Original Article

Diagnostic utility of fecal calprotectin as a biomarker of gut inflammation in neonates to predict necrotizing enterocolitis: A prospective study

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Abstract

Background: Necrotizing enterocolitis (NEC) is a neonatal emergency that affects preterm newborns during the 1st weeks of life. Diagnosis is made mainly by clinical criteria since no specific diagnostic tests are available. **Objective:** The objective was to evaluate fecal calprotectin (fCal) as a biomarker of gut inflammation to predict NEC in preterm neonates. **Methods:** Design: Diagnostic test evaluation. Inclusion criteria: 102 preterm neonates <36 weeks gestation and within 7 days of birth admitted in Level III neonatal intensive care unit (NICU) were recruited from January 2010 to May 2011. Exclusion criteria: Congenital anomalies and overt infection. Paired stool samples at day 3 and 7 were analyzed by lateral quantum blue rapid calprotectin assay. Cut-off values of fCal were determined among 30 term healthy infants. A structured questionnaire which included gestational age, symptoms at admission, and modified Bell's staging was used to record NEC episodes on day 3 and 7 of admission. Septic screen and radiological tools were done as per NICU protocol. **Results:** 48% were above 34 weeks gestation; 31.3% were of very low birth weight. As per modified Bell's staging on day 3 and 7, 22 and 11 neonates had 1a or above stage, respectively. 15 had features of NEC; of these, 12 were managed appropriately and discharged and 3 died. In the receiver-operated curve with fCal >279 μg/g as cutoff, the area under the curve was 0.652 (95% confidence interval: 0.516-0.789). Day 3 fCal levels were high in 65.7% neonates. Using NEC as outcome, sensitivity of the test was 93.3%; specificity was 39%; positive predictive value was 20.8% and negative predictive value was 97.14%. **Conclusion:** fCal has high sensitivity for diagnosing NEC in preterm neonates. However, further research is needed to establish its clinical usefulness.

Key words: *Fecal calprotectin, Gut inflammation, Lateral flow rapid assay, Necrotizing enterocolitis, Preterm neonates*

Necrotizing enterocolitis (NEC) presents a particular challenge to neonatologists worldwide with the impending possibility of surgical intervention with high mortality and uncertain prognosis [1-3]. Diagnosis is made purely on the basis of clinical criteria due to lack of a specific clinical presentation and a reliable diagnostic test. Bell et al. [4] presented an original system for the uniform clinical staging of neonates with NEC. The original criteria of Bell were modified by other authors to integrate therapeutic and prognostic aspects of the disease-systemic, gastrointestinal, and radiographic features [5,6]. Identifying prospective biomarkers would offer sufficient opportunities for early intervention.

Calprotectin is a calcium and zinc-binding protein normally seen as a component of cytosolic part of neutrophils and is a dominating granulocyte biomarker, originally discovered in 1980 by Fagerhol et al. [7]. Fecal calprotectin (fCal) is extremely stable in feces for more than 7 days with concentration approximately 6 folds higher than plasma levels. fCal is increasingly being evaluated over the last 10 years for pediatric and adult gastroenterological disorders such as screening for inflammatory bowel disease (IBD) [8,9] and has become the standard marker of inflammation in IBD [10]. The standard method to measure calprotectin is using an ELISA method. However, it requires a skilled personnel, advanced laboratory facilities, and many samples (40-80) before the analysis can be done. Therefore, this ELISA method can be used only in a high output facility. A novel method based on lateral flow technology for the determination of fCal has been developed by the Bühlmann laboratory in Basel, Switzerland. This can be performed by unskilled personnel like a nurse and does not require sophisticated laboratory facilities.

In an acute medical situation like NEC, since every hour count and a rapid result are needed for medical decision-making. For these reasons, we used lateral flow technology for the determination of fCal in our study. There are few published reports on the utility of fCal as a biomarker of gut inflammation in

very low birth weight (VLBW) infants [11]. However, not many studies have been done in Indian neonates to find out the cut-offs in preterm babies to screen NEC and detecting the diagnostic value of this biomarker, and hence this study was planned.

MATERIALS AND METHODS

This study was carried from January 2010 to May 2011 at the Level III NICU in Kempegowda Institute of Medical Sciences and Research Center, Bengaluru. Prior approval was obtained from the Institutional Ethics Committee. Written, informed consent was obtained from the parents before recruitment to the study. A pilot study was performed on 30 term neonates, and stool samples were obtained at day and analyzed for calprotectin. The values were tabulated, and the mean value was taken as the cut off for the diagnostic test. 102 premature neonates born at <36 weeks of gestation and age of life <7 days were included in the study. Preterm neonates with overwhelming active infection at admission either by culture proven sepsis and/or band cells and those with major congenital anomalies identified at birth were excluded.

Primary outcome was suspected or probable or definite NEC episodes during the study period, defined as clinical evidence of gastrointestinal and systemic illness, confirmed radiographically or intraoperatively. Secondary outcomes were suspected NEC or need for surgery for NEC during the study period; length of stay in hospital (days); days receiving intravenous fluids, blood/blood products, and NEC-related adverse events and/or death; by 28 days post-delivery or discharge.

Figure 1 depicts the recruitment process. Neonatal symptoms at the time of admission to NICU and the episodes of NEC at day 3 and 7 were evaluated as per the modified Bell's criteria. We have chosen day 3 and 7 to utilize the data for diagnostic testing as per the gold standard of modified Bell's staging. We have not taken the stool samples serially in view of the technical difficulty in collection of samples and also the cost and availability of rapid testing kits. Using a prospective design, we collected data on birth weight in g and gestational age according to Ballard scoring. Antenatal steroid usage was noted and the classical markers of inflammation such as white blood count, platelets, and C-reactive protein (CRP) were estimated and correlated with fCal. Hemogram, arterial blood gas analysis, blood urea, and serum electrolytes were also obtained. Ultrasound scan of abdomen and stool for occult blood test were carried out. NEC episodes were staged as per Modified Bell's criteria (Table 1). Neonates were followed-up for a month post discharge.

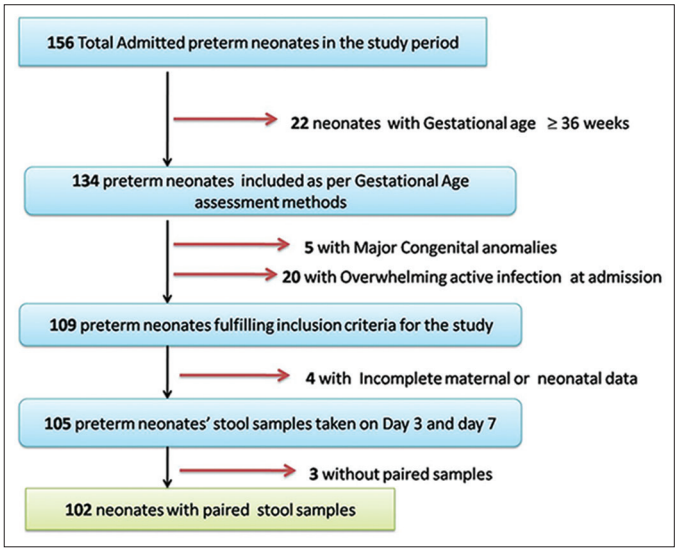

Figure 1: Recruitment of newborns-organizational flow chart

Paired stool samples were collected for calprotectin assay which was measured using a rapid assay by lateral flow quantum blue assay kits (Bühlmann, Basel, Switzerland). In this method, same monoclonal antibody as used in the ELISA is embedded on a nitrocellulose (NC) strip, but instead of being conjugated to an enzyme, gold particles are used [12]. After a drop (100 ul) is applied into the well, these antibodies bound to calprotectin molecules migrate down the NC paper to a secondary monoclonal antibody where it is arrested. The higher the fCal concentration, the more gold would be conjugated and stronger the red color of test band. For reading the test band, an electronic reader system that quantifies the fCal level in the stools was used. The correlation between the ELISA technique and the Quantum blue rapid test is typically between 0.93 and 0.96.

Data were analyzed using SPSS for Windows version 17.0. (Chicago, USA: SPSS Inc. Released 2008) fCal cut off levels were determined using the pilot data set by receiver operated curve (ROC) curve. Univariate analysis was carried out for the study variables using χ^2 and outcomes, namely composite outcome of NEC and/or death during hospitalization or within 28 days of birth (neonatal period) and modified Bell's at day 3 (0 vs. 1 and above). Spearman's correlation test was used to evaluate the relationship between fCal and serum CRP. Diagnostic test evaluation was carried out using the cut off for calprotectin in those with or without modified Bell's Staging 1a and above on day 3 and the composite outcome. ROC curves were drawn, and areas under the curve were plotted. Significance was set was 0.05.

RESULTS

Totally, 102 premature neonates were included in the study. 76 neonates were inborn and 26 were outborn. Gestation age by Ballard Scoring ranged from 28 to 34 weeks and birth weight ranged from 700 to 2300 g. 22 neonates had suspected NEC by Modified Bell's staging done at day 3; at day 7, 11 babies had Stage 1a or more. 15 preterm babies were noted to progress to NEC within a month of birth. Neonatal symptoms at admission were tachypnea in 31% and multiple symptoms such as abdominal distension, vomiting, and lethargy were noted in 13%. Antenatal

Table 1: Modified Bell's staging criteria for NEC

Stage	Systemic signs	Abdominal signs	Radiographic signs
IA suspected	Temperature instability, apnea, bradycardia, lethargy	Gastric retention, abdominal distention, emesis, heme-positive stool	Normal or intestinal dilation, mild ileus
IB suspected	Same as above	Grossly bloody stool	Same as above
IIA definite, mildly ill	Same as above	Same as above, plus absent bowel sounds with or without abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis
IIB definite, moderately ill	Same as above, plus mild metabolic acidosis and thrombocytopenia	Same as above, plus absent bowel sounds, definite tenderness, with or without abdominal cellulitis or right lower quadrant mass	Same as IIA, plus ascites
IIIA advanced, severely ill, intact bowel	Same as IIB, plus hypotension, bradycardia, severe apnea, combined respiratory and metabolic acidosis, DIC, and neutropenia	Same as above, plus signs of peritonitis, marked tenderness, and abdominal distention	Same as IIA, plus ascites
IIIB advanced, severely ill, perforated bowel	Same as IIIA	Same as IIIA	Same as above, plus pneumoperit-oneum

DIC: Disseminated intravascular coagulation; NPO: Nil per os or nothing by mouth, NEC: Necrotizing enterocolitis

factors like pregnancy-induced hypertension were present in 24 mothers of whom 5 had NEC; chorioamnionitis was present in 4 mothers and none of the neonates had NEC. 49 mothers had received antenatal steroids and 6 neonates developed NEC compared to NEC among 9 babies of 53 mothers who did not receive steroids. 3 of the 67 neonates who received no feeds orally for 3 days had features of NEC compared to 12 out of 35 who had been kept nil per orally for 4 or more days had NEC (odds ratio 11.13; 95% confidence interval [CI] = 2.880-43.017). Expressed breast milk was given to 65 neonates and 20 received formula feeds. Table 2 shows modified Bell's staging at days 3 and 7. CRP data are presented in Table 3.

fCal as a Biomarker

In the pilot study on term babies, the mean value of fCal was 279 µg/g of stool. This was used as a cutoff point to evaluate the utility of fCal at day 3 and day 7. fCal values on day 3 and day 7 are shown in Table 4. 43.1% had high fCal; 15 neonates progressed to NEC (primary and secondary outcomes). Higher fCal had poor outcome and of the 15 with NEC, 14 had high fCal. Of the 15 with NEC, only 2 who had Bell's Stage 2a and we could not analyze the relation of high fCal with stages of NEC.

In the evaluation of diagnostic test, ROC curve with area under the curve (AUC) is a combined measure of sensitivity and specificity and in this study (Figure 2), the AUC was 0.652 (95% confidence interval was 0.516-0.789).

With a cutoff value ≥280 µg/g, fCal had a sensitivity of 93.3% and specificity of 39%. It has positive predictive value of 20.8%, negative predictive value 97.14% and diagnostic accuracy of 47.05% (Table 5). Correlation of fCal with CRP is depicted in

Table 2: Modified Bell's staging at days 3 and 7

Modified Bell's staging	Frequency (%)	
	Day 3	Day 7
Stage 0	80 (78.4)	92 (91.2)
Stage 1a and more	22 (21.6)	11 (10.8)
Total	102 (100.0)	102 (100.0)

Table 3: CRP levels at day 3

CRP (in mg/L)	Frequency	Percent
≥6	17	16.6
<6	79	77.4
Not done	6	6.0
Total	102	100

CRP: C-reactive protein

Table 4: Day 3 fCal (in µg/g)

fCal value (µg/g)	Frequency (%)	
	Day 3	Day 7
≥280	67 (65.7)	44 (43.1)
<279	35 (34.3)	58 (56.9)
Total	102 (100.0)	102 (100.0)

fCal: Fecal calprotectin

Table 5: Diagnostic test characteristics of fCal with NEC as gold standard

Day 3 fCal value (in µg/g)	NEC		Total
	Present	Absent	
≥280	14	53	67
<279	1	34	35
Total	15	87	102

fCal: Fecal calprotectin, NEC: Necrotizing enterocolitis

Figure 3. There was no correlation of CRP and baseline fCal (r = 0.047, p = 0.647). Organization chart of progression of NEC in preterm neonates against fCal values is shown in Figure 4.

DISCUSSION

In this prospective study, we evaluated the risk factors to NEC and diagnostic utility of a new biomarker, fCal. High fCal levels were shown to correlate with an increased turnover of leukocytes in the intestinal barrier and granulocyte migration toward intestinal lumen. fCal levels have been reported to be much higher during the first few weeks of life both in healthy full-term and preterm infants than in healthy adults and children. Determining the cut off level is a challenge in utilizing fCal as a diagnostic marker. The high fCal may be related to the unusual physiology of neonatal gut.

A specific pattern of functioning in the 1st weeks of life is characterized by increased transmucosal leakage, as previously shown by Walker [13], a phenomenon that ends by the third trimester of life, in a process named "closure" as evidenced by intestinal permeability studies. In another prospective study, done by Campeotto et al. [14], in 69 full-term healthy newborns with three groups of feed types (standard infant formula, prebiotic infant formula, and breast fed group), fCal concentrations (median 167 µg/g) were higher than the reference values in the healthy adults. Rouge et al. [15] determined cut-off levels using an ELISA assay in 147 samples obtained prospectively from 47 preterm babies. Median fCal excretion was 138 µg/g. Dynamic change of fCal in VLBW infants (gestational age 23-30 weeks, birth weight <1500 g) was studied [16]. The fCal levels were not related to gestational age or feedings regimen and tend to decrease with increasing age (p = 0.121) and feeding volumes (p = 0.179) [16].

Carroll et al. [17] reported utility of fCal in 7 neonates with definite NEC and 7 age and gestational age-matched and sex-matched controls; mean fCal was 288·4 mg/L in patients with NEC compared with 98·0 mg/L in controls. In our study, there was a statistically significant association with high fCal and NEC as 14 of the 67 with elevated fCal and 1 of 35 with normal fCal progressed to develop NEC. ROC curve showed that the AUC was 0.652 with a 95% CI 0.516 to 0.789. With a cutoff value ≥280 µg/g, fCal had a sensitivity of 93.3% and specificity of 39%; PPV 20.8% and NPV 97.14% and diagnostic accuracy was 47.05%.

Excretion of fCal may vary between persons and between points of time of sampling as described in various studies [15,18-20]. Hence, correlating an fCal measurement to clinical findings of Bell's score and to the individual's previous fCal measurements may be the most appropriate way to utilize fCal in NEC. In a prospective case control study [21], median

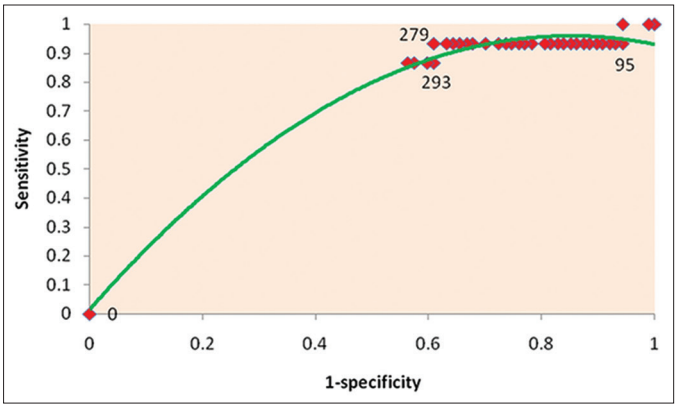

Figure 2: The receiver operating characteristic receiver operated curve for fecal calprotectin at day 3 and outcome

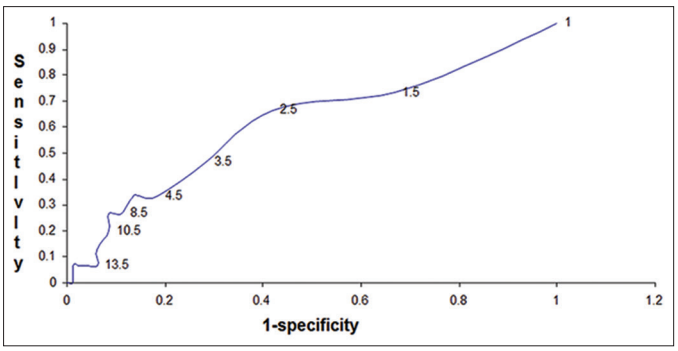

Figure 3: Correlation between C-reactive protein and baseline fecal calprotectin

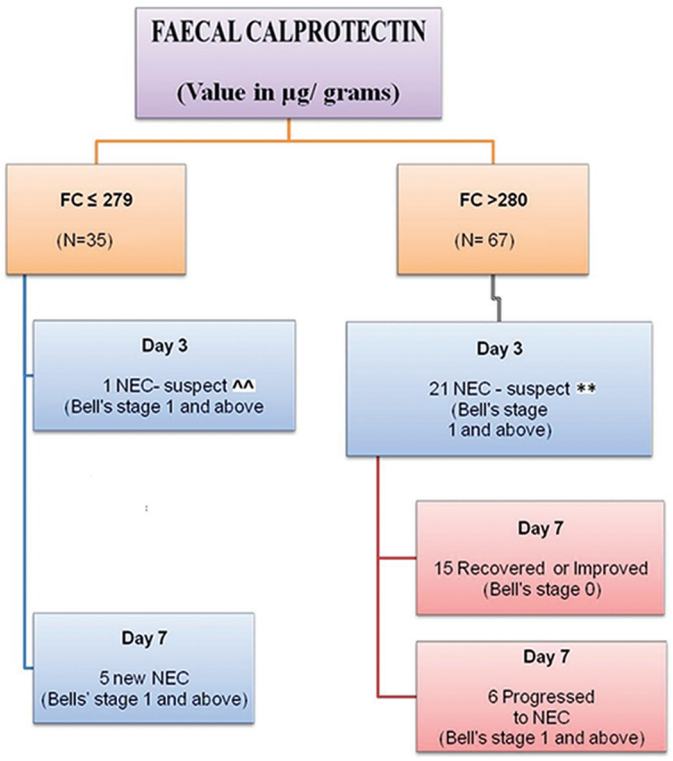

Figure 4: Organization chart of progression of necrotizing enterocolitis in preterm neonates against fecal calprotectin values. ^^Remaining 34 neonates had no features suggestive of necrotizing enterocolitis as per modified Bell's criteria. **Remaining 46 neonates did not have clinical features of necrotizing enterocolitis as per modified Bell's criteria

fCal concentrations were 1282 and 365 μg/g at diagnosis in infants with NEC and controls, respectively. fCal levels in NEC cases were significantly higher than those of the control group both in the first sample obtained at diagnosis and subsequent sample 3-5 days later. fCal value of 792 μg/g had a sensitivity of 76% and specificity of 92% for the diagnosis of definite NEC. There was correlation between calprotectin concentrations and severity of NEC [22].

Comparison of study groups with regard to fCal may be affected by many variations. In a previous study [23] on 126 preterm infants born at a median gestational age of 33 weeks to evaluate fCal as a marker of intestinal distress, ROC curve showed that with cutoff values of 363 μg/g, sensitivity was 0.65 and specificity 0.82; at cut off 636 μg/g, sensitivity was 0.72, specificity was 0.95 for the development of mild or severe enteropathy. Campeotto et al. [24] reported high levels of calprotectin (median 196 μg/g) in the stool samples, but did not correlate with the infants' gestational age, birth weight, gender, mode of feeding (breast or bottle), or postnatal age. Infants in the acute phase of a gastrointestinal event experienced a transitory increase in fCal levels, suggesting that it may serve as a noninvasively obtained biological marker of neonatal intestinal disturbance similar to the one observed in our study at day 3 of birth.

Development of NEC in VLBW babies and diagnostic value of fCal has been reported recently by Zoppelli et al. [25]. In this study, 1899 stool samples were obtained from 206 VLBW infants with a mean gestation of age 28.5 weeks. 19 of these infants developed NEC; of these 5 had fulminant NEC. fCal levels showed significant gestational and postnatal age dependent dynamics with particularly low levels in extremely premature infants. Sensitivity for discriminating moderate NEC from healthy infants and infants with intestinal distress was 0.89 for a cut-off of 180 and 210 μg/g, respectively, at the onset of symptoms. Specificity was 0.96 and 0.84. Fulminant NEC had low fCal and with a cut-off of <24 μg/g having a sensitivity of 0.84 and a specificity of 0.72. fCal can be a useful marker in identifying premature infants with gastrointestinal distress and NEC in particular. In our study, only 2 out of the 15 neonates with NEC had Bell's Stage 2a and because of this small number, we could not analyze the relation of high fCal with stage of NEC. Further multicenter studies are needed with large sample size to ascertain the diagnostic value before fCal can be recommended on a routine basis.

CONCLUSION

fCal has a diagnostic sensitivity of 93.3% and a negative predictive value of 97.14%. It is a useful addition to the existing methods to screen NEC in preterm infants.

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REFERENCES

1. Coit AK. Necrotizing enterocolitis. J Perinat Neonatal Nurs. 1999;12(4):53-66.
2. Stoll BJ. Epidemiology of necrotizing enterocolitis. Clin Perinatol. 1994;21(2):205-18.
3. Kosloske AM. Epidemiology of necrotizing enterocolitis. Acta Paediatr Suppl. 1994;3962-7.
4. Bell MJ, Ternberg JL, Feigin RD, Keating JP, Marshall R, Barton L, et al. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging. Ann Surg. 1978;187(1):1-7.
5. Walsh MC, Kliegman RM. Necrotizing enterocolitis treatment based on staging criteria. Pediatr Clin North Am. 1986;33(1):179-201.
6. Kanto WP Jr, Hunter JE, Stoll BJ. Recognition and medical management of necrotizing enterocolitis. Clin Perinatol. 1994;21(2):335-46.
7. Fagerhol MK, Dale I, Andersson T. Release and quantitation of a leukocyte derived protein (L1) Scand J Haematol. 1980;24393-8.
8. Røseth AG. Determination of faecal calprotectin, a novel marker of organic gastrointestinal disorders. Dig Liver Dis. 2003;35(9):607-9.
9. Limburg PJ, Ahlquist DA, Sandborn WJ, Mahoney DW, Devens ME, Harrington JJ, et al. Fecal calprotectin levels predict colorectal inflammation among patients with chronic diarrhea referred for colonoscopy. Am J Gastroenterol. 2000;95(10)2831-7.
10. Sandborn WJ. Why innovation in inflammatory bowel disease drug development will impact your practice. Clin Gastroenterol Hepatol. 2011;9(3):211-3.
11. Josefsson S, Bunn SK, Domellöf M. Fecal calprotectin in very low birth weight infants. J Pediatr Gastroenterol Nutr. 2007;44(4):407-13.
12. Kolho KL, Turner D, Veereman-Wauters G, Sladek M, de Ridder L, Shaoul R, et al. Rapid test for fecal calprotectin levels in children with Crohn disease. J Pediatr Gastroenterol Nutr. 2012;55(4):436-9.
13. Walker WA. Gastrointestinal host defence importance of gut closure in control of macromolecular transport. Ciba Found Symp. 1979;201-19.
14. Campeotto F, Butel MJ, Kalach N, Derrieux S, Aubert-Jacquin C, Barbot L, et al. High faecal calprotectin concentrations in newborn infants. Arch Dis Child Fetal Neonatal Ed. 2004;89(4):F353-5.
15. Rougé C, Butel MJ, Piloquet H, Ferraris L, Legrand A, Vodovar M, et al. Fecal calprotectin excretion in preterm infants during the neonatal period. PLoS One. 2010;5(6):e11083.
16. Yang Q, Smith PB, Goldberg RN, Cotten CM. Dynamic change of fecal calprotectin in very low birth weight infants during the first month of life. Neonatology. 2008;94(4):267-71.
17. Carroll D, Corfield A, Spicer R, Cairns P. Faecal calprotectin concentrations and diagnosis of necrotising enterocolitis. Lancet. 2003;361(9354):310-1.
18. Naismith GD, Smith LA, Barry SJ, Munro JI, Laird S, Rankin K, et al. A prospective single-centre evaluation of the intra-individual variability of faecal calprotectin in quiescent Crohn's disease. Aliment Pharmacol Ther. 2013;37(6):613-21.

19. Husebye E, Tøn H, Johne B. Biological variability of fecal calprotectin in patients referred for colonoscopy without colonic inflammation or neoplasm. Am J Gastroenterol 2001;96(9):2683-7.
20. Moum B, Jahnsen J, Bernklev T. Fecal calprotectin variability in Crohn's disease. Inflamm Bowel Dis. 2010;16(7):1091-2.
21. Aydemir O, Aydemir C, Sarikabadayi YU, Emre Canpolat F, Erdeve O, Biyikli Z, et al. Fecal calprotectin levels are increased in infants with necrotizing enterocolitis. J Matern Fetal Neonatal Med. 2012;25(11):2237-41.
22. Aydemir G, Cekmez F, Tanju IA, Canpolat FE, Genc FA, Yildirim S, et al. Increased fecal calprotectin in preterm infants with necrotizing enterocolitis. Clin Lab. 2012;58(7-8):841-4.
23. Campeotto F, Baldassarre M, Butel MJ, Viallon V, Nganzali F, Soulaines P, et al. Fecal calprotectin: Cutoff values for identifying intestinal distress in preterm infants. J Pediatr Gastroenterol Nutr. 2009;48(4):507-10.
24. Campeotto F, Kalach N, Lapillonne A, Butel MJ, Dupont C, Kapel N. Time course of faecal calprotectin in preterm newborns during the first month of life. Acta Paediatr. 2007;96(10):1531-3.
25. Zoppelli L, Güttel C, Bittrich HJ, Andrée C, Wirth S, Jenke A. Fecal calprotectin concentrations in premature infants have a lower limit and show postnatal and gestational age dependence. Neonatology. 2012;102(1):68-74.

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