Microbiota and Necrotizing Enterocolitis

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Abstract

Necrotizing enterocolitis (NEC) is an acquired gastrointestinal inflammatory condition with significant mortality and morbidity in preterm very low birth weight infants. The interplay between toll-like receptors, bacterial endotoxins, developmentally regulated excessive proinflammatory responses of the immature innate immune system, hypoxia, ischemia, reperfusion, free radicals, and the presence of substrates and bacterial endotoxins is thought to play an important role in the pathogenesis of NEC. The association (cause?) of various microbes (bacteria, viruses, and fungi) with NEC has intrigued researchers for many years. Availability of newer molecular methods (e.g., 16S ribosomal RNA gene-specific primers/pyrosequencing of fecal DNA) is expected to improve our understanding of the role of gut microbiota in the pathogenesis of NEC. Recent studies employing such methods to assess fecal microbiota are reviewed. Current evidence suggests that dysbiosis of the gut microbiota precedes the development of NEC in preterm infants. Further research is required to understand the significance of changes in the gut microbiome over the early postnatal period including the relative abundance of Gammaproteobacteria and the paucity of strict anaerobic bacteria that precedes NEC in preterm infants. Assessing the reproducibility of previous findings in large prospective studies with standardized methodology (e.g. sample processing, PCR primer, and DNA extraction) is important.

Introduction

Necrotizing enterocolitis (NEC) is a potentially devastating acquired condition of the gut in preterm infants. It occurs in 4–6% of preterm infants with very low birth weight (VLBW: <1,500 g) and 9–14% of infants with extremely low birth weight (ELBW: <1,000 g) [1–6]. NEC (≥stage II) is associated with significant
mortality (~25%) and morbidity including recurrent late-onset sepsis, prolonged dependence on parenteral nutrition, need for surgery, survival with intestinal failure, and long-term neurodevelopmental impairment [1–3, 7–13]. The outcomes are worse in ELBW infants needing surgical intervention for NEC [5, 9, 14]. The mortality could be as high as 100% in those with extensive full-thickness necrosis of the gut [5, 9, 14]. The socioeconomic burden of ≥stage II NEC is significant considering the prolonged hospital stay and long-term consequences of the illness [1, 15]. Advances in neonatal intensive care have resulted in an increase in the absolute number of survivors of extreme prematurity who are at high risk of NEC. Primary prevention of NEC will be difficult till prevention of preterm birth becomes a reality. The poorly understood pathogenesis of NEC makes it difficult to develop a cure for the illness [16, 17].

The interplay between various factors including the developmentally regulated inflammatory response of the immature innate immune system, hypoxia, ischemia, reperfusion, free radicals, and the presence of substrates and bacterial endotoxins is currently thought to play an important role in the pathogenesis of NEC [1, 17–22]. The roles excessive proinflammatory responses of the immature innate immune system, bacterial endotoxins, toll-like receptors, and dysbiosis of the gut microbiota play have become a research priority to improve the understanding of the pathogenesis of NEC [18, 19, 23–27]. This brief review is focused on the role of gut microbiota in the pathogenesis of NEC in preterm VLBW infants.

Role of Microbiota in the Pathogenesis of Necrotizing Enterocolitis

No specific pathogen has been consistently associated with NEC despite the wide agreement that gut microbes play an important role in the pathogenesis of this condition [28]. A range of microorganisms, including bacteria, viruses, and fungal species, has been associated with NEC in preterm infants, and the same microbe species are often found in gestation-matched healthy infants [27, 29–31]. Proving a causative relationship between a specific pathogen and NEC is thus difficult even during clustered outbreaks of the condition [32, 33]. In a prospective case-control study, Peter et al. [33] studied the association between NEC and specific pathogens. Over the study period, 18 preterm infants (<36 weeks) developed ≥stage II NEC with portal venous gas; 8 needed laparotomy, and 2 died. Gestation-matched infants without NEC were controls. In the week before NEC diagnosis, potentially pathogenic bacteria were identified in stools of all cases and 79% of controls (p < 0.05). There was no significant difference in the occurrence of specific pathogens or groups of pathogens in cases versus
controls [33]. In the context of association versus causation, it is important to note that randomized trials of oral antibiotics to prevent NEC suggest a causal relationship between gut bacteria and NEC, and, importantly, NEC does not occur in the sterile in utero environment [34–36].

The concept of “opportunistic pathogens” suggests that a microbe that is nonpathogenic/nonvirulent in healthy term infants can become pathogenic in an immunocompromised preterm infant [37]. Leach et al. [37] investigated this issue by analyzing the meconium and fecal samples collected over the first month of life in preterm infants (24–32 weeks of gestation) by the 16S ribosomal RNA (rRNA) gene sequencing method. During the study period, 4 infants developed NEC (cases) and 18 without NEC served as controls. Fecal S100A12 concentrations, measured by immunoassay, increased significantly after NEC development. The fecal microbiome did not significantly differ in cases versus controls. However, potentially pathogenic bacteria were detected significantly more often in cases than controls ($p = 0.0007$), suggesting that they may contribute to the pathogenesis of NEC [37]. Investigators have reported that Cronobacter sakazakii, a gram-positive endospore-forming obligate anaerobe, is an opportunistic pathogen that displays increased virulence in a compromised host and has strain–specific effects [38–42]. The role of C. sakazakii in neonatal NEC is supported by studies in a rat pup model of the illness [43, 44]. Its effects are dose dependent, with higher doses ($10^7$ CFU) causing enterocyte apoptosis and destruction of the villus tips due to induction of proinflammatory cytokine release (e.g. IL-6) and inducible nitric oxide synthase to increase nitric oxide levels [44–46].

Studies Assessing Gut Microbiota in Preterm Infants with Necrotizing Enterocolitis


(1) In 2016, Warner et al. [47] reported a large prospective observational study comparing the gut bacteria in preterm VLBW infants who developed $\geq$stage II NEC (cases) versus those who did not (controls). The controls (1–4 per case) were matched for gestation, birth weight, and date. The primary (122 infants/2,492 stool samples) and secondary (44 infants/1,094 stool samples) cohorts were enrolled in different hospitals. They used bacterial 16S rRNA gene-specific primers and pyrosequencing of fecal DNA. A total of 28/122 infants in the primary cohort developed NEC (cases); 94 infants served as controls. The...
fecal microbiota differed significantly between cases and controls after the first month of age. Development of NEC was positively associated with Gammaproteobacteria and negatively with strictly anaerobic bacteria, especially Negativicutes. In the secondary cohort, a total of 18/44 infants developed NEC (cases), and 26 infants served as controls. Combined data from all cohorts (166 infants/3,586 stools/46 NEC cases) showed increased proportions of Gammaproteobacteria \(p = 0.0011\) and lower proportions of Negativicutes \(p = 0.0013\) and the Clostridia-Negativicutes combination \(p = 0.0051\) in cases versus controls. These associations were strongest in the primary and overall cohort for infants born <27 weeks of gestation [47].

(2) In 2016, Ward et al. [48] reported uropathogenic *Escherichia coli* (UPEC) colonization as a risk factor for NEC and subsequent mortality. The early gut microbiota of preterm \((n = 144)\) and term \((n = 22)\) infants was studied using deep shotgun metagenomic sequence analysis. A pan-genomic approach was used to functionally subtype the *E. coli* and identify genes associated with NEC and mortality that indicate colonization by UPEC. Metagenomic multilocus sequence typing analysis defined NEC-associated strains as sequence types often associated with urinary tract infections, including ST69, ST73, ST95, ST127, ST131, and ST144 [48]. Further studies are needed to confirm whether there is a causal link between UPEC and NEC.

(3) In 2016, Heida et al. [49] studied the prognostic factors for NEC development in high-risk neonates who did (11 cases) versus those who did not (22 controls matched for gestation/birth weight) develop the illness. The 16S rRNA gene sequencing method was used. The presence and abundance of *Clostridium perfringens* (8.4%) and *Bacteroides dorei* (0.9%) in meconium were significantly increased in cases versus controls \(p < 0.001\). In postmeconium samples, the abundance of staphylococci was negatively associated with NEC; *C. perfringens* was more prevalent in NEC cases. Early enteral feeding and, in particular, breast milk were correlated with an increase in lactate-producing bacilli in postmeconium samples [49].

(4) In 2016, Hourigan et al. [50] reported serial microbiome changes (16S rRNA gene sequencing) in twins discordant for NEC, with similar intrauterine and early environmental exposures. A decrease in bacterial diversity and an increase in Proteobacteria were noted a week preceding the signs of NEC in the twin who developed the illness [50]. These findings suggest that early gut microbiota may play an important role in the pathogenesis of NEC.

(5) In 2016, Cortese et al. [51] hypothesized that a cross talk exists between the host epigenome and the initial microbiota colonizing the gut at a critical stage. By exposing immature enterocytes to probiotic and pathogenic bacteria, they showed >200 regions of differential DNA modification, which were specific
for each exposure. In a mouse model, they demonstrated that antenatal glucocorticoid treatment altered the host epigenome. The effects on the expression of genes associated with inflammatory responses and intestinal barrier function were studied by quantitative polymerase chain reaction (qPCR). The DNA modification changes in 5 candidate genes were verified by quantitative methylation-specific PCR. Using 16S RNA sequencing-based phylogenetic analysis, they showed that epigenome changes conditioned early microbiota colonization leading to differential bacterial colonization at different taxonomic levels. These findings suggest that microbial colonization may alter epigenetic signatures of the immature gut establishing inflammatory changes and compromising barrier properties predisposing to NEC [51].

(6) In 2016, Abdulkadir et al. [52] analyzed 72 longitudinal stool samples from 20 infants (10 NEC cases and 10 controls) by qPCR. Controls were matched for birth weight, gestation, delivery mode, and gender. There was no significant difference in the total bacterial load in cases versus controls. There were also no significant temporal changes in the total bacterial load within NEC infants before versus after NEC diagnosis, and in healthy controls [52]. These findings suggest that fecal bacterial load may not be a reliable surrogate for tissue bacterial load in NEC.

(7) In 2015, Cassir et al. [53] analyzed the gut microbiota in stool samples from NEC cases and controls (15 each) by 16S rRNA pyrosequencing and culture-based methods. A Clostridium butyricum-specific qPCR assay was developed. Stool samples from preterm infants with NEC (n = 93) and controls without NEC (n = 270) were tested. The whole genome of 16 C. butyricum strains was sequenced and analyzed. C. butyricum was specifically associated with NEC using molecular and culture-based methods (15/15 vs. 2/15; p < 0.0001) or qPCR (OR: 45.4; 95% CI: 26.2–78.6; p < 0.0001). Culture supernatants of C. butyricum strains from NEC infants (n = 14) showed significant cytotoxic activity (p = 0.008). A homologue of the β-hemolysin toxin gene shared by Brachyspira hyodysenteriae, the cause for swine dysentery, was identified in all NEC cases. The corresponding protein was secreted by a NEC-associated C. butyricum strain [53].

(8) In 2015, Zhou et al. [54] studied the longitudinal changes in the gut microbiome preceding NEC in preterm infants. Using the 16S rRNA method, they analyzed 312 samples in 12 cases that developed NEC and 26 gestation-matched controls that did not. The gut microbiome evolved rapidly during the first 2 months of life. The day of life was the major factor contributing to the colonization process. Depending on the postnatal age at development of NEC (early vs. late onset), the pattern of microbial progression was different in cases versus controls. The differences were most obvious between early-onset NEC and...
controls. Closer to the onset of the illness, *Clostridium sensu stricto* was significantly more abundant in early-onset NEC than in controls. In late-onset NEC, the Gammaproteobacteria *Escherichia/Shigella* showed an increasing pattern prior to the illness and were significantly higher in cases than controls 6 days before NEC. *Cronobacter* (Gammaproteobacteria) was significantly higher in late-onset NEC cases than controls 1–3 days before NEC [54]. Overall, these results indicate that the specific pathogen associated with NEC may vary by the infant’s postnatal age at onset of NEC.

(9) In 2015, McMurtry et al. [55] compared the gut microbiota of infants with NEC (21 cases) to matched controls without NEC (74 controls) using 454 pyrosequencing analyses of 16S rRNA genes that were PCR amplified from stool DNA specimens. NEC severity was categorized as mild, severe, and lethal. Bacterial diversity as well as the relative abundance of Actinobacteria and Clostridia was significantly lower in NEC specimens than controls. The absence of Clostridia was significantly associated with NEC. Microbial diversity and Clostridia abundance and prevalence decreased with increasing severity of NEC. The investigators concluded that low fecal bacterial diversity may be indicative of NEC and its severity, and that the presence of taxa such as Clostridia may play a role in attenuating inflammation leading to NEC [55].

(10) In 2015, Raveh-Sadka et al. [56] studied the spread of potential pathogens among hospitalized preterm infants in the context of NEC. They compared microbial communities between infants who did (34 cases) or did not (5 controls) develop NEC using strain-resolved comprehensive bacterial community analysis. The strains colonizing each infant were distinct, and none was common to infants who developed NEC. The investigators commented that the paucity of shared gut colonizers suggested the existence of significant barriers to the spread of bacteria among infants [56].

(11) In 2015, Sim et al. [57] studied the gut microbiota preceding NEC in preterm infants (n = 369) by next-generation sequencing of 16S rRNA gene regions. Fecal samples were analyzed from 12 infants with definite NEC, 8 with suspected NEC, and 44 controls. Before diagnosis, a clostridial operational taxonomic unit was overabundant in samples from infants with established NEC (p = 0.006). Culture confirmed the presence of *C. perfringens* type A. Fluorescent amplified fragment length polymorphism typing showed that no isolates were identical. Samples from NEC cases without profuse *C. perfringens* showed an overabundance of a *Klebsiella* operational taxonomic unit [57]. The utility of *Clostridium* and *Klebsiella* operational taxonomic units as biomarkers for early diagnosis of NEC needs to be confirmed.

(12) In 2014, Brower-Sinning et al. [58] studied the diversity of mucosal bacteria in resected gut samples from preterm infants with (n = 16) and without (n =
10) NEC, using 16S rRNA gene sequencing. The total bacterial burden was higher in NEC samples. Both NEC and non-NEC samples showed high interindividual variability and an abundance of opportunistic pathogens. The NEC samples showed an abundance of strict anaerobes and a decreased diversity of the bacterial community, and no uniform pattern of microbial colonization [58].

(13) In 2013, Claud et al. [59] studied the gut microbiota of preterm infants that did develop NEC (5 cases) versus those who did not (5 controls) using the 16S rRNA gene sequencing method. Over time, the gut microbiota of control infants developed and became closer to that in healthy breast milk-fed term infants. The gut microbiome differed between cases and controls, starting from 3 weeks before NEC diagnosis. The majority of the differentially abundant genes in cases were associated with carbohydrate metabolism and mapped to the Enterobacteriaceae family [59]. These findings are helpful in understanding the temporal changes in the gut microbiome and in the substrate availability for growth of different bacteria.

(14) In 2013, Normann et al. [60] reported no significant differences in gut microbiota composition in NEC cases versus matched controls. In a prospective study, they analyzed fecal flora by barcoded pyrosequencing in extremely preterm infants (10 cases of NEC and 20 controls matched for gender, gestation, and mode of delivery). The gut microbiota was dominated by Enterococcus, Bacillales, and Enterobacteriaceae in cases, with a high relative abundance of Bacillales and Enterobacteriaceae preceding the diagnosis of NEC. The flora was dominated by enterococci in control samples. A low diversity of gut microbiota was found with no significant differences between NEC cases and controls. In 16 healthy controls, Firmicutes (Enterococcus and Bacillales) dominated the fecal flora during the first weeks after birth and were then succeeded by Enterobacteriaceae [60]. Further studies are needed to confirm these findings.

(15) In 2012, Smith et al. [61] analyzed fecal samples (n = 482) from 163 preterm infants (<30 weeks of gestation) during the first month of life by culture and PCR denaturing gradient gel electrophoresis (DGGE). A total of 21/163 infants developed NEC. Very few bacterial species could be cultured from the samples. Gram-positive bacteria dominated the samples in the NEC group, whereas in the control group a mixed flora of gram-positive and -negative bacteria were isolated. Molecular analysis using PCR-DGGE profiles did not confirm these differences. The investigators suggested that intestinal gram-positive bacteria may play a role in the development of NEC in preterm infants [61].

(16) In 2011, Mai et al. [62] compared the diversity of microbiota and prevalence of specific bacterial signatures in preterm infants (≤32 weeks of gestation or birth weight ≤1,250 g; 9 NEC cases and 9 gestation-matched controls) using

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high-throughput 16S rRNA sequencing. Weekly stool samples \((n = 110,021)\) were collected starting with the first stool and continued until discharge. Microbiota composition differed in control samples collected 1 week but not <72 h before the diagnosis of NEC. Between the 1-week and <72-h samples, Proteobacteria increased (34%), Firmicutes decreased (32%), and some of the molecular signatures were increased in NEC cases. One of the more frequently detected bacterial signatures \((p < 0.01)\) matched closest to Gammaproteobacteria. Although this sequence was close to the Enterobacteriaceae family, it did not match any sequence in GenBank by >97%. Further data are required to confirm if this microbe contributes to the development of NEC [62].

(17) In 2009, Wang et al. [63] compared fecal microbiota in preterm infants who did (10 cases) versus those who did not (10 controls, including 4 twin pairs) develop NEC by 16S rRNA gene sequencing and terminal restriction fragment length polymorphism analysis. Gut microbiota showed low diversity in the samples from all infants. Infants with NEC showed a further decrease in diversity, increased abundance of Gammaproteobacteria, and a decrease in other bacterial species. They had received antibiotics for a longer duration prior to developing NEC. These findings support the role of a diminished diversity of the gut microbiome and prolonged exposure to antibiotics in the development of NEC [63].

**Discussion**

Overall, the current evidence indicates that in contrast to the healthy adult gut microbiota, the early postnatal gut microbiota of preterm infants is simple, very diverse, and dynamic, and plays an important role in the development of NEC [28]. Given the number of factors involved in shaping the early gut microbiota in preterm infants, including the mode of delivery, gestational and postnatal age, type of milk feeds, exposure to antibiotics in the early pre- and postnatal period, and exposure to gastric acid inhibitors, it is not surprising that the results of gut microbiota studies in preterm infants are difficult to interpret [64–69]. Differences in the methodology for assessing the gut microbiota further complicate the issue. However, experts point out that the results are mostly similar when diverse methods (e.g. culture-based or molecular methods) are used in the same study [69–75]. Investigators report that culture-based methods miss relatively few, if any, bacterial species when analyzing the early microbiota [76]. Most studies have assessed fecal microbiota, and results of mucosal biopsies and lumenal content analysis seem to suggest/confirm the adequacy of fecal sampling [71, 77, 78].
<table>
<thead>
<tr>
<th>First author</th>
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<tr>
<td>Warner et al. [47], 2016</td>
<td>16S rRNA, Pyroseq</td>
<td>46</td>
<td>120</td>
<td>NEC was associated with significantly increased Gammaproteobacteria and decreased Negativicutes</td>
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<td>Ward et al. [48], 2016</td>
<td>16S rRNA, DSMSA</td>
<td>27</td>
<td>89</td>
<td>Colonization with uropathogenic <em>Escherichia coli</em> was a risk factor for NEC and subsequent mortality; the strains included ST69, ST73, ST95, ST127, ST131, and ST144</td>
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<td>Heida et al. [49], 2016</td>
<td>16S rRNA</td>
<td>11</td>
<td>22</td>
<td>Significantly increased presence and abundance of <em>Clostridium perfringens</em> and <em>Bacteroides dorei</em> in meconium in cases vs. controls; postmeconium samples: abundance of staphylococci negatively associated with NEC; <em>C. perfringens</em> more prevalent in NEC cases</td>
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<td>Hourigan et al. [50], 2016</td>
<td>16S rRNA</td>
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<td>Abdulkadir et al. [52], 2016</td>
<td>16S rRNA, qPCR</td>
<td>10</td>
<td>10</td>
<td>No significant difference in TBL in cases vs. controls; no significant temporal changes in TBL within cases before vs. after NEC diagnosis, and in controls</td>
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<td><em>Clostridium butyricum</em> specifically associated with NEC using molecular and culture-based methods</td>
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<td>Strains colonizing each infant in the unit were distinct, and none was common to infants who developed NEC; the paucity of shared gut colonizers suggested significant barriers to the spread of bacteria among infants</td>
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<td>16S rRNA, FAFLP typing</td>
<td>12</td>
<td>44</td>
<td>A clostridial OTU was overabundant in samples from infants with NEC before diagnosis; culture confirmed <em>C. perfringens</em> type A; FAFLP typing showed that no isolates were identical; samples from NEC cases before diagnosis without profuse <em>C. perfringens</em> showed an overabundance of <em>Klebsiella</em> OTU</td>
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Further research is required to understand the significance of temporal changes in the gut microbiome in the early postnatal period, specific bacterial molecular signatures, and the relative abundance of Gammaproteobacteria as well as the paucity of strict anaerobic bacteria that precede NEC in preterm infants. The possibility that changes in the gut microbiota may be a consequence rather than a cause of NEC in preterm infants also needs to be considered [79]. Warner and Tarr [80] have recently reviewed studies [22, 47–49, 54–58, 62] in this field that used 16S rRNA/metagenomic sequencing on at least 100 stools from ≥100 matched controls. Assessing the reproducibility of previous findings (Table 1) in large prospective studies is important. Finally, issues with sample processing, the choice of the PCR primer, laboratory contamination, and differences in DNA extraction methods need to be considered in interpreting results of bacteriological assessments [81–83].

**Disclosure Statement**

The author declares no potential source of conflict of interest in relation to this work.
References


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