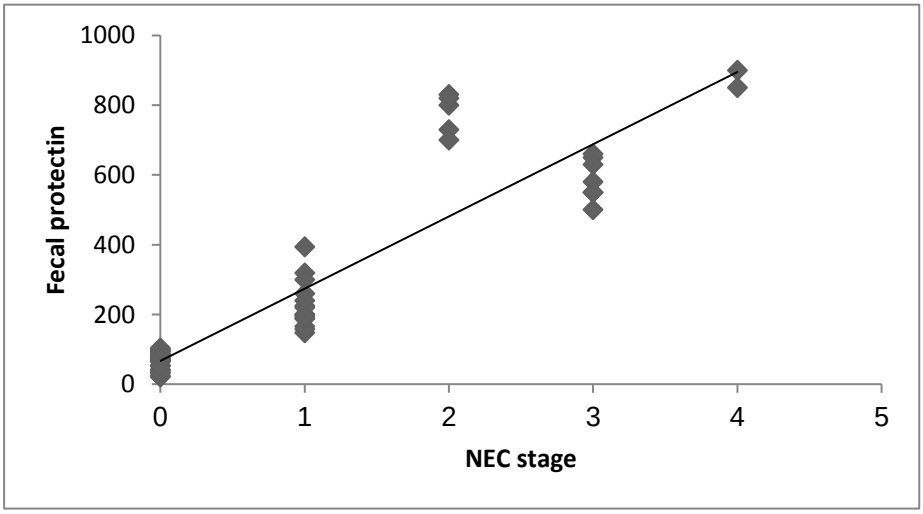


**Figure 1.** Scatter plot of the relationship between NEC stage and fecal calprotectin level

**Table 5.** Fecal calprotectin level (ug/g) among cases and controls

| Fecal calprotectin | NEC group | Control group | P-value |
|---|---|---|---|
| Mean±SD | 453.6±258.6 | 65.3±24.9 | < 0.001[1*] |

* Statistically significant at P < 0.01

**Table 6.** Spearman's correlation coefficient between fecal calprotectin level and NEC stages, gestational age, postnatal age, and neonatal weight

| | Fecal calprotectin level | |
|---|---|---|
| | r | P-value |
| NEC stage | 0.911 | < 0.001* |
| Gestational age | -0.239 | 0.07 |
| Postnatal age | -0.157 | 0.239 |
| Neonatal weight | 0.138 | 0.3 |

*Statistically significant at P < 0.05

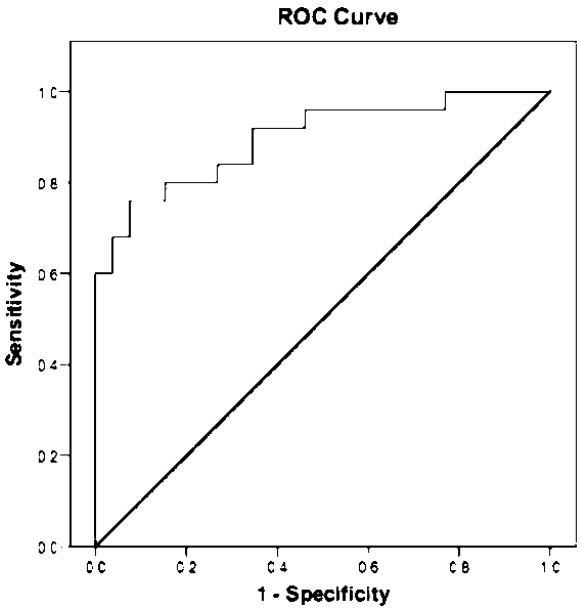


**Figure 2.** ROC curve of fecal calprotectin level for the diagnosis of NEC

## Discussion

In spite of the advances in neonatal guidelines and care, NEC is still a potentially critical disease in premature infants. This disease is of a multifactorial etiology leading to the necrosis and inflammation of the neonatal intestine (3). Calprotectin is an abundant calcium-binding protein found in neutrophilic granulocytes (14). The patients in the current study had a mean gestational age of 33.8 weeks, mean birth weight of 1.49 kg, and mean age of 5 days at diagnosis. The controls had a mean gestational age of 34.1, mean birth weight of 1.51, and mean age of 6 days at the time of sampling.

In the study performed by Carroll et al., the participants had a mean gestational age of 30 weeks, mean age of 12 days at diagnosis. In addition, four were boys and three were girls (21). The study carried out by Aydemir et al. (3) evaluated patients with a mean gestational age of 28.3±2.5 weeks and a mean birth weight of 1.048 kg. Ehab et al. (18) reported the patients had a mean gestational age of 32 weeks, mean birth weight of 1.5 kg, and mean age of 9 days at diagnosis.

Twenty of the patients in the case group of the current study (68.9%) were suspicious of NEC (Bell's stage IA and IB) and nine (31.1%) had definite NEC (Bell's stage IIA or IIB). Carroll et al. (21) found 43%, 14%, and 43% of their patients as Bell's stage IB, Bell's stage IIB, and Bell's stage

IIIA or IIIB, respectively. In the study completed by Ehab (18), two (14%) of the subjects in the case group were suspicious of NEC (Bell's stage I), eight (53%) had definite NEC (Bell's stage IIA or IIB), and five (33%) had advanced NEC (Bell's stage IIIA or IIIB). Approximately 53% of the patients died and 67% improved.

We observed a highly significant increase in fecal calprotectin level in patients with NEC, in comparison with the controls with a mean fecal calprotectin concentration of 453.6 µg/g in patients with NEC, compared to 65.3 µg/g in control group with a P-value of <0.001. Carroll et al. (21) found a marked increase in the fecal calprotectin of patients with NEC (288.4 mg/l), compared to the controls (98 mg/l).

Aydemir et al. (3) found that fecal calprotectin elevated in infants with NEC where the median fecal calprotectin level was 1282 ug/g in the case group versus 365 ug/g in the controls (P < 0.001). Therefore, serial measurements may be useful as a non-invasive prognostic marker for the progression of the disease. Ehab et al. (18) found a highly significant rise in the fecal calprotectin level of patients with NEC versus control group.

Nissen et al. (22) declared that because NEC primarily affects preterm infants, fecal calprotectin measurements could be a valuable tool for the investigation of preterm infants suspicious of having NEC. Reisinger et al. (23) detected that the combination of non-invasive measurement of intestinal fatty acid-binding protein and fecal calprotectin seems promising for diagnosing NEC at an early time point. Regarding the sequels of NEC, our study showed a positive correlation between NEC stage and level with r = 0.911, P-value < 0.001. The mean level of fecal calprotectin was 226.9 µg/g in stage IA and reached the maximum in patients of stage IIB (875 µg/g).

A study by Aydemir et al. (3) found that infants with NEC had augmented fecal calprotectin concentrations and there was a correlation between calprotectin concentrations and the severity of NEC. These authors found that fecal calprotectin level in the firstly collected stool samples was 1285, 1251, and 365 µg/g in stage II, stage III, and control group, respectively.

On the other hand, it was reported in the secondly collected samples as median fecal calprotectin level of 541, 2341, and 226 µg/g in stage II, stage III, and control group, respectively. Consequently, in the second samples, the fecal calprotectin concentration was significantly higher in stage III, compared to stage II and could be concluded that fecal calprotectin is a useful marker for the diagnosis and severity of NEC in preterm infants.

Ehab et al. (18) found that the level of fecal calprotectin reached the maximum level in patients with Bell's stage IIIB (307.6±4.1 mg/dl). In contrast, Selimoğlu et al. (24) observed that the mean fecal lactoferrin and fecal calprotectin were not different between preterm and full-term newborns (P=0.235 and P=0.845, respectively). Furthermore, they were not different between the subjects diagnosed with NEC or not (P=0.545 and P=0.968, respectively).

In the current study, nine patients of the case group (31%) were artificially fed, none were breastfed, 20 patients (69%) were on mixed feeding. In the control group, four patients (13.8%) were artificially fed, 12 patients (41.4%) were breastfed, and 13 patients (44.8%) were on mixed feeding.

Jung et al. (25) revealed that the average fecal calprotectin concentration was 4.34±1.89 and 5.66±1.65 mg/dL in the groups of artificial feeding and mixed feeding, respectively. These data demonstrated that the concentration of fecal calprotectin was higher in newborns that were both formula-fed and breastfed, compared to only formula feeding newborns.

In the present study, no significant correlation was found between fecal calprotectin level and gestational age (r=0.239, *P*=0.07). The study conducted by Rouge et al. (26) involved 47 premature newborns and verified the correlation between gestational age and fecal calprotectin levels. There was a weak negative linear relationship between gestational age and calprotectin level with statistically significant *P*-value of 0.001. Moreover, Moussa et al. in 2015 (27) found the same correlation, especially in cases with increased ischemia modified albumin (28).

Jung et al. (25) analyzed newborns according to stratified gestational age. They demonstrated a clear positive linear relationship between the two variables of gestational age and the level of fecal calprotectin in newborns born at < 26 weeks gestational age. There was a significant negative linear relationship between the two variables in newborns born after or at week 26 to < 30 weeks. In the current study, a fecal calprotectin value of 153 µg/g was found to be 96.6% sensitive and 100% specific.

Josefsson et al. (29) showed that fecal calprotectin could not identify neonates with NEC in a population of neonates with very low birth weight. They proposed a cutoff level of above 2000 µg/g for identifying a severe intestinal

inflammation. It was shown in the study carried out by Thuijls et al. (30) that fecal calprotectin measurements were useful in the diagnosis of NEC. The ideal cutoff value for fecal calprotectin to discriminate neonates with NEC from those with suspected NEC and other final diagnoses was 286.2 µg/g. The specificity was reported as 93% and sensitivity as 86%.

Aydemir et al. (3) stated that a fecal calprotectin value of 792 µg/g was 76% sensitive and 92% specific for the diagnosis of definite NEC. Unfortunately, fecal calprotectin levels measured at initial diagnosis were not useful to predict which infants will progress to stage 3 of NEC. Furthermore, the utility of fecal calprotectin measurement in the diagnosis of NEC may be limited because stool samples cannot be obtained in some patients.

## Conclusion

Fecal calprotectin is a promising marker in the diagnosis of NEC.

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## Conflicts of Interests

The authors have no conflict of interest to declare.

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