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Independence of Gut Bacterial Content and Neonatal Necrotizing Enterocolitis Severity

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Abstract

Introduction—Necrotizing enterocolitis (NEC) is a common and devastating gastrointestinal disease of premature infants. NEC severity varies widely. Recent data have demonstrated a strong link between gut microbial dysbiosis and development of NEC. We tested the hypothesis that alterations in the gut microbiome at the time of diagnosis predict the severity of NEC.

Methods—We used prospectively collected fecal samples from very low birth weight infants who developed NEC, stratifying by NEC severity. Fecal bacterial DNA was sequenced using 16S rRNA pyrosequencing. A generalized Wald-type test based on the Dirichlet multinomial distribution was used to test for differences in microbial communities.

Results—Of 489 infants at risk, 30 NEC cases had 410 fecal samples collected in the 28 days prior to the onset of NEC available for analysis. There were no differences the pre NEC gut microbial community between infants treated medically vs. surgically, or those with NEC totalis. Furthermore, neither treatment of NEC significantly changed the gut microbiome post NEC among the survivors.

Conclusion—We found no evidence that the gut microbiome, prior to the onset of disease, differentiates the clinical course of NEC. These data suggest that factors other than the gut microbiome may dictate disease severity.

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Keywords

Necrotizing Enterocolitis; Microbiome; Small Bowel Resection; Level Of Evidence–Level 4

1. Introduction

Necrotizing enterocolitis (NEC) is a catastrophic gastrointestinal disease of the newborn affecting 4–13% of premature very low birth weight (VLBW) infants (<1500g). NEC often results in perforation and irreversible bowel injury requiring resection (1, 2). The overall mortality from NEC remains 20–30%, and disease severity varies from a single segment or discrete segments of bowel, to panintestinal with the preponderance of the bowel being nonviable (NEC totalis)(3). The management of NEC is guided by multiple factors, but includes Bell's staging (4), infant weight, and clinical condition. Less severe NEC is initially treated medically, while advanced cases require surgical intervention consisting of either percutaneous peritoneal drain (PPD) and/or laparotomy and bowel resection. Mortality in the subset of patients that undergoes surgery ranges from 40–60%, decreases inversely with birth weight, and varies with procedure. (5) NEC is notorious for its swift progression and clinicians are often challenged by the minimal time from diagnosis to death. (6) Although many characteristics have been associated with increased risk of developing NEC, no single factor predicts the clinical course of established NEC.

Literature over the past 30 years has articulated a significant role for the gut microbiota in the pathogenesis of NEC (7). We have previously established an orderly progression of preterm infant gut microbial maturation with changes occurring at predictable postmenstrual ages (8), and subsequently, that NEC is preceded by gut dysbiosis with an increase in Gammaproteobacteria and decreases in Negativicutes and Clostridia. (9) Knowing the orderly progression of the healthy microbiome as well as the dysbiosis that precede NEC, we sought to test the hypothesis that these pre-NEC microbiome changes would predict disease severity, and secondarily, be associated with the effect of medical and surgical treatment on the recovery of the gut microbiome.

2. Methods

2.1 Patient Selection

Infants admitted to the St. Louis Children's Hospital NICU between July 2009 and September 2013 weighing 1,500 grams at birth with a predicted survival of >1 week were eligible for inclusion. All samples were collected to 36 weeks postmenstrual age, and frozen (–80°C) until analyzed, as detailed previously.(9) The study was approved by the Washington University Institutional Review Board (#201503102) and written consent was obtained prior to processing any of the collected fecal samples of enrolled infants.

Cases were defined as infants whose clinical course and radiographs fulfilled Bell's stage 2 or 3 criteria. Medical NEC was defined as nonsurgical treatment of NEC, in a child who survived > 24 hours after diagnosis. Surgically treated NEC was defined as infants who underwent laparotomy and resection or peritoneal drain placement with or without

subsequent resection. NEC totalis was defined as infants with extensive bowel involvement based on direct visualization at the time of laparotomy or autopsy or infants who died within 24 hours of diagnosis before an operation could take place.

For the pre-NEC analysis, samples were selected from 28 days prior to the development of NEC until the day before NEC diagnosis. The week of NEC is designated as week zero and includes samples between seven and one day prior to NEC, samples from days 8 to 14 prior to NEC onset are designated as originating in Week -1, and samples from days 15 to 21 prior to NEC onset are designated as originating in Week -2, and samples from days 22 to 28 prior to NEC are designated as origination in Week -3. If a patient produced more than one specimen within an interval, proportions were collapsed into composite values for that individual for that week. Intervals with <4 subjects having adequate samples were excluded from analysis.

For the post-NEC analysis, we first sought to examine the effect of NEC on the gut microbiota over time. We compared samples from a 14-day interval before diagnosis to a 14-day interval after diagnosis in the same infant. Next, we sought to compare the effect of NEC on the gut microbiota between medically and surgically treated cases, compared to non-NEC controls matched on gestational age (+/- one week) and birth weight (+/- 100 grams). Comparisons were made in five 15-day intervals based on day of life (DOL) beginning at birth and continuing to DOL 75 or discharge home, whichever came first. Multiple samples from individual subjects were again collapsed into composite values and time intervals with <4 subjects excluded.

2.2 DNA Extraction and Sequencing

Fecal DNA was extracted using the Qiagen (Hilden, Germany) DNA Stool Mini kit and the automated QIAcube (Qiagen). Sequencing of the bacterial 16S rRNA was performed using amplicons specific to the V3-5 regions at the McDonnell Genome Institute at Washington University. Details regarding methods for DNA sequencing, library construction, and taxonomic grouping have been previously published.(9)

2.3 Statistical Analysis

Sequences results were analyzed using the generalized Wald-type test based on the Dirichlet multinomial distribution to determine if microbial communities differed between cohorts. (10) Briefly, seven-day intervals were created with day zero being the day of diagnosis and progressing before or after NEC depending on analysis. Bacterial populations at the class level were averaged for all samples collected from each subject during an interval. For cases, samples were included up to the day before NEC diagnosis. If a subject had no sample (or inadequate reads in a sample), no data were entered for that sample.

For demographic comparisons we used χ^2 and Fisher's exact to compare categorical variables and Student's t-test and one-way analysis of variance (ANOVA) for continuous variables. Significance was assigned to p values less than 0.05.

3. Results

3.1 Pre NEC Analysis

Of 489 infants at risk, 35 developed NEC and 30 of these had sufficient samples for inclusion. No significant differences were evident between the medically treated, surgically treated and the NEC totalis groups with respect to demographics (race, gender), clinical characteristics (gestational age birth weight, APGAR scores, mode of delivery or singleton pregnancy) or age of NEC onset (Table 1).

From these subjects, a total of 410 pre-NEC fecal samples from the 28-day period prior to NEC were used for comparing the microbial community of medical, surgical, and NEC totalis patients. There were 108 samples from infants with medically treated NEC (7–10 infants per analysis interval), 226 samples from infants with surgically treated NEC (6–13 infants per analysis interval), and 150 samples from infants with NEC totalis (6–7 infants per analysis interval).

No statically significant differences in the gut microbiome were found between infants with medically treated NEC, surgically treated NEC and those with NEC totalis at any point during the four week total analysis interval prior to NEC diagnosis (Figure 1). As previously described, Gammaproteobacteria remained the predominant class at all time points (8). Likewise, no relationship was evident between the gut microbial community structure and NEC mortality, with no statistical differences between survivors (combined medical and surgical survivors), and those who died from NEC totalis. **(data not shown).**

3.2 Post-NEC Analysis

To examine the impact of NEC and its treatment on each subject's gut microbial community we compared samples collected before NEC to samples collected after NEC from the same infant, among the 10 subjects who survived their episode of NEC and who had sufficient pre and post NEC samples for study. To account for the patterned progression of community assembly over time that naturally occurs in preterm infants, we restricted the analysis to 14 days pre and 14 days post NEC. This analysis included 173 samples from five subjects per treatment group. When comparing the microbiome from the same subject before and after NEC, we observe no consistent changes in any of the microbial classes that dominated prior to NEC **(data not shown).**

To determine if infants return to the predictable, orderly progression of microbiome development outlined in previous work(8) we included samples from 16 subjects who survived their episode of NEC and had sufficient post NEC material. (Figure 2) This post-NEC analysis therefore included 22 samples from medically treated NEC (n=4–5 per analysis interval), 659 samples from control subjects matched to the medical cohort (n=76–84 per analysis interval), 64 samples from surgically treated NEC (n=4–7 per analysis interval), and 592 samples from control subjects matched to the surgical cohort (n=43–60 per analysis interval).

We compared characteristics of both the medical and surgical groups to their respective controls and also to each other. Significantly lower APGAR scores at one minute (1.2 ± 0.17

vs. 4.1 ± 0.26 , $p=0.008$) and 5 minutes (4 ± 0.77 vs. 6.1 ± 0.22 , $p=0.03$), and more African-American infants (100% vs. 50%, $p=0.01$) were present in the medical ($n=6$) than the medical controls ($n=103$). We did not identify significant differences in the post NEC microbiome of infants with medically treated NEC or surgically treated NEC when compared to controls. (Figure 3)

4. Discussion

In this study, we found that the fecal microbial community was indistinguishable between infants with NEC that were managed with antibiotics alone, those who required surgical resection, and babies with NEC totalis. Thus, three cohorts of comparable infants with similar microbiomes had vastly different clinical outcomes. Further, our data would suggest that neither medical nor surgical treatment of NEC significantly alters the intestinal microbiome. These findings advocate for other host factors likely playing a more critical role in determining the severity of NEC.

Recent advances in high-throughput sequencing of the 16S ribosomal rRNA genes of luminal gut bacteria have established a significant association between the composition and function of the intestinal microbiota and various inflammatory responses that are associated with a wide spectrum of diseases and conditions including malnutrition, diabetes, obesity, and inflammatory bowel disease.(11–14) However, one limitation of our study is that we did not evaluate gut microbes adherent to the intestinal mucosa or that have already invaded into the bowel wall. Therefore, a simple analysis of fecal samples may not reflect the injured site. Similarly, a resected piece of intestine, after a patient is subjected to multiple interventions after NEC diagnosis including nasogastric suction, withholding of feeds, and commencement of broad-spectrum antibiotics, is also not quite representative of the pre-NEC adherent mucosal bacteria.(15) A role for enteroinvasive bacteria in the pathogenesis of NEC was suggested in a prior study from our laboratory in which bacterial DNA was identified within the bowel wall of infants with NEC at the time of operative resection(16). In that cohort, there was significantly more bacterial DNA in the bowel wall of infants with acute NEC compared with the same infants at the time of ostomy closure after the NEC had clinically resolved. In another study, the bacterial population was characterized in formalin-fixed NEC tissue employing laser-capture-micro-dissection combined with fluorescence in situ hybridization and 16S rRNA sequencing from the bowel of 24 neonates with surgically-treated NEC.(17) In that report, a large variability of bacteria was found in most of the analyzed specimens. However, no single or combination of known potential pathogenic bacteria species was predominant in the samples. Since all patients in this study had surgical NEC, there was no opportunity to compare gut mucosal-associated microbial communities with medically treated patients or those with NEC totalis. The invasive nature of tissue acquisition makes it a much less likely candidate for NEC screening in the at-risk neonates even if such material could offer predictive insight.

Our results may also be related to the utilization of 16s rRNA analysis, which is limited to identification of community structure at a genus or species level. Strain specific genotypic variation within the same genus and species of bacteria, not evident on 16S rRNA analysis may impact both pathogenicity and host- microbial interactions(18). Additionally,

differences exist not only between microbial community structure, but also its repertoire of genetic expression. These microbial community attributes may impact disease severity, but will not be detected by 16S rRNA analysis, identifiable by alternative methods, especially metagenomic shotgun sequencing.

Consistent with our discovery that the clinical course of NEC could not be predicted by the gut microbiome at the time of diagnosis, clinical resolution of NEC after treatment with either antibiotics or surgical resection did not appear to affect the gut microbiome. This was surprising, in light of the known influence of the administration of specific antibiotics(19–21) as well as intestinal resection(22–25) on the gut microbiota. On the other hand, recent data have demonstrated that the intestinal mucosal-bound microbiome remains unaffected by prolonged antibiotic use during ischemic and inflammatory events in neonates (15), and, in aggregate, by antibiotics administered to preterm infants (8). It is likely that the small numbers of infants with sufficient samples who were medically or surgically managed were too small to make statistical comparisons. Also sample limitation might be biased toward those subjects healthy enough to produce samples post NEC, further limiting power to detect difference. It might also be considered that changes in the microbiome may occur later than the 1–2 week postoperative time points that we studied. For all of these reasons, we hesitate to draw conclusions about the differences between the effects of medical and surgical treatment on NEC. Nevertheless, the NEC resolved clinically, but without identifiable changes in the gut microbiome.

Our findings suggest that NEC is a process involving an existing abnormal microbial community, but that community structure does not appear to substantially alter the severity of the outcome, given the baseline dysbiosis that predisposes to NEC. An additional variable that we did not analyze was the extent to which host response, manifested as IgA coating of bacteria, could affect severity of NEC. Another function worthy of consideration is host mucin. Secretory IgA has been found to localize with bacteria on the outer layer of colonic mucus the colon of humans and mice.(26) Intestinal mucin expression has been shown to be reduced in experimental models of NEC(27, 28). The ability to generate mucus may therefore play a role in the clinical outcome of NEC. Continued investigation of host-microbial interactions in the context of NEC is critical for a more comprehensive understanding of this important condition. If there is no evidence for varying microbial subsets of NEC as determinants of outcome (NEC severity or recovery), then perhaps outcome is ordained by the duration of the interval between injury and awareness that clinical NEC has evolved. If so, this would make a strong case for intensive surveillance for NEC, before the clinical appearance of advanced inflammatory injury is manifest.

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Figure 1.

The microbiome does not predict NEC severity. Fecal samples from premature, very low birth weight infants who developed NEC were collected and sequenced using 16s pyrosequencing. Cases were stratified into groups based on disease severity and compared in 7-day intervals beginning at the week of NEC diagnosis (Week 0). Day 7–14 prior to NEC designated as one week prior to NEC, 15–21, two weeks prior to NEC and 22–28, 3 weeks prior to NEC. $N < 4$ for Medical group at 3 weeks prior to NEC and was therefore excluded from analysis. Bars show SEM for each class within each group.

Figure 2. Study design for post-NEC analysis comparing medical and surgical NEC to their respective controls.

Figure 3.

The microbiome of infants with medically treated or surgically treated NEC is not significantly different from healthy infants 14 days after NEC. Stool samples were collected from infants with NEC and compared to gestational age and weight matched controls. Samples were grouped into 15-day time intervals based on day of life. $N < 4$ in both medical and surgical NEC groups prior to day of life (DOL) 30 and in the medical NEC group after DOL 60 and were therefore excluded from the analysis. Bars show SEM for each class within each group.

Table 1

Characteristics of Infants With NEC Included in Pre-NEC Analyses.

Gramaproteobacteria	Bacilli	Negativitrites	Bacteroidetes	Chloridria	other	LC Gamma	LC Bacil	LC Negat	LC Bacte	LC Clost	LC Other	UC Gamma	UC Bacil	UC Negat	UC Bacte	UC Clost	UC Other	SE Gamma	SE Bacil	SE Negat	SE Bacte	SE Clost	SE Other
	0.07	0.11	0.00	0.04	0.00													0.054	0.02	0.04	6.49	0.02	0.00
0.755438	7163	3863	0104	9641	3791	1												319	2519	4215	E:05	2959	2556
	0.11	0.00		0.07	0.05													0.046	0.03	0.00	1.43	0.02	0.01
0.745803	853	4662	2.07E-05	8028	2956	1												742	6604	3377	E:05	3488	5765
	0.02	0.01	0.03	0.29	0.00													0.086	0.00	0.00	0.03	0.08	0.00
0.625485	5944	3864	9247	3417	2043	1												677	9796	8739	9139	9446	0493
51.64%	37.89%	3.69%	0.14%	4.12%	2.61%	0.08%	0.02%	0.00%	0.00%	0.00%		99.94%	99.79%	31.36%	0.68%	31.20%	14.74%	4.89%	5.12%	1.13%	0.10%	1.11%	0.62%
66.68%	28.08%	0.10%	0.09%	1.45%	3.61%	0.25%	0.07%	0.00%	0.00%	0.00%		99.88%	99.13%	0.38%	0.03%	16.45%	26.46%	3.20%	3.13%	0.06%	0.08%	0.53%	0.76%
68.65%	13.35%	1.34%	5.71%	9.57%	1.38%	5.87%	0.01%	0.00%	0.00%	0.00%		98.99%	83.52%	3.55%	58.76%	55.20%	12.44%	3.30%	2.66%	0.92%	1.89%	1.94%	0.43%
68.79%	24.72%	0.27%	0.16%	2.71%	3.36%	2.04%	0.02%	0.00%	0.00%	0.00%		99.96%	96.29%	1.71%	1.42%	30.83%	18.74%	6.39%	5.64%	0.10%	0.10%	1.58%	1.21%
62.29%	32.72%	0.05%	0.01%	1.14%	3.80%	1.90%	0.29%	0.00%	0.00%	0.00%		99.45%	96.83%	0.25%	0.07%	10.67%	10.92%	5.18%	5.11%	0.02%	0.01%	0.45%	1.67%
69.08%	10.77%	9.90%	5.95%	2.28%	2.02%	3.47%	0.01%	0.00%	0.00%	0.00%		99.31%	44.11%	86.88%	67.47%	30.87%	9.86%	4.85%	2.21%	3.79%	2.61%	1.17%	1.11%
62.98%	31.65%	0.32%	0.04%	0.25%	4.77%	6.53%	0.02%	0.00%	0.00%	0.00%		99.93%	71.36%	2.84%	0.28%	1.90%	22.79%	5.68%	4.71%	0.20%	0.02%	0.15%	1.46%
34.69%	43.43%	2.11%	4.46%	1.13%	14.18%	0.09%	0.14%	0.00%	0.00%	0.00%		95.00%	99.80%	10.21%	40.41%	6.25%	60.68%	10.64%	10.33%	0.88%	4.00%	0.55%	5.62%
85.12%	11.19%	0.21%	0.01%	0.30%	3.17%	33.63%	1.03%	0.00%	0.00%	0.00%		98.95%	54.71%	1.20%	0.07%	1.82%	11.05%	9.04%	7.72%	0.17%	0.01%	0.27%	1.65%

Data is presented as mean (min-max), (SEM) or n (%)

* medical vs. surgical