# Postnatal Dynamics and Clinical Associations of Fecal Calprotectin in Very Preterm Infants: Implications for Necrotizing Enterocolitis and Feeding Intolerance

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INTRODUCTION: **To elucidate the postnatal dynamics and clinical associations of fecal calprotectin (FC) in very preterm infants, with a focus on necrotizing enterocolitis (NEC) and feeding intolerance (FI).**

METHODS: **We performed a prospective observational cohort study in infants with a gestational age of <32 weeks or birth weight <1,500 g with weekly feces collection. The relationships between FC, NEC, and FI were investigated, adjusting for demographic and clinical factors.**

RESULTS: **A total of 1,086 fecal samples were collected from 194 preterm infants. Postnatal FC levels of non-NEC infants were highly variable and followed an age-dependent patterned progression. FC levels were elevated in patients with NEC before and at NEC onset, distinguishing them from non-NEC infants and those at sepsis onset. Among infants without NEC or sepsis, those with FI exhibited lower FC concentrations throughout hospitalization and displayed a significant delay in reaching high FC levels after meconium compared with non-FI infants. The age to reach the first high nonmeconial FC levels was positively associated with the time to achieve full enteral feeding.**

DISCUSSION: **Postnatal FC dynamics among premature infants followed a patterned progression but were disturbed in patients with NEC and FI. Because of the high variations, the use of FC levels in NEC diagnosis should be implemented with caution in clinical practice. FC may help understand FI and feeding progression in very preterm infants. Further research is needed to validate these findings and explore the potential clinical applications of FC in this population.**

**KEYWORDS:** preterm neonate; fecal calprotectin; necrotizing enterocolitis; feeding intolerance

**SUPPLEMENTARY MATERIAL** accompanies this paper at http://links.lww.com/CTG/A951

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## INTRODUCTION

Calprotectin, a 36-kDa protein from the S100 calcium-binding family, is abundant in neutrophils' cytosolic proteins and is secreted at inflammation sites (1). Over recent decades, fecal calprotectin (FC) has emerged as a reliable biomarker for assessing gastrointestinal (GI) inflammation in adults and older children (2). However, during the neonatal period, physiological FC dynamics are complex and poorly understood, characterized by elevated levels with substantial interindividual and intraindividual variability (3–6), which poses challenges for clinical application (7).

Necrotizing enterocolitis (NEC) is a severe inflammatory intestinal disease and remains a leading cause of morbidity and mortality in neonatal intensive care units (NICUs) (8,9). Early clinical presentations of NEC are difficult to differentiate from sepsis or other GI diseases, and no specific biomarkers are currently available for diagnosis. Over the past 20 years, several studies have investigated calprotectin level changes at NEC onset [elevated (10–14), decreased (15), or unchanged (16–18)], considering FC as a noninvasive marker for NEC screening. However, these studies were limited by small sample sizes and varying patient and sample characteristics (17,19,20). The diagnostic uncertainty of FC in detecting NEC can lead to prolonged cessation of enteral feeding, extended courses of parenteral nutrition, and increased antibiotic use in clinical practice.

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Feeding intolerance (FI) is another prevalent GI issue among premature infants, characterized by atypical symptoms such as recurrent abdominal distention, vomiting, and significant residual gastric volume after enteral feeding. FI in very preterm infants is closely related to an immature GI system and altered gut microbiota (21). As recent studies suggest that calprotectin plays a crucial role in the postnatal development of the immune system and gut microbiota (22), investigating the association between FC and enteral feeding conditions in premature neonates is warranted.

To improve clinical utilization of FC, we longitudinally collected fecal samples from a prospective cohort of very preterm infants with and without GI disorders to elucidate the postnatal dynamics and clinical associations of FC levels.

## MATERIALS AND METHODS

### Study design and study population

This study was conducted in the NICU of the Children's Hospital of Fudan University between January 2018 and October 2020. In the prospective cohort, infants with a gestational age of <32 weeks or birth weight <1,500 g admitted to the NICU within 24 hours of life during the study period were enrolled and followed prospectively. Exclusion criteria included significant congenital anomalies, inborn metabolic errors, and hospitalization for fewer than 7 days. Infants with fewer than two samples were also excluded from the study. The study protocol was reviewed and approved by the Ethics Committee of the Children's Hospital of Fudan University (approval number [No. 2016(137)]). Informed consent was not obtained from the participants.

### Definitions

NEC was defined as neonates with stage ≥II NEC according to the Bell criteria (23). Sepsis included both culture-proven and culture-negative sepsis. Culture-proven sepsis was defined by a positive blood culture, whereas culture-negative sepsis required meeting the following criteria: (i) infection-related clinical manifestations; (ii) abnormal white blood cell count, C-reactive protein level, or procalcitonin level; (iii) antibiotics used or intended for ≥5 days; (iv) negative blood culture; and (v) no evidence of concurrent focal infection (24,25). Early-onset sepsis was defined as sepsis diagnosed within 72 hours of postnatal age (26). FI was diagnosed when all the following criteria were met: (i) gastric residual volume >50% of the previous feeding volume; (ii) emesis or abdominal distention, or both; and (iii) decreased, delayed, or discontinued enteral feeding (21,27). Gestational age was determined using the hierarchy of best obstetric estimates or gestational age estimation using the Ballard score (28). Small for gestational age was defined as birth weight <10th percentile for the gestational age according to the Chinese neonatal birth weight values (29). Full enteral feeding was defined as receiving enteral feeding volumes of 145 mL per kilogram per day for 3 consecutive days (30).

### Fecal sample collection, preparation, and FC measurement

Fecal samples were collected weekly during hospitalization for all enrolled infants. For neonates with NEC or sepsis, fecal samples at the disease onset (within 24 hours of the diagnosis of NEC or sepsis) were also collected if available. Fecal samples were immediately stored at 0°C and transferred to −80°C within 1 hour. Meconium was defined as the stool passed within 72 hours after birth.

FC levels were determined using a monoclonal ELISA kit (EK-CAL, Bühlmann, Switzerland) following the manufacturer's instructions at the end of the study. FC levels were expressed as micrograms per gram (μg/g) of feces. FC levels below the detection threshold (30 μg/g) were replaced with the minimum value (30 μg/g). Samples with FC values larger than the detection range (1,600 μg/g) were further retested after dilution. Among older children and adults, high or low FC levels were often defined as ≥100 μg/g or <100 μg/g, respectively (2). In this study, we evaluated the applicability of this cutoff value among preterm infants without NEC or sepsis.

### Statistical analysis

Statistical analyses, including data normality tests, Student *t* tests, ANOVA, Wilcoxon tests, $\chi^2$ tests, Spearman correlations, and graphical outputs, were performed using R version 4.0 and RStudio (ver. 1.2.5001). All preterm infants were divided into NEC and non-NEC groups based on whether NEC occurred during hospitalization. In the non-NEC group, we excluded infants with sepsis and divided the remaining infants into two subgroups according to whether FI occurred. We described the longitudinal changes in FC levels according to postnatal age. The FC levels were subjected to log10 transformation to better approximate a normal distribution before subsequent analysis, and the geometric mean of the log-transformed values was explored.

Multiple linear regression models were used to determine the clinical associations of meconial calprotectin. Mixed-effects linear regressions were applied to determine the clinical associations of postnatal FC levels with infant ID as a random intercept. To compare the differences between NEC and non-NEC groups in the pre-NEC period and at NEC onset, we used propensity score matching to identify samples of non-NEC neonates with characteristics comparable to those of infants with NEC at two time points, respectively. Subsequently, non-NEC neonate samples were matched to samples of infants with NEC with the closest propensity score of 5:1, using the nearest-neighbor greedy matching algorithm without replacement. Samples of patients with sepsis at sepsis onset were matched to samples of infants with NEC at NEC onset with the closest propensity score of 1:1.

The continuous variables included in the models mentioned above were postnatal age (days), gestational age (weeks), birth weight (g), Apgar scores at 5 minutes, and days of antibiotic use or probiotic use within the past week (days). The binary variables included NEC, sepsis, FI, sex, delivery mode, prolonged premature membrane rupture, multiple pregnancy, small for gestational age, use of prenatal antibiotics, gestational diabetes mellitus, gestational hypertension, and exclusive breastfeeding. *P* values were adjusted using the false discovery rate approach. A two-tailed *P* value of <0.05 indicated statistical significance.

## RESULTS

A total of 194 very preterm infants were included in this study (Figure 1), and 1,086 fecal samples were analyzed. There was no significant difference in demographic characteristics and clinical data between infants with and without NEC (Table 1). The median (IQR) age at NEC onset was 22 (18–32.3) days in the NEC group. Among infants without NEC or sepsis, there were 64 and 76 with and without FI, respectively, and the demographic and clinical variables are shown in Supplementary Table 1 (see Supplementary Digital Content, http://links.lww.com/CTG/A951).

### Postnatal dynamics and clinical associations of FC among very preterm neonates without NEC

Among non-NEC preterm infants, the overall dynamics of FC levels in early life followed a patterned progression. FC levels were high in the meconium (median [IQR] 560 [183–1,601]

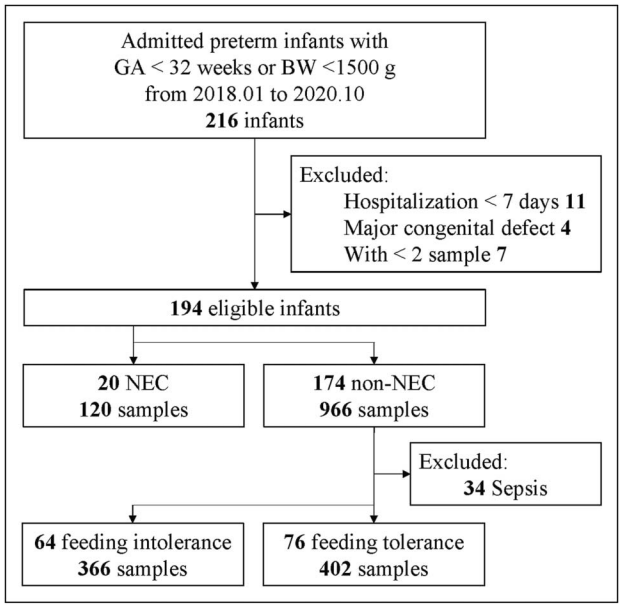


**Figure 1.** Study population. BW, birth weight; GA, gestational age; NEC, necrotizing enterocolitis.

μg/g), followed by a downward trajectory until 2 weeks of age (median [IQR] 30 [30–54] μg/g) and a gradual increase afterward (median [IQR] 129 [34–332] μg/g) (see Supplementary Table 2, Supplementary Digital Content, http://links.lww.com/CTG/A951). The postnatal FC dynamics according to different grouping variables were examined (Figure 2a–c and Supplementary Figure 1 [see Supplementary Digital Content, http://links.lww.com/CTG/A951]). In the multiple linear regression model, meconial calprotectin levels were positively associated with Apgar scores at 5 minutes and history of prenatal antibiotics use, and were negatively associated with postnatal age, male sex, and multiple pregnancy (Figure 2d). Among all samples collected at ≥2 weeks of postnatal age, the FC levels were positively associated with birth weight and postnatal age and were negatively associated with the recent use of antibiotics in mixed-effects linear regressions (Figure 2e).

### Changes in FC levels during NEC progression

Compared with the non-NEC neonates, the postnatal FC levels in patients with NEC were elevated throughout hospitalization (Figure 3a). We further examined the temporal change of FC levels according to the time of NEC diagnosis (Figure 3b). With fluctuations, the overall FC levels of patients with NEC rose during the pre-NEC period (defined as within 7 days before NEC diagnosis; median [IQR]:186 [45–807] μg/g), peaked at NEC onset (defined as within 24 hours of NEC diagnosis; median [IQR]: 2,536 [1,020-6,230] μg/g), and remained relatively high afterward. After adjusting for postnatal age and demographic and treatment factors (see Supplementary Table 3, Supplementary Digital Content, http://links.lww.com/CTG/A951), the FC levels of patients with NEC were significantly higher than those of the matched samples of non-NEC infants during the pre-NEC period ($P = 0.02$, Figure 3c) and at NEC onset ($P < 0.001$; Figure 3d), respectively. With a cutoff of 529 and 1,237 μg/g, the FC level distinguished NEC from non-NEC patients in the pre-NEC period and at NEC onset with a sensitivity of 93.3% and 93.3% and a specificity of 38.9% and 75%, respectively (Figure 3f). The positive predictive

**Table 1. Demographics and clinical variables of infants with and without NEC**

| Variable | All (n = 194) | NEC (n = 20) | Non-NEC (n = 174) | *P* value |
|---|---|---|---|---|
| Infant characteristics | | | | |
| Gestational age (wk), mean (SD) | 30.0 (1.7) | 30.6 (1.2) | 30.0 (1.7) | 0.130 |
| Birth weight (g), mean (SD) | 1,310 (186) | 1,385 (210) | 1,302 (182) | 0.059 |
| Male, n/N (%) | 90 (46.4) | 7 (35.0) | 83 (47.7) | 0.400 |
| Small for gestational age, n/N (%) | 16 (8.2) | 1 (5.0) | 15 (8.6) | 0.898 |
| Apgar scores at 5 min, mean (SD) | 8.7 (1.1) | 8.7 (1.1) | 8.7 (1.1) | 0.875 |
| Maternal characteristics | | | | |
| Multiple pregnancy, n/N (%) | 71 (36.6) | 8 (40.0) | 63 (36.2) | 0.930 |
| Vaginal delivery, n/N (%) | 62 (32.0) | 5 (25.0) | 57 (32.8) | 0.652 |
| Gestational diabetes mellitus, n/N (%) | 36 (18.6) | 3 (15.0) | 33 (19.0) | 0.898 |
| gestational hypertension, n/N (%) | 16 (8.2) | 0 (0) | 16 (9.2) | 0.324 |
| PPROM, n/N (%) | 48 (24.7) | 4 (20.0) | 44 (25.3) | 0.806 |
| Use of prenatal antibiotics, n/N (%) | 38 (19.6) | 5 (25.0) | 33 (19.0) | 0.729 |
| Care practice[a] | | | | |
| Exclusive breastmilk feeding, n/N (%) | 157 (81.8) | 13 (65.0) | 144 (83.7) | 0.081 |
| Probiotics use (d), n/N (%) | 0.4 (2.1) | 1.2 (4.3) | 0.4 (1.6) | 0.084 |
| Antibiotics use (d), mean (SD) | 6.8 (5.8) | 8.8 (5.2) | 6.6 (5.8) | 0.107 |

NEC, necrotizing enterocolitis; PPROM, prolonged premature rupture of membrane.
[a]Care practice (probiotics use and antibiotics use) in the NEC group and the non-NEC group was calculated as the accumulated d of treatment before NEC onset and that before the mean age of NEC onset, respectively.

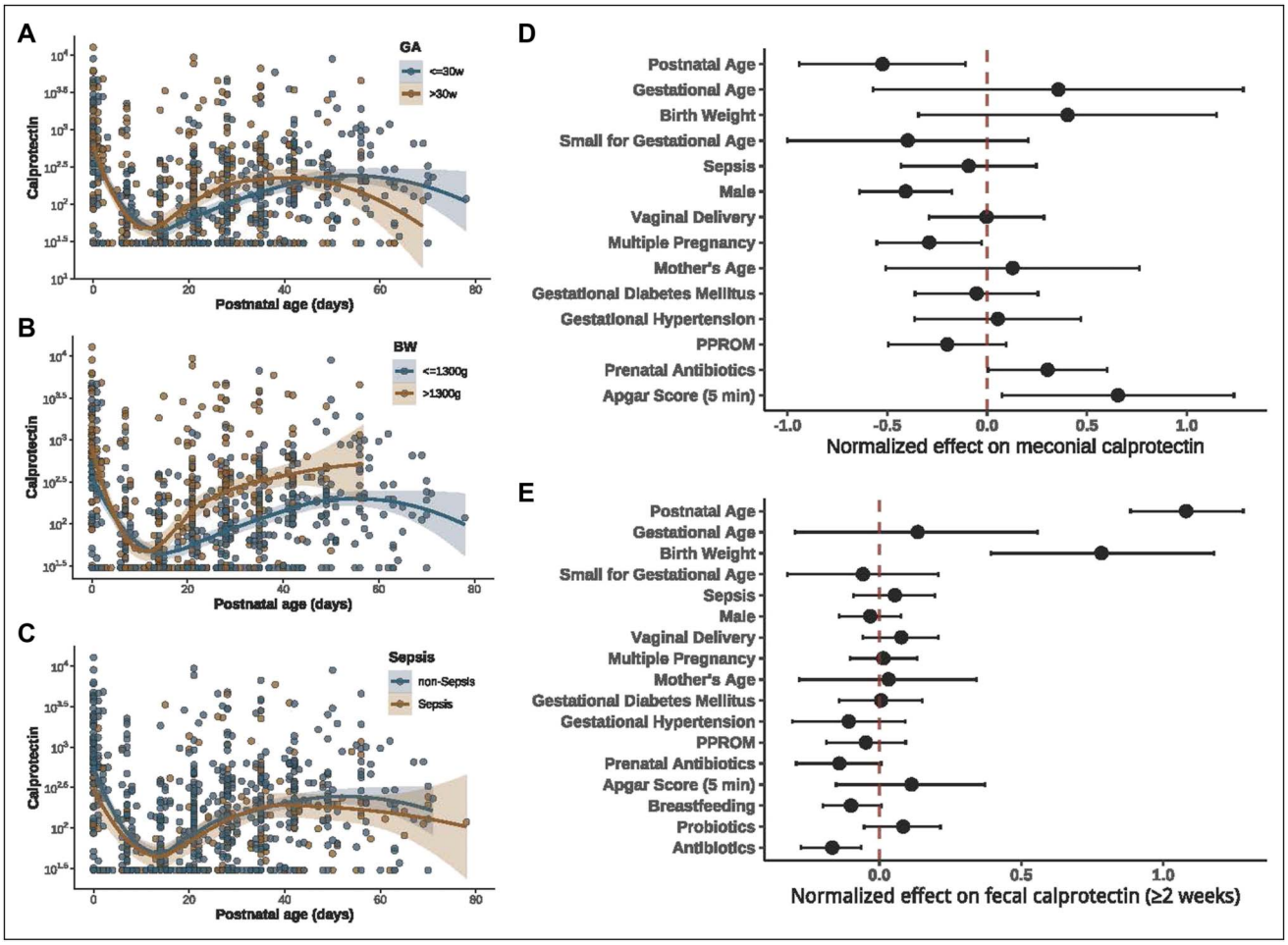


**Figure 2.** Postnatal dynamics and clinical associations of FC levels among very preterm neonates without NEC. Scatterplots of the changes in FC levels according to postnatal age among non-NEC preterm neonates. Each point represents a sample, colored by different grouping variables (**a**–**c**). Effects of different clinical factors on FC levels of meconial samples (**d**) and samples collected at ≥2 weeks (**e**) in preterm infants without NEC. Error bars indicate 95% confidence intervals. BW, birth weight; FC, fecal calprotectin; GA, gestational age; NEC, necrotizing enterocolitis; PPROM, preterm premature rupture of membrane.

value was 88.3% and 94.9% and the negative predictive value was 50.0% and 69.2%, respectively (see Supplementary Table 4, Supplementary Digital Content, http://links.lww.com/CTG/A951).

Although no difference in FC levels was observed between those with or without sepsis among non-NEC infants (Figure 2c), the FC levels of matched samples from non-NEC neonates at sepsis onset was significantly lower than those of neonates at NEC onset (Figure 3e). With a cutoff of 238 μg/g, the FC level distinguished neonates at NEC onset from at sepsis onset with a sensitivity of 83% and a specificity of 83% (Figure 3f).

### Associations between FC levels and feeding conditions in very preterm infants without NEC or sepsis

Among neonates without evidence of inflammation during hospitalization (NEC or sepsis), the associations between FC levels and their feeding conditions were further examined. Compared with the non-FI group, infants with FI had significantly lower FC concentrations in meconium and in fecal samples within 3–5 weeks of age (Figure 4a). After adjusting for demographic and treatment factors, FC levels of the FI groups remained significantly lower than those of the non-FI group at or beyond 2 weeks of age ($P = 0.004$). Most very preterm infants without NEC or sepsis reach high FC levels (defined as FC ≥ 100 μg/g) at least once after the meconium (119/140; 85%). Among them, infants with FI displayed a significant delay in the increase to high FC levels after meconium compared with the non-FI infants (Figure 4b).

Neonates with FI were also associated with extended days to reach full enteral feeding compared with the non-FI group ($P < 0.001$, Figure 4c). Meconial calprotectin levels were negatively associated with the time to reach full enteral feeding ($P < 0.001$, Figure 4d); however, the association was insignificant after adjusting for age and perinatal factors ($P = 0.124$). Among infants who reached high nonmeconial FC levels, the age to reach first high FC levels was significantly positively associated with days to reach full enteral feeding in both univariate (Figure 4e) and multivariate analyses ($P = 0.02$).

## DISCUSSION

In this study, we provided a comprehensive analysis of the postnatal dynamics and clinical associations of FC in very preterm infants, with particular attention given to the alterations of FC dynamics in infants with NEC and FI while considering other clinical factors.

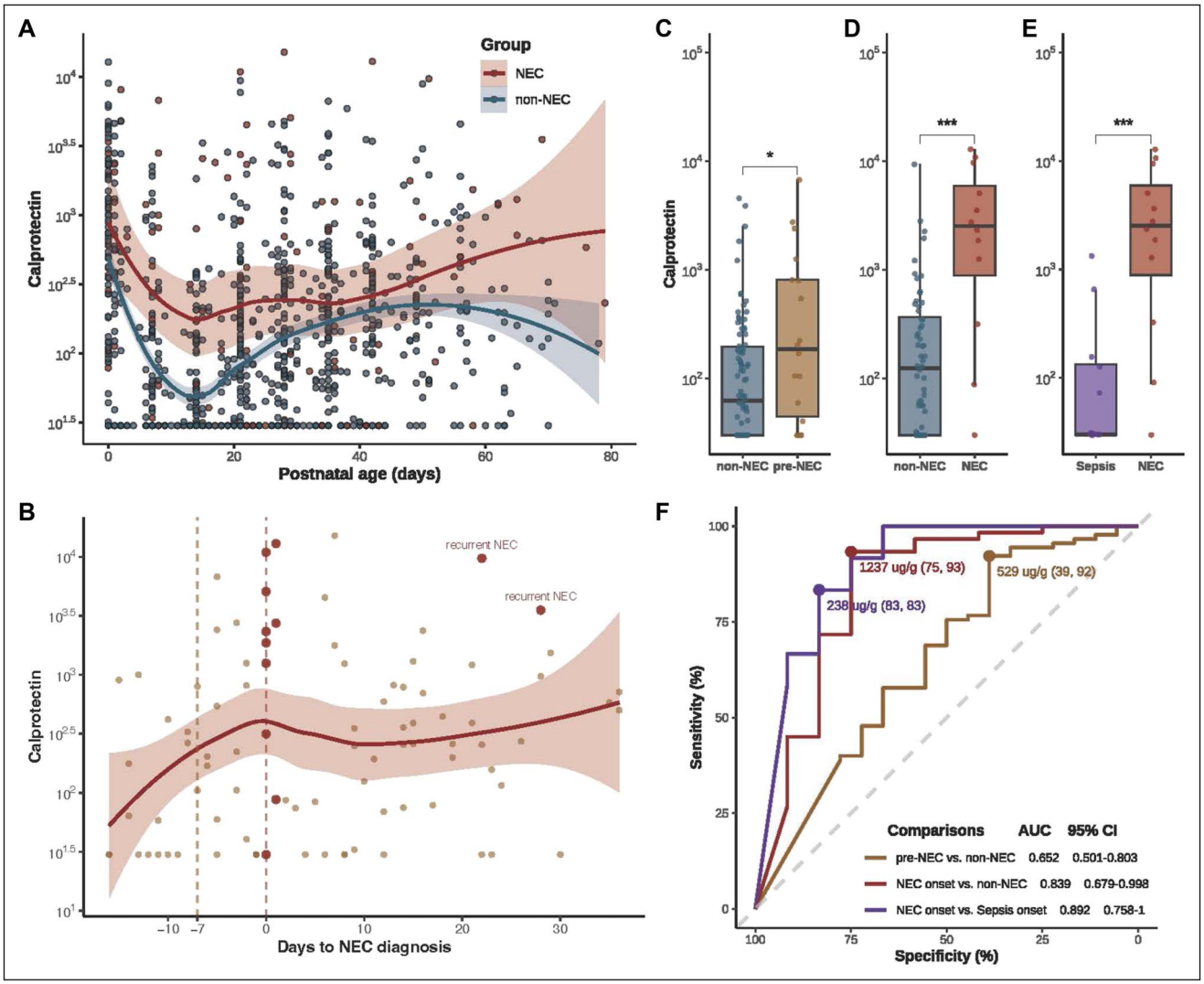


**Figure 3.** Temporal change and clinical utility of FC levels in NEC progression. (**a**) Scatterplot of the changes in FC levels according to postnatal age. Each point represents a sample. Smoothed lines in blue and red result from LOESS and indicate a longitudinal change of FC levels in non-NEC and NEC groups, respectively. (**b**) Change of FC levels according to the time of NEC onset. Smoothed lines result from LOESS and indicate a longitudinal change of all data. Meconial samples or samples collected more than 2 weeks before NEC onset were not shown. (**c** and **d**) Comparisons of FC concentrations of matched samples of patients with NEC and non-NEC infants in the pre-NEC period (yellow) and at NEC onset (red), respectively. (**e**) Comparisons of FC concentrations of matched samples of patients at sepsis onset (purple) and at NEC onset (red), respectively. (**f**) ROC curves analyzing the performance of FC as a biomarker for detection of NEC. Lines in yellow and red represent the AUC curve of FC levels at the pre-NEC period and at NEC onset, respectively. AUC, area under curve; CI, confidence intervals; FC, fecal calprotectin; LOESS, locally estimated scatterplot smoothing; NEC, necrotizing enterocolitis.

Our findings are consistent with previous observations, where elevated levels and drastic changes of FC dynamics were reported among very preterm infants during early life (7,31–34). The age-dependent patterned progression of FC dynamics highlights the significant impact of postnatal age, particularly during the first month of life, which accounts for the high variations of FC levels. High FC levels, which used as indications of intestinal inflammation among adults and elder children, were often defined as that of ≥100 μg/g (2). In this study, however, most preterm infants without NEC or sepsis had at least 1 nonmeconial FC level that exceed this level. A cutoff level of 538 μg/g in infants aged 1–6-month-old has been proposed using the same method (34). These observations emphasizes the need to re-evaluate the cutoff values for high FC levels at different postnatal ages, which should be aware of in clinical practice. On the other hand, our multivariate analyses identified distinct influencing factors for FC levels at different age periods. Apgar scores at 5 minutes, birth weight (but not gestational age), and history of antibiotics use were the major demographic factors influencing the changes in FC levels of meconium and fecal samples at or older than 2 weeks of age. The impacts of major clinical factors on the neonatal FC level should also be aware.

We observed elevated FC levels in infants with NEC before NEC onset and peaking at NEC onset compared with non-NEC infants. Using cutoffs for NEC prediction and diagnosis (514 and 1,086 μg/g), the specificities were low (38.9% and 75%) while the sensitivities were high (93.3% and 93.3%), suggesting FC as a potential screening marker for NEC but not for diagnosis. Interestingly, FC levels distinguished patients at NEC onset from sepsis onset with a relatively low cutoff of 238 μg/g and a

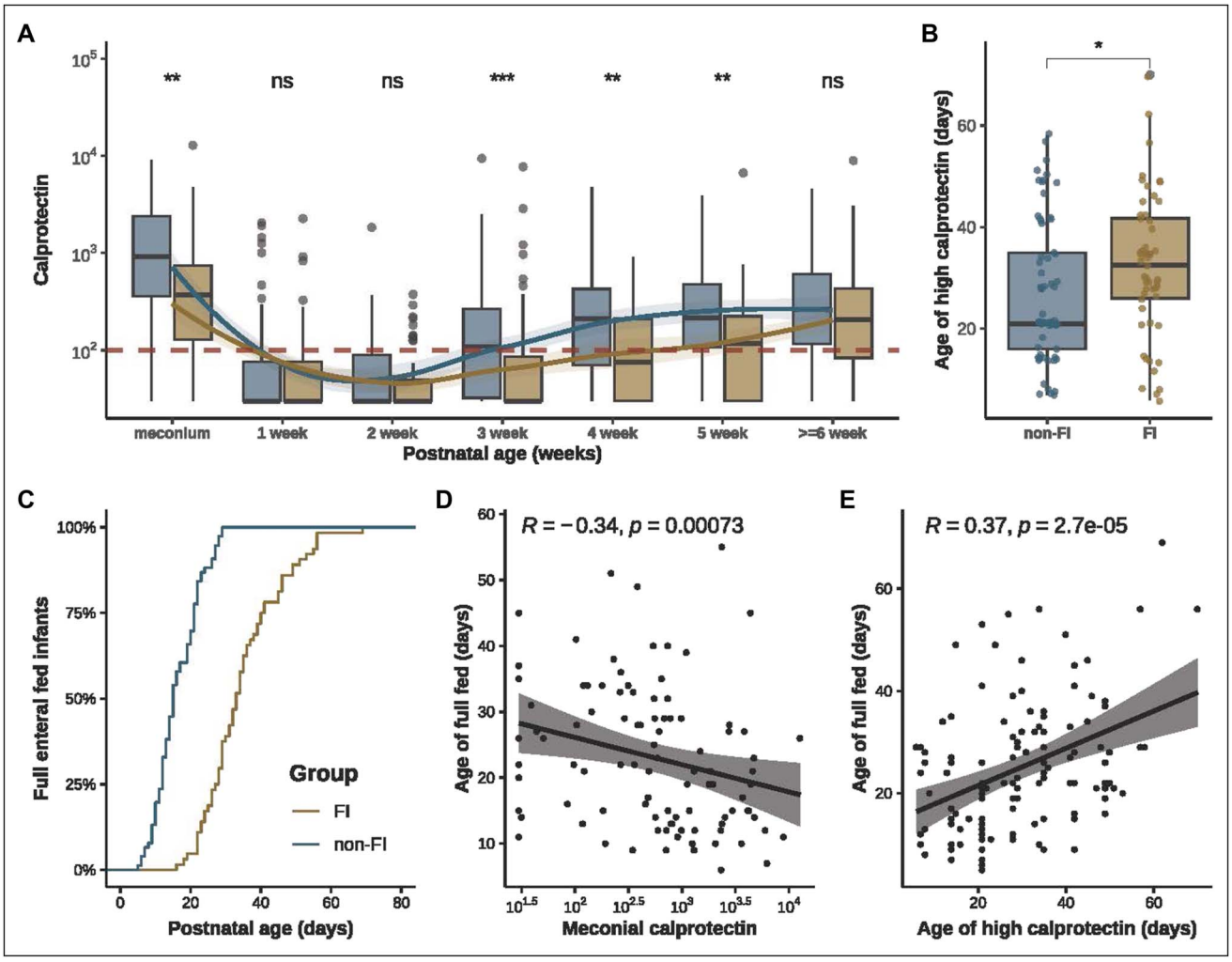


**Figure 4.** Associations between FC levels and feeding condition in very preterm infants without NEC or sepsis. (**a**) Difference in FC levels between infants with and without feeding intolerance at different postnatal ages. Boxplots and lines in yellow and blue represent infants with and without feeding intolerance, respectively. (**b**) Comparisons of the age of first high FC level after meconium between infants with and without FI. (**c**) Cumulated plots on percentage of infants to reach full enteral feeding in the FI and non-FI groups. (**d** and **e**) Spearman correlations between age of full enteral feeding (y-axis) and meconial calprotectin (**d**) or age of first high calprotectin level (**e**), respectively. FC, fecal calprotectin; FI, feeding intolerance; NEC, necrotizing enterocolitis.

specificity of 83%, indicating a possible role for FC in differentiating NEC from neonatal sepsis, which is often challenging in clinical practice. However, considering the high variations in FC levels and the temporal resolution of FC measurement, the results should be implemented with caution in clinical practice. Further validation with more cases and a more intensive sampling routine are warranted.

Despite the high prevalence and substantial disease burden, the pathogenesis of FI in preterm infants remains understudied (21,27). Calprotectin has recently been reported to play a crucial role in postnatal gut microbiota and immune system development, with higher FC levels during infancy associated with a reduced incidence of sepsis and lower BMI (15). However, research on FC levels in premature infants under different feeding conditions is limited because of the complex clinical context during the neonatal period and wide variance (35). In this study, we analyzed the changes of FC levels among premature infants without evidence of inflammation. Compared with feeding-tolerant infants, those with FI experienced significantly prolonged days to reach enteral feeding, accompanied by lower FC levels during hospitalization and delayed postnatal FC level increases. We reported for the first time a negative association between meconial FC levels and time to reach full enteral feeding, as well as a positive association between age to reach first high FC levels and time to reach full enteral feeding. These observations suggest that high FC levels during the neonatal period may be more indicative of a physiological rather than pathological state and could be associated with the maturation of GI systems. The results imply the potential of FC measurements as a valuable tool for anticipating the risk of FI and predicting the trajectory of feeding progression in preterm infants. Further research with more intensive sampling is warranted to validate the clinical utility of this framework, establish precise cutoff values, refine sampling frequencies, and evaluate its effectiveness in different clinical settings.

Our study's strengths include a relatively large sample size, longitudinal sampling throughout hospitalization, and in-depth analyses with detailed clinical information. However, there are several limitations. First, we only included very preterm infants

with gestational age <32 weeks or birth weight <1,500 g. Second, NEC and sepsis are rapid-onset conditions that may not be easily captured by weekly measurements, particularly when the previous measurement might have been taken 5–6 days before noticeable clinical changes occur. The limited temporal resolution of our FC data may have contributed to the significant variability and challenges in establishing predictive models for NEC. Serial measurements of fecal calprotectin on a daily basis are warranted to generate more accurate predictive models and evaluate FC levels' usability in early NEC screening. Third, although we used a widely accepted reagent, the absence of international standards in measuring FC concentrations may limit the generality of the results. Notably, the method used in this study (EK-CAL, Bühlmann, Switzerland) may yield higher FC levels compared with other calprotectin assay methods (36). This should be considered when determining cutoff values in clinical practice.

In summary, our findings offer comprehensive information on postnatal dynamics and clinical associations of FC among very preterm infants with and without GI disorders. FC levels were elevated during NEC progression and may help distinguish NEC from sepsis. Because of the high variations, the use of serial FC measurements as a screening tool for NEC should be implemented with caution in clinical practice. Further research on the associations between FC and feeding conditions is warranted.

### CONFLICTS OF INTEREST

**Guarantor of the article:** Yun Cao, MD, PhD.
**Specific author contributions:** Y.C., Y.Y.: conceived and designed the research. L.H., L.Z., Q.Z., S.L., and J.H.: collected the samples. L.H., Y.H.: carried out laboratory and data analyses for fecal samples. The manuscript was drafted by L.H. and Y.H., edited by Y.C., Y.Y., and S.J., and approved by all authors.
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**Potential competing interests:** None to report.
**IRB approval statement:** This study protocol was reviewed and approved by the Ethics Committee of the Children's Hospital of Fudan University, approval number [no. 2016(137)].

## Study Highlights

### WHAT IS KNOWN

- ✓ Fecal calprotectin is widely used for the diagnosis of inflammatory bowel disease.
- ✓ Fecal calprotectin is reported to be elevated in preterm neonates with necrotizing enterocolitis, but its utility is limited because of varied results.

### WHAT IS NEW HERE

- ✓ Postnatal fecal calprotectin in preterm infants follows age-dependent pattern progressions.
- ✓ Fecal calprotectin is elevated before and on necrotizing enterocolitis onset.
- ✓ Fecal calprotectin is decreased in feeding intolerance infants.

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