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# Enteral Feeding and Antibiotic Treatment Do Not Influence Increased Coefficient of Variation of Total Fecal Bile Acids in Necrotizing Enterocolitis

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## Abstract

**Introduction:** Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in preterm infants. In animal models, the accumulation of ileal bile acids (BAs) is a crucial component of NEC pathophysiology. Recently, we showed that the coefficient of variation of total fecal BAs (CV-TBA) was elevated in infants who develop NEC compared to matched controls. However, neither the type of enteral nutrition nor antibiotic treatments—parameters that could potentially influence BA levels—were used to match pairs. Thus, we assessed the relationships between exposure to enteral feeding types and antibiotic treatments with NEC status and CV-TBA.

**Materials and methods:** Serial fecal samples were collected from 79 infants born with birth weight (BW) ≤1800 gm and estimated gestational age (EGA) ≤32 weeks; eighteen of these infants developed NEC. Total fecal BA levels (TBA) were determined using a commercially available enzyme cycling kit. Relationships between CV-TBA and dichotomous variables (NEC status, demographics, early exposure variables) were assessed by independent samples t-tests. Fisher's exact tests were used to assess relationships between NEC status and categorical variables.

**Results:** High values for CV-TBA levels perfectly predicted NEC status among infants in this study. However, feeding type and antibiotic usage did not drive this relationship.



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**Conflict of interest:** None

**Conclusions:** As in previous studies, high values for the CV-TBA levels in the first weeks of life perfectly predicted NEC status among infants. Importantly, feeding type and antibiotic usage —previously identified risk factors for NEC—did not drive this relationship.

### Keywords

Antibiotics; Bile acids; Baby; Enteral nutrition; Infant; Necrotizing enterocolitis; Newborn; Neonate

## Introduction

Worldwide, necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency of preterm infants with a birth weight (BW) of below 1500 gm.[1,2] Characterized by an inflammatory, hemorrhagic necrosis of the distal ileum and colon,[3] the clinical presentation of NEC ranges from abdominal distension to intestinal gangrene and bowel perforation.[4] In the United States alone, thousands of pre-term infants develop NEC with mortality rates ranging 20–40%.[1,5–7] Disease-associated costs are significant: preterm infants diagnosed with NEC remain hospitalized for an average of 43 days[8] with yearly costs estimated in billions of US dollars.[9] Patients with necrotic bowel often go on to develop short bowel syndrome, which is also associated with significant complications and prolonged medical expenses. In addition, surgical intervention in NEC is a strong predictor of neurodevelopmental morbidity.[10] The pathophysiology of this disease remains poorly understood, and non-surgical treatment strategies are mainly supportive. Currently, no predictive tests are approved to identify which infants will develop NEC, and by the time NEC is diagnosed clinically, intestinal damage has already occurred.

Bile acids (BAs) are required for emulsification, absorption, and transport of fats, sterols, and fat-soluble vitamins in the intestine and liver. Furthermore, BA homeostasis is a complex process involving coordinated synthesis from cholesterol in the liver, transport from the liver to the intestine, followed by transport back to the liver. If enterohepatic circulation is interrupted, accumulation of cytotoxic BAs can result in damage to the intestinal epithelium.[11,12] Also, BA-induced cellular disruption—largely a result of their detergent-like properties—can cause further damage through the release of inflammatory mediators. We have previously shown that the accumulation of ileal BAs is crucial to NEC pathophysiology.[13–15] Our most recent publication—using nine matched subject pairs, each with five paired samples based on the day of life when the samples were collected—showed a statistically significant increase in the coefficient of variation of total fecal BAs (CV-TBA) in infants who develop NEC compared to matched controls. Notably, there was a perfect prediction of NEC, and the increases in CV-TBA occurred well prior to clinical NEC diagnosis.[16]

Compared to premature infants who are breastfed, formula-fed preemies are 6–10 times more likely to develop NEC[17] and have higher fecal BA levels.[18] Formula feeding is also required to develop experimental NEC.[19,20] In addition, while no specific pathogen has been conclusively associated with NEC,[21–29] the disease cannot be developed in germ-free conditions,[30,31] and colonization with specific species of gut bacteria is also required

for formation of more cytotoxic secondary BAs.[32–34] Given that neither enteral nutrition type nor antibiotic treatments were used to match pairs in our previous publication,[16] and that these parameters could influence BA levels, it is possible they could also affect CV-TBA. Therefore, using a larger, unmatched cohort and without a standardized window for sample collection, we assessed relationships between exposure to enteral feeding types and antibiotic treatments, NEC status, and CV-TBA.

## Materials and Methods

### Study Participants

Following approval by the University of Arizona Institutional Review Board, premature infants were enrolled prospectively *via* informed, written parental consent at Banner University Medical Center Tucson. All research was performed in accordance with relevant regulations. The inclusion criteria—BW less than or equal to 1800 gm, estimated gestational age (EGA) less than or equal to 32 weeks, and below 30-days old prior to initiation of enteral feeding—were chosen because NEC occurs almost exclusively in premature infants, the most premature infants are more likely to develop the disease, and most cases occur after the initiation of enteral feeding.[4,35,36] Exclusion criteria included conditions not related to prematurity, including blood–culture positive sepsis or genetic syndromes and were based on eliminating subjects that could develop NEC-like syndromes due to other confounding problems not related to the most common risk factors for NEC. Definitions of NEC diagnosis and time of diagnosis were defined as any subject with Bell's Stage above or equal to II (modified Bell's staging criteria) and radiographic evidence of NEC, respectively. Feeding and antibiotic exposures were defined as a subject being given of any formula of any brand or type, donor or maternal breast milk, breast milk fortifier, any antibiotics, or specific antibiotics during the range of samples used for BA analysis.

### Sample Collection and Analysis

Post-meconium fecal samples were collected from the diaper for up to four weeks after initiation of enteral feeding. Samples were placed in sterile microtubes, frozen in the NICU at −20°C and transported to the laboratory weekly where they were stored until processing. For analysis, samples were thawed, weighed, and mixed with an equal volume of nanopure water. After homogenization, samples were centrifuged to separate fecal water from the solids and the fecal water was frozen until BAs were assayed.[18,37] The Diazyme Total Bile Acids Assay Kit (Diazyme Laboratories, Poway, California, USA) was utilized to measure all BAs *via* an enzymatic cycling method with spectrophotometric readout.[13,14]

### Statistics

For each infant, TBA levels across all stool samples were summarized in terms CV-TBA, calculated for each infant by dividing SD-TBA by mean-TBA. Relationships between NEC status and categorical variables (demographic and exposures) were described in terms of counts and percentages and assessed using Fisher's exact tests. Relationships between NEC status and continuous variables (CV-TBA, EGA, BW, sample number, and sample DOL start and end) were assessed by independent samples *t*-tests assuming unequal variances, as

were relationships between CV-TBA and other dichotomous variables (demographics, early exposure variables).

## Results

Among the 79 infants included in this study, 18 developed NEC within the first 39 days of life and the other 61 infants were selected as controls. Observation periods, EGA and BW were similar for control infants and those with NEC, as control infants were selected based on similar EGA and BW ranges to their NEC counterparts and were followed for similar times as NEC infants (Table 1). Comparisons of exposure prevalence between infants with NEC and unmatched controls for types of enteral feeding and antibiotic treatment during the range of samples used for analysis are shown in Table 2. No infants were exclusively formula fed, and in both groups, most patients received BM (maternal and/or donor), with a much smaller percentage receiving formula and BM as formula is given only when there is no consent for donor breast milk and maternal milk is not available. For this dataset, none of these factors showed a relationship to NEC.

Figure 1 shows the distributions of CV-TBA between groups. Notably, similar to what was shown previously using matched pairs,[16] CV-TBA has no overlap: all infants who developed NEC had CV-TBA greater than 0.8, and all infants who did not develop NEC, had CV-TBA less than 0.8.

Table 3 shows CV-TBA means and standard deviations (SDs) among all patients receiving (YES) or not receiving (NO) exposures to formula, breast milk (BM), breast milk fortifier (BM fortifier), formula and BM, any antibiotics, or specific antibiotics. Among these comparisons, there were no statistically significant relationships to CV-TBA. Figure 2 illustrates what is shown descriptively in the table: that the overall distribution and range of CV-TBA was similar for control and NEC infants fed formula (Fig. 2A) or given any antibiotics (Fig. 2B) compared to those who were not exposed to formula and antibiotics (Figs 2A and B, respectively). These points taken together demonstrate that the relationship between NEC and CV-TBA was independent of the relationship between CV-TBA and formula feeding or antibiotic treatment in this sample.

## Discussion

As previously shown[16] high values for the coefficient of variation (CV) of TBA levels perfectly predicted NEC status among infants in this study. Specifically, no control infants had CVs greater than 0.78, and no infants with NEC had CVs lower than 0.84, thus any threshold of detection set between 0.78 and 0.84 would have resulted in 100% sensitivity and 100% specificity in this sample. Importantly, feeding type and antibiotic usage—previously identified risk factors for NEC—did not drive this relationship.

By the time NEC is diagnosed clinically, intestinal damage has already occurred. An early marker is critical for reducing both morbidity and mortality. Current standard of care relies on monitoring preterm infants—particularly those with very low birth weight (VLBW; those born at less than 1500 g)—for clinical signs of NEC, such as feeding intolerance, vomiting, apnea, abdominal distension, or blood in stools.[38–40] The biomarkers

that have been suggested for use in monitoring or diagnosing NEC—for example, C-reactive protein,[41] serum amyloid A,[42] calprotectin,[43] proinflammatory cytokines,[44–46] heart rate variability and peripheral oxygen saturation[47–49]—are similar to those found in sepsis, making differentiation between the two diagnoses problematic. Prediction of NEC by analyzing fecal microbiota,[50] proteomic,[51,52] or metabolomic[53,54] methods are more specific, but involve complex and expensive techniques that are not readily available in a clinical laboratory. Moreover, many of these proposed methods do not allow prediction with enough lead time for meaningful intervention. Increases of CV-TBA, however, occur well prior to NEC diagnosis.[16]

A common option for exploring the influence of formula feeding on the relationship between CV-TBA and NEC development would be through the inclusion of formula feeding as a covariate with CV-TBA in a logistic regression model. However, given the complete separation in CV-TBA values by NEC status, a valid logistic regression model is not possible.[55] Visually, by displaying CV-TBA values by formula feeding and NEC status, we show the complete separation of CV-TBA values by NEC status, and that the overall distribution and range of CV-TBA was similar for infants fed formula compared to those who were not fed formula and infants given antibiotics versus those not given antibiotics. Similarly, the relationship between CV-TBA and development of NEC was also independent of whether the infant received both formula and breastmilk, BM fortifier, or specific antibiotic treatments (Table 3).

Other statistical characteristics of TBA levels (mean and SD) were strongly predictive of NEC (data not shown), but did not yield complete separation, i.e., there was overlap in the range of values between infants who developed NEC and control (data not shown). While it could be argued that because the CV is a function of the mean, specifically that the mean is in the denominator of the formula, that children with higher mean TBA levels would tend to have lower CVs, even if SDs were similar. This would not explain, however, our finding that high CVs were predictive of NEC. In fact, infants with NEC tend to have higher mean TBA levels, which would drive CVs downward rather than upward.

Because CV-TBA shows perfect prediction of NEC and is not influenced by enteral feeding or antibiotic treatment types, it is a promising candidate as a biomarker to predict development of this devastating disease. Further research is needed to assess these findings in a larger cohort and to fully develop and assess predictive models in order to initiate a multicenter trial to validate this biomarker.

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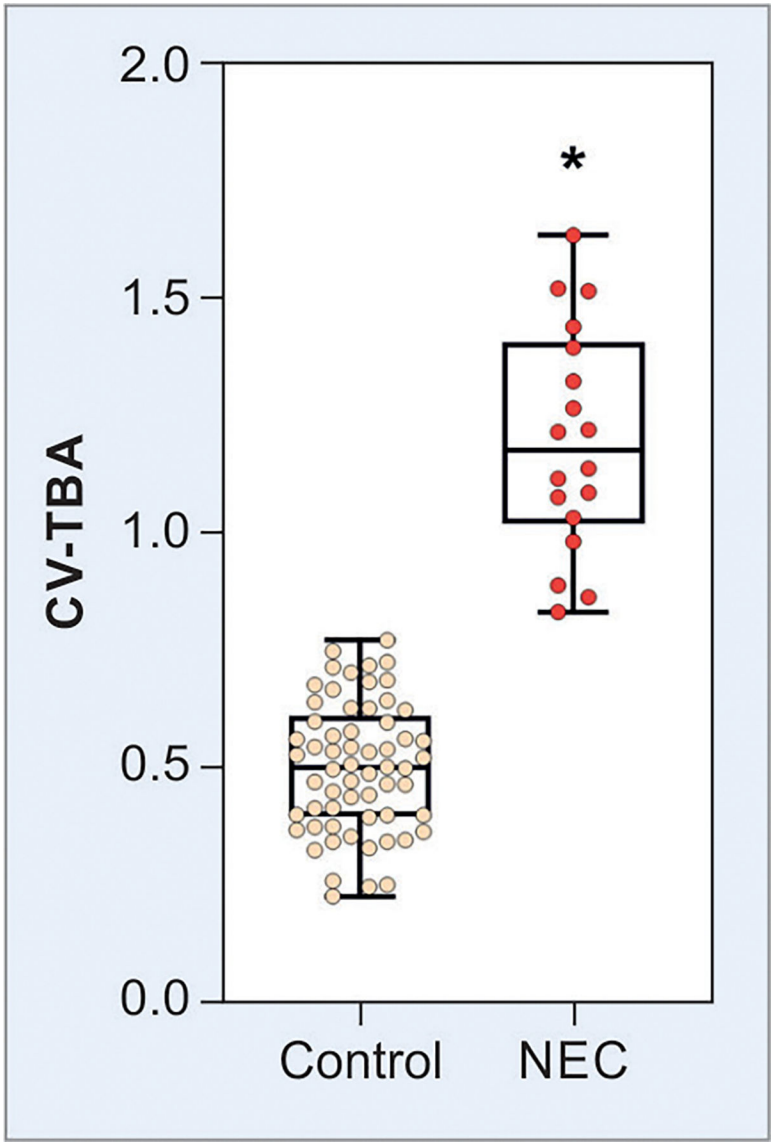
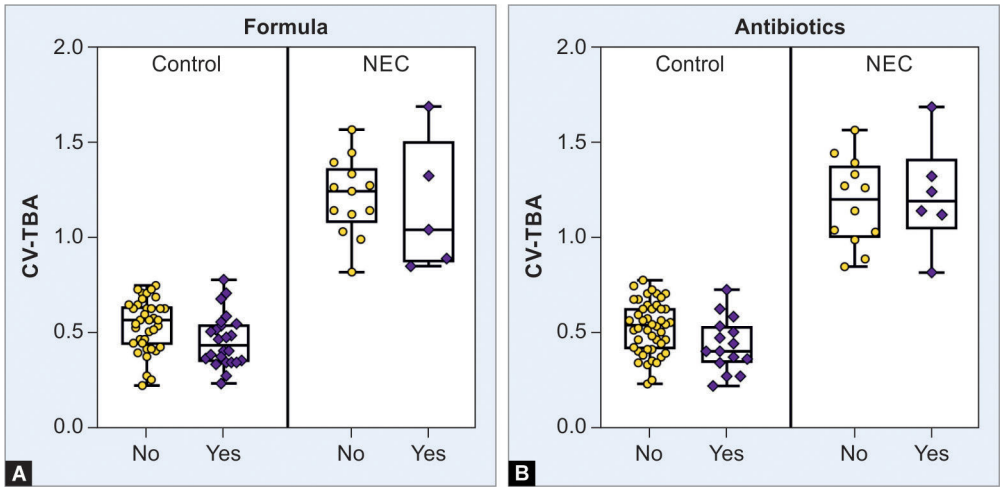



**Fig. 1:**
CV-TBA by NEC status. Each point represents an individual subject's CV-TBA. *$p < 0.0001$




**Figs 2A and B:**

Distribution of CV-TBA by (A) Formula feeding and NEC status and (B) Antibiotic treatment and NEC status. Each point represents an individual subject's CV-TBA

**Table 1:**

Characteristics of cohort and samples analyzed

| | Control (n = 61) | NEC (n = 18) | |
|---|---|---|---|
| | Mean ± SD | Mean ± SD | p-value |
| EGA (weeks) | 27.6 ± 2.6 | 27.4 ± 2.6 | 0.7[1] |
| BW (gm) | 1058 ± 303 | 968 ± 308 | 0.3[1] |
| % Male | 57 (*n* = 35) | 44 (*n* = 8) | 0.4[2] |
| Sample # | 19.8 ± 3.2 | 20.0 ± 4.4 | 0.9[1] |
| Sample DOL start | 8.3 ± 3.5 | 7.8 ± 3.1 | 0.5[1] |
| Sample DOL end | 29.3 ± 4.3 | 28.6 ± 3.8 | 0.5[1] |

[1] *t*-test, unequal variances assumed.

[2] Fisher's exact test. BW, birth weight; DOL, day of life; EGA, estimated gestational age

**Table 2:**

Feeding practices and antibiotic use by NEC status

| | Control (n = 61) | NEC (n = 18) | |
|---|---|---|---|
| | % (n) | % (n) | p-value* |
| *Feeding practice* | | | |
| Formula | 39.3 (24) | 27.8 (5) | 0.4 |
| BM | 95.1 (58) | 88.9 (16) | 0.3 |
| Formula + BM | 34.4 (21) | 22.2 (4) | 0.4 |
| BM fortifier | 95.1 (58) | 94.4 (17) | 1.0 |
| *Antibiotics* | | | |
| Any antibiotics | 24.6 (15) | 33.3 (6) | 0.5 |
| Gentamycin | 24.6 (15) | 33.3 (6) | 0.5 |
| Ampicillin | 23.0 (14) | 33.3 (6) | 0.4 |
| Vancomycin | 3.3 (2) | 5.6 (1) | 0.5 |
| Other | 1.6 (1) | 5.6 (1) | 0.4 |

*Fisher's exact test

**Table 3:**

CV-TBA[1] among all patients by feeding type and antibiotic exposure

| Exposure (n) | Yes | No | p-value |
|---|---|---|---|
| Formula (29) | 0.58 (0.32) | 0.72 (0.33) | 0.08 |
| BM (75) | 0.66 (0.33) | 0.70 (0.36) | 0.80 |
| Formula + BM (54) | 0.70 (0.33) | 0.59 (0.34) | 0.15 |
| BM fortifier (75) | 0.67 (0.34) | 0.56 (0.20) | 0.30 |
| Any antibiotics (21) | 0.66 (0.40) | 0.67 (0.31) | 0.90 |
| Gentamycin (21) | 0.66 (0.40) | 0.67 (0.31) | 0.90 |
| Ampicillin (20) | 0.67 (0.41) | 0.66 (0.31) | 0.90 |
| Vancomycin (3) | 0.72 (0.53) | 0.66 (0.33) | 0.90 |
| Other (2) | 1.11 (0.81) | 0.66 (0.32) | 0.60 |

[1] Mean (SD).

[*] *t*-test assuming unequal variances