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Stooling Pattern and Early Nutritional Exposures Associated with Necrotizing Enterocolitis in Premature Infants

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Necrotizing enterocolitis (NEC) remains one of the major unsolved morbidities associated with prematurity. It is the most common gastrointestinal emergency presenting in premature infants and the leading cause of aberrant nutrition, growth and neurodevelopment in this vulnerable patient population^{1–5}. Feeding is typically a precursor to NEC, which is then characterized by insidious disease onset and rapid disease progression. Scientific advances related to early intestinal colonization, the human microbiome, and identification of sensitive and specific biomarkers of NEC will result in the development of novel interventions for more effective detection and treatment of this disease^{6–10}. However, while we wait for the findings generated by these studies to be replicated and then translated to effective patient care interventions, there is a need to better understand clinical signs and symptoms in proximity to disease onset.

Gut motility and stooling pattern has been shown to be influenced by inflammatory bowel disease in infants¹¹. While the stooling pattern of premature infants has been shown to differ from that of infants born at term¹², little is known about the stooling pattern of premature infants who develop NEC. The purpose this study was to investigate the onset of stooling, as well as the stooling pattern over time in infants with NEC and age-matched controls during the early neonatal period. Findings relevant to an improved understanding of the clinical signs and symptoms associated with NEC will not only aid in our ability to more accurately anticipate disease, but will also be important in developing tailored interventions based on presentation of clinical signs and symptoms in tandem with findings generated by microbiome and biomarker studies.

Background and Significance

NEC and Infant Stooling Pattern as a Symptom of Disease

The only consistently identified clinical risk factor for NEC is prematurity¹. The premature infant bowel is prone to NEC as a result of aberrant intestinal colonization, inadequate barrier function, poor circulation, an immature immune response, as well as hypomotility^{1,13,14}. These factors not only predispose the premature bowel to inflammatory disease, but are likely to result in a stooling pattern that is unique from that of infants born at

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term gestation^{12,15–17}. Studies have shown that the first passage of meconium is delayed and that the transition from meconium to transitional stools is prolonged in the premature infant¹². Interestingly, the type of feeding, classification as small for gestational age, and the number of days the infant required respiratory support did not delay the passage of meconium stools in this analysis¹². Studies have shown that the delayed passage of stool in the premature infant is a function of both illness severity and gestational immaturity¹⁷. When antenatal exposures including betamethasone and magnesium sulfate were investigated, it was shown that betamethasone exposure resulted in an earlier passage of stool while magnesium sulfate exposure did not affect the time of first stool in premature infants¹⁷. Finally, a more recent study has shown that the establishment of regular bowel habits, defined as the passage of at least two bowel movements per day¹⁵, in the preterm infant is inversely correlated to gestational age and to the first passage of meconium¹⁸. In this analysis, the time of first meconium passage and time to achieve regular bowel movements were not influenced by the initiation of feeding or type of feeding, oxygen therapy, gender, birth weight, mode of delivery, presence of sepsis, or type of ventilator support¹⁸.

NEC and Human Milk as a Protective Factor of Disease

Several protective factors for NEC have been explored, but to date, only feeding with human milk has been shown to reduce the risk of this disease in premature infants^{19–23}. Human milk is thought to protect against NEC as a result of the potent immunological factors unique to human milk and in turn, the role it plays in ensuring early optimal bacterial colonization of the infant bowel^{1,19}. Unfortunately, mother's breast milk is not always available in sufficient quantities to premature infants and as a result, they do not receive the full immunologic and microbiologic benefit from this nutritional exposure. Quantifying the percentage of human milk within the premature infant diet necessary to confer a protective effect against NEC is an important step towards better understanding the unique association between early nutritional exposures and NEC.

While it has been shown that the preterm infant bowel pattern is different from that of the term infant (14–16), and part of the Bell Staging criteria that have been used to differentiate disease severity in NEC cases includes changes in stooling²⁴, analysis of the differences in stooling within the preterm infant patient population in the context of a GI disease such as NEC is not well understood. Thus, we conducted this investigation exploring the initiation of stooling, early stooling pattern, and frequency of stooling in proximity to disease onset in a retrospective case-control study involving 258 premature infants born prior to 29 weeks gestational age.

Methods

Participants and Study Design

In this retrospective case-control study, we assessed the early nutritional exposures and stooling patterns of 258 infants born prior to 29 weeks of gestation. 129 infants developed NEC and 129 unaffected infants served as controls. Infants were drawn from a larger study (HL 67669) of 1,178 extremely low gestational age infants born between 1997 and 2009 at

Brigham and Women's Hospital. NEC cases were matched 1:1 to NEC cases by gestational age cohort (less than 27 weeks gestation, and 27 to 29 weeks gestation). Each case was matched with the next infant born who met the gestational age matching criteria and did not develop NEC. Infants who were diagnosed with spontaneous intestinal perforation were not included in this study.

Definition of NEC

Case group assignment required diagnosis of NEC, as defined by Bell Stages I to III^{24,25}. Of the 129 infants who met Bell Stage criteria for NEC diagnosis, classification into Stages I, II, and III was as reported in Table 1. While Bell Stage I, the mildest form of the disease, has often been excluded in studies on NEC, we purposely included infants representative of all disease severities (Bell Stages I, II, and III). This allowed us to develop a better overall understanding of GI symptoms associated with all types of disease severity.

Data Collection and Analysis

All data were obtained from the medical record following research strategies ensuring valid and reliable retrospective data collection²⁶. Demographic data collected includes gestational age at birth, birth weight, and gender. Perinatal data collected includes mode of delivery, Apgar scores, and resuscitation efforts required at time of birth. Neonatal data collected includes use of an umbilical arterial catheter, day enteral feeding was initiated, type and volume of first enteral feeding, type and volume of feeding per day, and number of stools per day. Detailed feeding and stooling data was collected on a daily basis until NEC diagnosis for cases or, for controls, until the day of diagnosis for the matched case, for up to 28 days following birth. All feeding data represented either maternal breast milk or infant formula; donor milk was not yet part of clinical care at the time of this study.

Data up to day of disease diagnosis or, for controls, to the day of the matched case's diagnosis, is included. Medians, percentages, and non-parametric tests of significance were used to assess differences in demographic, perinatal, neonatal, feeding, and stooling characteristics between cases and controls. For continuous and ordinal data, Mann-Whitney U tests were used to compare the case group to the control group, and when comparing groups by NEC stage, significance was assessed by Kruskal-Wallis tests. For categorical data, Chi-Squared tests were used. Cumulative initial stool by postnatal day was calculated for cases and separately for controls.

Linear Mixed Models with unstructured covariance were used to estimate stooling changes in the week prior to disease diagnosis. Day of NEC diagnosis was modeled as Time=0, the day prior Time=-1, and so on for days prior to disease. The control group was used for comparison using the diagnosis postnatal day of the matched cases. Differences in slope between case and control groups were estimated. Because our findings showed that infants stool more frequently as they age, we cohorted the infants by their postnatal week of NEC diagnosis and analyzed each of these groups separately. Data was collected only until postnatal day 28. Thus, those infants diagnosed with NEC after their fourth postnatal week could not be included in this analysis. All statistical analyses were performed using SAS software, version 9.3 (SAS Institute Inc.; Cary, NC).

RESULTS

Our study population was comprised of 109 (42%) girls and 149 (58%) males. The median birth weight was 810 [680–1000] grams and median gestational age was 26 [24.7–27.3] weeks. The majority of infants were born via cesarean section (70.2%). Median Apgar scores at one and five minutes were 5 and 7, respectively. There were no statistically significant differences among NEC cases and controls in the demographic, perinatal, or neonatal data, including feeding data such as postnatal day enteral feeding was initiated, the volume of the first feeding, and type of first feeding (Table 1). Descriptive feeding and stooling data is presented in Table 2.

Stooling Pattern

The premature infants included in this study passed their first stool, on average, two days following birth. Infants who were diagnosed with NEC did not differ significantly from controls on the day of first stool or the mean or max number of stools per day after the first week following birth. However, infants who developed medical NEC (Stage I and II) had a greater maximum number of stools per day ($p=0.04$) and a greater mean number of stools per day ($p=0.02$) when compared to healthy controls during the first week following birth. In controls, the maximum stools in a day during the first week was 2[1–3] and the mean number of stools per day was 0.6 [0.3–1.0]. The mean stools per day for controls gradually increased over the first four weeks following birth to an average of 2.6 stools per day during week 4. In NEC cases, the mean number of stools per day during the first week following birth ranged from 0.42 [0.14–1.10] in infants with Stage III NEC to 1.00 [0.42–1.60] in infants with Stage II NEC. This difference in the number of stools per day was statistically significant ($p=0.02$), but only in the first week following birth. There were no statistically significant differences in the mean or maximum number of stools per day in the second, third, or fourth weeks following birth. This data is presented in Table 2.

Pairwise comparisons were conducted comparing the mean stools per day of each case to their matched control. Anticipating that stooling could change with proximity to NEC diagnosis, we grouped the infants based on the postnatal week of NEC diagnosis and analyzed each group separately. These findings are reported in Table 3 and showed no significant differences between the mean numbers of stools per day in infants who developed NEC and their matched controls.

The cumulative distribution of stooling onset in cases and controls was evaluated. As seen in Figure 1, there were no major differences observed among cases and controls. Most cases (60%) and controls (65%) passed their first stool within three days following birth and the vast majority of both cases (95%) and controls (91%) passed their first stool within the first week following birth. When the stooling pattern was evaluated in proximity to NEC diagnosis, analyses were conducted based on whether or not the infant was diagnosed with NEC during week 1, 2, 3, or 4. Those developing NEC later were not included in this analysis. This data is shown in Figure 2, which illustrates the average stools per day for the days prior to NEC diagnosis, with 0 indicting NEC diagnosis day. For controls, day of diagnosis is based on each control's matched case, as described earlier. Overall, the number of stools in both NEC cases and controls increased as the infant attained greater gestational

age, but there were no statistically significant differences in the number of stools between NEC cases and controls in the week prior to NEC diagnosis for infants diagnosed in the first three weeks (Figures 2A–2C). For infants diagnosed in week 4, there was a statistically significant but not clinically meaningful increase in the number of stools as the infants approached diagnosis when compared to their matched controls (Figure 2D). On average, in the seven days prior to diagnosis, the cases increased at a rate of 0.02 stools per day while the controls decreased at a rate of 0.07 stools per day ($p=0.03$).

Early nutritional exposures: Feeding with maternal breast milk

The majority of the subjects had received their first feeding by day 5 [3.0–9.0] following birth. Control infants were more frequently fed breast milk at the time of first feed (69.6%) when compared to infants who developed NEC ($p=0.03$). When the percentage of feeding with breast milk was compared over time prior to diagnosis with NEC, infants who developed Stage I and II NEC received a lower percentage of their feedings from breast milk (85% and 88%, respectively) when compared to controls (100%) ($p=0.02$).

The number of days infants were not fed (NPO) during the first week following birth differed significantly only among Stage II NEC cases and controls, and only in the first postnatal week. On average, infants in the control group remained NPO for 5 days, while infants who eventually developed Stage II NEC were NPO for 3 days during the first week following birth ($p=0.05$).

DISCUSSION

This descriptive analysis of the onset and pattern of stooling among low gestational age infants with and without NEC shows that during the first four weeks following birth, few differences exist in the day of first stooling, maximum and mean number of stools per day, and the number of stools per day in the week prior to disease onset. The differences that were identified primarily occurred in the first week following birth. During this time, infants who ultimately developed Stage II NEC stoolled slightly more frequently. This may be explained by the finding that these infants were NPO for slightly fewer days when compared to their controls. However, in the pairwise analysis, when comparing cases to their matched controls and separating groups by week of NEC onset, we did not identify any notable differences in the mean number of stools per day (Table 3). Because this pairwise analysis compared infants to those within their same gestational age cohort, and since NPO is likely influenced by gestational age, perhaps both gestational age and NPO are good candidates for future study of stooling first postnatal week. We did not see any difference in the day of first stooling between cases and controls.

It was interesting to observe that the vast majority of infants passed their first stool in the week following birth. For the small number of infants who did not stool in this time frame, we explored some of the clinical reasons why they might have had delayed motility (i.e. maternal magnesium, GI disease). In these 20 infants, we found that approximately half ($n=9$) of their mother's received magnesium. We also found that 40% ($n=8$) developed NEC on or before postnatal day 5. This very early onset and treatment for NEC was likely to have contributed to a delay in stooling in these infants.

In analyzing our stooling data in proximity to disease onset, we found that overall, infants had a greater number of stools each day as they attained postnatal age. This was not surprising and was a similar finding to the work others have reported¹⁵. What was interesting to note was that overall, there were no major differences in the number of stools between cases and controls as the infants approached disease diagnosis. This was especially true for infants who were diagnosed with NEC two and three weeks following birth. For the infants who were diagnosed during week 1, controls trended towards a greater number of stools when compared with cases on the same day, but this trend was not significant. For the infants who became ill during week 4, the infants with NEC had a slight increase in the number of stools as they approached disease while the controls were slightly decreasing in number of stools during that week. This change was statistically significant, but again, the overall difference in the number of stools was small, changing by less than a tenth of a stool per day on average for both groups. While these differences were small, it is possible that they may be explained by a differing pathophysiology contributing to NEC that occurs in the very early vs. later neonatal period. It is scientifically plausible that early onset NEC has more to do with fetal biology and a maternal contribution to the disease, while late onset NEC is the result of neonatal factors such as nutritional and nosocomial exposures in the NICU. These factors may influence the stooling pattern as a result of subtle differences in early intestinal colonization and overall function of the bowel as the infant matures.

Infants who developed NEC were more frequently fed infant formula for their first feeding, as expected given the results of previous studies. A statistically significant difference was also identified in the percentage of feeding with maternal breast milk prior to disease onset ($p=0.02$). Interestingly, in this study, even infants who developed NEC received a relatively high percentage of maternal breast milk, on average (i.e. 85% in Bell Stage I, 88% in Bell Stage II, and 100% in Bell Stage III). While there was a much larger range in the percentage of maternal breast milk among the cases when compared to the controls, these observations suggest that the percentage of maternal breast milk necessary for protection against NEC may be as high as 90%. Further study that helps to pinpoint the amount of breast milk required to adequately protect against NEC is needed.

In conclusion, the stooling pattern among low gestational age infants with or without NEC has not previously been studied. The findings of our analyses show that increased stooling frequency during the first week may be associated with later disease, although we only detected an increase among babies who developed Stage II NEC, and the increase might be too small to be clinically useful. Overall, feeding, stooling, and other neonatal factors we investigated of infants who develop NEC are similar to those who are unaffected by this GI disease. These findings are relevant to clinical practice in the NICU, where our clinical suspicion for NEC is often heightened by changes in the stooling pattern. While more study may be needed, the results reported here suggest that there are no major differences in the onset of stooling or the stooling frequency in infants who do or do not develop NEC. This was somewhat surprising because NEC is an inflammatory disease of the bowel where GI signs and symptoms would be expected. However, this data suggests otherwise. As a result, concern for NEC should not be focused on GI signs and symptoms alone. In addition, this is important information for researchers aiming to use stool samples to investigate bowel

disease, inflammatory disease (such as sepsis), or long-term morbidity (such as allergy, asthma, and growth), in premature infants. Investigators should not anticipate NEC to affect their ability to collect stool samples from the majority of premature infants.

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Figure 1.
Distribution of Stooling Onset, NEC Cases and Controls

a. Infants who were diagnosed with NEC in week 1 (n=129)

b. Infants who were diagnosed with NEC in week 2 (n=99)

c. Infants who were diagnosed with NEC in week 3 (n=60)

d. Infants who were diagnosed with NEC in week 4 (n=39)

Figure 2.

Stooling pattern in proximity to NEC onset day, as indicated by 0 on x-axis

x-axis: days prior to disease diagnosis day, which is indicated by 0; y-axis: number of stools per day

Table 1

Descriptive demographics, perinatal and neonatal factors

	Total Sample (n=258)	NEC Cases (n=129)	Control Subjects (n=129)	<i>p</i>
Demographics				
Gestational Age	26 [24.7–27.3]	26 [24.6–27.4]	26.3 [25.0–27.3]	0.30
Birth Weight	810 [680–1000]	795 [680–1000]	850 [700–990]	0.35
Gender				
Female	109 (42.2)	51 (39.5)	58 (44.9)	0.38
Male	149 (57.8)	78 (60.5)	71 (55.1)	
Perinatal Factors				
Mode of Delivery				
Vaginal	77 (29.8)	43 (33.3)	34 (26.4)	0.22
Cesarean Section	181 (70.2)	86 (66.6)	95 (73.6)	
Apgar score at 1”	5 [3–7]	5 [3–7]	5 [3–6]	0.11
Apgar score at 5”	7 [6–8]	7 [6–8]	7 [6–8]	0.11
DR Resuscitation:				
Chest Compressions	25 (10)	12 (9.8)	13 (10.2)	0.89
Epinephrine	14 (9.3)	6 (4.9)	8 (6.3)	0.62
Neonatal Factors				
Umbilical Arterial Catheter (UAC)	171 (67.1)	89 (69.5)	82 (64.6)	0.39
Day enteral feeding initiated	5 [3.0–9.0]	5 [3.0–8.5]	5.5 [3.0–9.0]	0.77
Volume of first enteral feeding	1.3 [1.0–1.7]	1.2 [1.0–1.7]	1.3 [1.0–1.7]	0.96
Type of first enteral feeding				
Breast Milk	155 (60.1)	68 (52.7)	87 (67.4)	0.03
Other (formula)	83 (32.2)	46 (35.6)	37 (28.6)	
Not fed/missing	20 (7.7)	15 (11.6)	5 (3.8)	

Median [IQR] reported for: gestational age, birth weight, Apgar scores, day enteral feeding initiated, volume of first enteral feeding

n (%) reported for gender, mode of delivery, DR resuscitation, UAC, type of first enteral feeding

Table 2

Descriptive feeding and stooling data, reported by Bell Stage

	Controls (n=129)	NEC Cases (n=129)			p
		Bell Stage I (n=42)	Bell Stage II (n=31)	Bell Stage III (n=56)	
Days NPO					
Week1	5 [3-7] (n=129)	5 [3-6] (n=42)	3 [2-6] (n=31)	6 [4-7] (n=56)	0.05
Week2	0 [0-4] (n=100)	0 [0-3] (n=35)	0 [0-1] (n=29)	1 [0-3] (n=36)	0.28
Week3	0 [0-2] (n=60)	0 [0-3] (n=20)	0 [0-2] (n=21)	0 [0-2] (n=19)	0.89
Week4	0 [0-1] (n=39)	0 [0-1] (n=13)	0 [0-1] (n=14)	1 [0-1] (n=12)	0.85
Proportion of breast milk feeding prior to NEC	1.0 [0.75-1.0]	0.85 [0.07-1.0]	0.88 [0.36-0.99]	1.0 [0.79-1.0]	0.02
DIFFERENCE in breast milk feeding during Week 1 (% of feeding from MBM)		0 [-0.35-0.24] p=0.07 n=34	-0.06 [-0.33-0.25] p=0.45 n=28	0 [0-0.99] p=0.89 n=30	
First postnatal day of stooling	2 [2-4]	3 [2-5]	2 [2-3]	3 [2-5]	0.23
Max number of stools/day					
Week1	2 [1-3] (n=129)	3 [2-3] (n=42)	3 [1-4] (n=31)	2 [1-3] (n=56)	0.04
Week2	4 [2-5] (n=99)	3 [2-4] (n=34)	4 [2-5] (n=29)	3 [2-5] (n=36)	0.91
Week3	4 [3-5] (n=60)	4 [2-5] (n=20)	4 [3-5] (n=21)	4 [3-5] (n=19)	0.78
Week4	4 [3-5] (n=39)	5 [4-6] (n=13)	4 [4-5] (n=14)	5 [4-6] (n=12)	0.35
Mean number of stools/day					
Week1	0.6 [0.3-1] (n=129)	0.9 [0.4-1.4] (n=42)	1.0 [0.42-1.6] (n=31)	0.4 [0.14-1.1] (n=56)	0.02
Week2	1.9 [0.9-3] (n=99)	1.4 [1.0-2.5] (n=34)	2.1 [1.0-3.0] (n=29)	1.3 [0.5-3.0] (n=36)	0.74
Week3	2.6 [1.6-3.5] (n=60)	2.4 [1.0-2.8] (n=20)	2.4 [1.6-3.1] (n=21)	2.8 [1.3-4.4] (n=19)	0.49
Week4	2.6 [1.8-3.4] (n=39)	2.8 [2.4-3.4] (n=13)	2.4 [1.8-3.8] (n=14)	3.1 [2.0-3.5] (n=12)	0.93

All variables reported as Median [IQR]

For all weekly analyses, only data up until the time of NEC diagnosis (or for controls, the day of their matched case's diagnosis) is included in these analyses, as indicated by (n=)

MBM: Mother's Breast Milk

Table 3

Pairwise comparison of differences between mean number of stools per day, reported by week of NEC onset and by NEC stage

	DIFFERENCE in number of stools/day Median [IQR] <i>p</i>	Stage I Median [IQR] <i>p</i>	Stage II Median [IQR] <i>p</i>	Stage III Median [IQR] <i>p</i>
DIFFERENCE in mean number of stools/day reported by Bell Stage and Week of disease diagnosis				
Week1 (n=129)	0.0 [-0.6, 0.7] .38	0.2 [-0.3, 0.9] .09	0.1 [-1.0, 1.0] .99	0.0 [-0.6, 0.6] .90
Week2 (n=99)	0.0 [-1.0-1.0] .77	0.0 [-1.0, 0.5] .30	-0.1 [-1.0, 1.0] .79	0.1 [-1.4, 1.9] .47
Week3 (n=60)	0.0 [-1.1, 1.1] .73	0.0 [-0.7, 1.6] .99	0.1 [-2.1, 1.0] .35	.21 [-1.0, 1.0] .90
Week4 (n=39)	0.3 [-0.7, 1.7] .16	0.9 [-0.3, 2.3] .12	0.1 [-1.2, 1.7] .89	.21 [-0.6, 1.0] .48