Necrotizing enterocolitis and the microbiome: Current status and future directions

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Abstract

Decades of research have failed to define the pathophysiology of necrotizing enterocolitis (NEC), a devastating pediatric gastrointestinal disorder of preterm infants. However, recent evidence suggests that host-microbiota interactions, in which microbial dysbiosis is followed by loss of barrier integrity, inflammation, and necrosis, are central to NEC development. Thus, greater knowledge of the preterm infant microbiome could accelerate attempts to diagnose, treat, and prevent NEC. Here, we summarize clinical characteristics of and risk factors for NEC, the structure of the pre-event NEC microbiome, how this community interfaces with host immunology, and microbiome-based approaches that might prevent or lessen the severity of NEC in this very vulnerable population.

Introduction

Necrotizing enterocolitis (NEC) is one of the most catastrophic disorders in all of gastroenterology and a major contributor to morbidity and mortality in infants born preterm. NEC presents suddenly, mostly in the first two months of life; the treatment of severe cases (surgical resection of the affected gut) and the case fatality rates (15-40%) range have not changed in decades[1,2].

In recent years, massively parallel sequencing of stool from longitudinal cohort studies of preterm infants has greatly expanded our understanding of the pre-NEC microbiome. Case-control studies, in which stools are collected prospectively from dozens of neonates, have established associations of particular taxa with the subsequent development of NEC. Current data strongly suggest that NEC is driven by aberrant host-microbial community interactions rather than by any single organism within this community. This association is broadly analogous to what is observed in inflammatory bowel disease, in which dysbiotic, low-diversity microbiota interacting with host tissues, immunity, and risk alleles, results in tissue injury. Here, we review our current concept of the NEC microbiome, highlight how technology is transforming the field, and emphasize the need to refine our understanding of clinically actionable microbiome signatures that predict disease risk prior to onset.

Approach

We identified relevant literature using search terms "necrotizing enterocolitis" with and without "microbiome" or "microbiota", "microbiota development" and "preterm microbiome" in PubMed and Google Scholar. We excluded non-English publications and studies that focus on NEC in children born at term. We emphasized studies in which sequencing is used to define the pre-event gut microbiome.

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Clinical aspects of NEC

NEC is a necroinflammatory gastrointestinal disease, largely of preterm infants, with significant morbidity and mortality. NEC occurs only after birth, during an interval in which bacterial communities rapidly populate the newborn gut. Non-specific early signs of NEC include feeding intolerance, abdominal distention, and/or bloody stools[3], which often progress rapidly to intestinal perforation and systemic hypotension requiring immediate medical and surgical intervention[3,4]. Bell's scoring system is widely used to describe NEC severity and guide treatment; most studies consider Bell's stage II and III, with typical clinical and radiographic (pneumatosis, portal venous air) findings[5], to represent bona fide NEC. Treatment of NEC escalates with disease severity and ranges from abdominal decompression by suction, bowel rest, broad-spectrum intravenous antibiotics, and total parenteral nutrition in the mildest cases, to exploratory laparotomy and bowel resection in severely affected infants[3,5]. Surgical intervention is lifesaving in only ~50% of cases in which it is attempted[5], but even if resection is successful, the resulting short bowel syndrome can cause lifelong complications. NEC is also accompanied by systemic inflammation and damage to extra-intestinal organs including the brain, which hinder neurodevelopment among infants who survive the initial gut injury[6].

NEC risk factors

The pathophysiology of NEC is incompletely understood, and reliable strategies for its early detection have not emerged. Therefore, attempts to understand and predict NEC currently revolve around studying risk factors for its development. Preterm birth remains the single greatest risk factor for developing NEC[5]. There is an inverse gestational risk, with infants born after the briefest gestations having both the highest incidence and highest mortality. In very low birthweight (VLBW) infants (<1.5 kg at birth), NEC incidence ranges between five and 13%[1,2]. Prolonged antibiotic use in the first week of life and feeding of formula in lieu of maternal milk are additionally and consistently associated with subsequent development of NEC[7]. Congenital and especially cardiac defects, transfusions, indomethacin treatment of patent ductus arteriosus, and gastric acid suppression, are less consistently associated with NEC occurrence[5].

Multiomic analyses of clinical samples from human cohort studies, combined with experimental evidence from animal models, suggest that NEC has multifactorial causes. Immune immaturity, underdeveloped gut function (particularly motility and barrier integrity), and aberrant microbial colonization all likely contribute to intestinal injury, excessive inflammatory responses, and NEC development [3,5]. At the time NEC is manifest, infants have increased concentrations of circulating pro-inflammatory cytokines, including TNF-a, IL-8, IL-12, and IL-18[8]. Host pathways invoked in NEC pathogenesis include those associated with activation of Toll-like receptors (TLRs). These receptors and their associated signaling pathways play critical roles balancing inflammatory responses to bacteria and homeostasis, including tissue repair and maintenance of barrier integrity. TLR4 and its downstream pathways has received particular attention (reviewed in [9]), as bacterial lipopolysaccharide (LPS) binds TLR4 and activates downstream signaling[10]. Moreover, NEC has been associated with variants of human genes (e.g., *NFKB1* and *SIGIRR*) whose products are engaged in signaling cascades downstream of TLR4 activation[11,12]. SIGIRR inhibits LPS-mediated effects on TLR4. Excessive TLR4 activation causes epithelial cell death, reduces mucosal restructuring, and constricts mesenteric vessels, contributing to local ischemia that is also typical of NEC[10]. Intriguingly, pharmacological inhibition and genetic knockout of TLR4 in mouse models protect

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against early life intestinal injury, providing further evidence for this transmembrane protein's importance in NEC pathogenesis[10].

Gut microbes and NEC risk in preterm infants

Despite years of attempts, no single species/subspecies has emerged as the cause of NEC. However, multiple lines of evidence suggest that the gut microbiome plays a major role in NEC development. First, NEC does not occur *in utero*, an interval during which the gut harbors few, if any, viable bacteria. Second, as noted above, risk factors that increase the likelihood that NEC will develop (antibiotics, formula feeding, and possibly acid suppression) affect gut bacterial communities. Third, animal models of early life gut injury suggest that intestinal immune immaturity plays an important role in tissue injury, and that microbial modulation can alter this outcome[13].

The concept that gut community perturbations are the most substantial risk factor for NEC has been enabled by massively parallel sequencing technology, where a single nucleic acid extraction from a specimen is sequenced and microbial content profiled (**Table 1**). Community profiling first demonstrated that the earliest gut communities of healthy, term infants are dominated by *Bifidobacterium*, *Bacteroides*, *Escherichia* and *Parabacteroides*[14,15]. Following initial colonization, the gut microbiota rapidly gains diversity, undergoing individualized developmental trajectories that are structured by environmental factors including diet, co-habitation, and antibiotic exposure[14,15].

In comparison, the gut microbiota of preterm infants is compositionally distinct and less diverse than that of term-born infants[16,17]. Its constituents are mainly influenced by post-menstrual age (gestational age at birth plus day of life) of the infant, and has a characteristic progression, though with considerable inter-day variability[18]. Immediately after birth, the gut bacterial community is dominated briefly by Bacilli, which are soon outnumbered by Gammaproteobacteria, including *Klebsiella*, *Escherichia*, and other *Enterobacteriaceae*. Gammaproteobacteria predominance steadily cedes to obligate anaerobic populations (Clostridia and Negativicutes) in the absence of NEC. This transition occurs more rapidly in infants who are born after longer gestations, while anaerobic colonization is delayed to later days of life among those who are born after the shortest gestation. This is noteworthy given the timing of NEC development: the shorter the gestation, the later in life NEC develops[19]. Following hospital discharge, the preterm gut microbiota rapidly gains diversity, and by two years of life, these communities are taxonomically indistinguishable from those of term infants[17,20,21]. Nevertheless, recent data demonstrate subtle microbiota 'scars' of preterm birth that persist after taxonomic recovery, including long-term gut carriage of multidrug-resistant *Enterobacteriaceae*[16].

Gut bacterial diversity as a risk factor for NEC was first proposed 19 years ago[22], and has been confirmed in several studies since[23–25], though with some exceptions[26,27]. However, when considering diversity, it is important to note that neonatal gut microbial populations are highly non-complex: only four bacterial classes represent >90% of the preterm infant stool. Hence, there are limits to the degrees of freedom available for populations to differ. In other words, overrepresentation or underrepresentation of a single taxon obligates reciprocal changes in the proportions of a highly constrained number of other taxa. In these situations, it is difficult to ascribe a host phenotype to a variation in diversity as opposed to the expansion or contraction of a single taxon.

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It remains unclear whether pre-onset microbiome diversity is truly a risk factor for NEC, or if discrepancies in patient cohorts and procedures, such as the use of different 16S rRNA sequencing primers in different studies, have effectively confounded a genuine biological interaction. However, the most replicable finding across preterm infant cohorts is that NEC is associated with pre-event enrichment of Proteobacteria, particularly *Enterobacteriaceae*, and with corresponding underrepresentation of Firmicutes and Bacteroidetes[28,29] (**Table 2**). This same result has been repeatedly observed, though various genera within *Enterobacteriaceae* (*Klebsiella*, *Escherichia*, and *Enterobacter*) are implicated in different cohorts[25,27,30,31]. While Proteobacteria are overrepresented in NEC infants immediately prior to onset, obligate anaerobes, specifically *Veillonella*, were significantly associated with control status in one of the largest longitudinal studies of NEC microbial risk performed to date[24].

Although overrepresentation of *Enterobacteriaceae* is the most commonly reported microbiome signature of NEC, this value has limited predictive value for the many neonates whose infant gut microbiota is dominated by that family from birth. Thus, discovery of more refined microbiome signatures of NEC is a top priority. Recent efforts have begun to leverage technology with higher taxonomic and genomic resolution. For example, Olm et al. used whole-genome shotgun sequencing, not 16S rRNA sequencing, to extensively evaluate metagenomic features from a prospective cohort of NEC cases and controls, of which a small subset was collected prior to NEC onset[31]. This approach enables assessment of numerous genome-resolved features, including individual genes, bacterial strains and plasmids, viruses and eukaryotes, and even growth rates for their relative association with NEC. Using these high-resolution data, they built a machine learning classifier, which identified bacterial replication rate, *Klebsiella* abundance, and genes encoding fimbriae and several secondary metabolites as the best predictors of NEC. Despite the unprecedented integration of genome features, the resulting classifier achieved a median accuracy of 64%, only 14% better than random chance. Future classifiers may be improved by additional data, such as metatranscriptomics, and by including more pre-onset samples from NEC cases and matched controls.

How aberrant host-microbiota interactions might drive NEC

The postulated association between Proteobacteria and NEC prior to onset is particularly intriguing in light of these bacteria's interactions with the gut's innate immune system. As the dominant Gram-negative bacterial group in the preterm infant gut microbiota, Proteobacteria are chief candidates for stimulating pro-inflammatory immune responses via TLR4 signaling[28]. This lends support to the hypothesis that NEC results from microbiota dysbiosis and overstimulation of TLR4, resulting in massive inflammation, loss of barrier integrity, local ischemia, and tissue death, as described above[32]. Nevertheless, blooms of Proteobacteria are insufficient to cause this cascade, because NEC does not exclusively occur in infants in whom *Enterobacteriaceae* dominate the gut microbiota. Conversely, infants

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whose gut microbial communities are dominated by *Enterobacteriaceae* do not always develop NEC.

Recent exploration of the role of immunoglobulin A (IgA) in NEC pathophysiology offers new and compelling insights by synergizing host and microbial biology. In older children and adults, IgA is secreted in large quantities by intestinal B cells, where it binds epithelium-associated (and thus potentially invasive) bacteria in a proximity-dependent manner. For roughly the first 40 days of life (DOL), however, maternal breastmilk is the primary source of IgA. Gopalakrishna et al. applied IgSeq, in which IgA-bound and IgA-unbound bacteria are sorted by flow cytometry and then 16S sequenced, to a longitudinal NEC case-control cohort with samples from this interval[33]. Consistent with previous reports, the authors observed a relative enrichment of *Enterobacteriaceae* and reduction in obligate anaerobes prior to NEC onset. However, the association of NEC development and an increase in the relative abundance of IgA-unbound *Enterobacteriaceae* was even stronger. While the absolute abundance of *Enterobacteriaceae* did not differ statistically between cases and controls, the proportion of *Enterobacteriaceae* bound by IgA was lower in neonates who later developed NEC. Remarkably, milk from IgA-deficient (*Rag1$^{-/-}$* or *Igha$^{-/-}$*) dams did not protect from experimentally induced NEC in a mouse model of disease, in contrast to milk from wild-type dams. Although the number of specimens analyzed was modest (<100 samples) and the underlying mechanisms remain unclear, this study supports a protective role for maternal IgA that at least partly explains why formula feeding increases the risk of developing NEC.

Microbiota-directed treatment of NEC

As discussed above, an increasing body of evidence connects microbial dysbiosis to development of NEC. Consequently, microbiota-directed therapies have been proposed to prevent NEC. General concepts for microbiota-directed therapies include (I) nutritional supplementation, (II) avoidance of interventions that are likely to promote dysbiosis (e.g., antibiotics), (III) probiotics, prebiotics, and synbiotics, and (IV) fecal microbiota transplants.

Human breastmilk components, including oligosaccharides, lactoferrin, secretory IgA, and antioxidants and growth factors, have been suggested to reduce an infant's risk of developing NEC[5]. Consequently, donor breastmilk is now widely given to preterm infants when their mother's milk is unavailable[5]. Experimental evidence suggests that human breastmilk acts by controlling expansion of detrimental microbes and by attenuating TLR4 signaling[33]. Dietary supplementation with donor milk or specific human milk components, including arginine, reduces NEC incidence in animal models[34], but human milk, and especially an infant's mother's own milk, continue to offer the best opportunity to reduce the risk of developing NEC[35].

Antibiotics are also correlated with NEC development, with the most frequent association being prolonged administration during the first week of life[36–38]. When used in appropriate situations, antibiotics are among the most valued interventions available to neonatologists. However, as more is learned about unintended adverse effects of these agents, including but not limited to increased risk of NEC development, it is prudent to develop mechanisms to reduce antibiotic administration in all situations in which it offers little or no benefit.

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Probiotics, defined here as "living micro-organisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition [39]," frequently receive interest as interventions to favorably alter gut microbial communities and prevent NEC. Probiotics could "educate" the developing immune system, outcompete detrimental microbes, and support intestinal barrier function[40], attributes that theoretically would prevent NEC. Studies vary by probiotic, dose and duration, and results. Two high-quality, large, double-blind randomized controlled trials exemplify the challenges in interpreting the literature: in one Australian/New Zealand consortium, a combination of *Bifidobacterium infantis*, *Streptococcus thermophilus*, and *Bifidobacterium lactis* reduced the NEC rate in preterm infants (birth weights < 1,500 g) from 4.4% to 2%[41]. However, NEC rate in the controls in this study was low, the result was only modestly significant, and the benefit was confined to infants whose birth weights were <1,000 grams. In a multicenter study from the United Kingdom, *Bifidobacterium breve*[42] administration did not lower NEC incidence. Despite meta-analyses favorable to the use of probiotics to prevent NEC, we believe that the conclusions of many of the primary studies in which probiotics appear to prevent NEC are weakened by methodological and/or statistical concerns. We also note that the beneficial effects inferred from these meta-analyses do not apply to infants weighing <1,000 grams at birth, a group with the highest incidence of, and case fatality rate from, NEC. The challenges of producing high quality evidence to test the efficacy of probiotics have recently been reviewed[43].

Finally, animal studies suggest the potential of inoculating the preterm digestive system with complex bacterial communities via fecal microbiota transplantation (FMT)[44–46], echoing postulated benefits of probiotics on bacterial community structure and diversity, intestinal immunity, and tissue damage from pro-inflammatory TLR4 signaling. However, in view of challenges and safety concerns (selection of ideal donor microbial community, risk of pathobiont translocation from gut to bloodstream), it would be difficult to conduct a trial of FMT in the preterm population.

Conclusion

NEC remains a major unsolved challenge. Recent efforts and technological advances have dramatically improved our understanding of how the microbiome contributes to the pathophysiology of NEC, but key questions remain. The main challenge now for NEC microbiome research is translating results of large, associative case-control studies into mechanistic insight and clinically actionable targets. Many studies have identified general microbiota trends before NEC ensues, and common themes are overrepresentation of Gammaproteobacteria/Proteobacteria and, increasingly, underrepresentation of specific obligate anaerobes (**Table 2**). Recent data illustrate the potential of genome-resolved microbiome profiling for identifying species, functions and genes associated with NEC[31]. No study to date, however, has combined high-resolution microbiome characterization with concurrent host profiling, a prerequisite for identifying causal relationships in the pathophysiology of NEC. We look forward to the integration of deep multi-omic profiling of bacterial communities with robust characterization of host biology, using large longitudinal pre-onset sample collections, to develop classifiers that enable personalized risk assessment, early diagnosis, and timely intervention.

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Conflict of interest

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Table 1. Studies of gut microbiota assembly in term and preterm infants without necrotizing enterocolitis (NEC) (adapted from [29]**)**

Study, Year, sequencing technology	Participants, Specimens	Conclusions
Palmer et al., 2007 [47], 16S rRNA	14 term infants, 26	Intestinal microbiota trajectories are highly individual, environmental exposure shapes gut microbiota trajectories
Koenig et al, 2011 [48], 16S rRNA and WGS	1 term infant, 60	Discrete steps of bacterial succession are structured by diet and health
Eggesbø et al. 2011 [49], 16S rRNA	85 term infant, 24	Gammaproteobacteria and Bifidobacteria dominate the intestinal microbiome throughout the first month of life
LaRosa et al, 2014 [18], 16S rRNA	58 preterm infants, 922	Community population is a function of post-menstrual age; inter-day instability in structure
Stewart et al, 2015 [21], 16S rRNA	29 preterm infants, 57	The preterm infant gut microbiome develops a complexity comparable to term infants following NICU discharge
Bäckhed et al., 2015 [15], WGS	98 term infants, 294	Species shifts represent nonrandom transitions in infants' guts; Cessation of breastmilk rapidly matures intestinal microbiome
Gibson et al., 2016 [16], WGS	84 preterm infants, 401	Antibiotics most commonly administered in the NICU, have non-uniform effects on the microbiota; distinct antibiotic treatments enrich for specific antimicrobial resistance genes
Yassour et al, 2016 [50], 16S rRNA and WGS	39 term infants, 1069	Antibiotic exposure reduces both species and strain-level diversity in the developing gut microbiome, and transitionally increases the antibiotic resistance gene burden
Bokulich et al, 2016	43 term infants, 578	Antibiotic exposures, cesarean section, and formula feeding

[51], 16S rRNA		delay microbiome development and alter microbiota diversity
Stewart et al., 2018 [14], 16S rRNA and WGS	903 term infants, 12,005	Gut microbiota progress through developmental, transitional and stable phases over the first four years of life, shaped by birth mode, diet and environmental exposure
Baumann-Dudenhoeffer et al, 2018 [52], WGS	60 near term infants, 402	Distinct early-life microbiome signatures correlated with breastfeeding, formula ingredients, and maternal gestational weight gain. Commensal microbiota gene content adjusts to counterbalance components relatively lacking in human milk
Gasparrini et al., 2019 [17], WGS	41 preterm and 17 near term infants, 437	Early life antibiotic exposure is associated with an enriched intestinal resistome, prolonged carriage of multidrug resistant *Enterobacteriaceae* and distinct patterns of intestinal microbiome assembly
Abbreviation: WGS – whole genome sequencing.		

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Table 2. High-throughput sequencing studies characterizing the gut microbiota of infants that develop necrotizing enterocolitis (NEC) (adapted from [29]**)**

Study, Year, Sequencing technology	Participants (NEC and total)	Specimens	Conclusions
Morrow et al., 2013 [26], 16S rRNA	11 of 32	58	Low community diversity, abundance of Firmicutes or Proteobacteria, associated with NEC risk
Torrazza et al., 2013 [27], 16S rRNA	18 of 53	119	Abundance of Proteobacteria or Actinobacteria associated with NEC risk
Brower-Sinning et al., 2014 [23], 16S rRNA	18 of 19	26	High abundance of anaerobes and low community diversity in intestinal tissues associated with NEC risk
Warner et al., 2016 [24], 16S rRNA	46 of 120	2720	Gammaproteobacteria associated with NEC risk and Negativicutes associated with NEC protection; lack of diversity is associated with NEC risk
Ward et al., 2016 [30], WGS	16 of 165	262	Uropathogenic *E. coli* strain types associated with NEC risk
Dobbler et al., 2017 [25], 16S rRNA and WGS	11 of 40	132	Enterobacteriaceae and low community diversity associated with NEC risk
Olm et al., 2019 [31], WGS	34 of 160	1163	*Klebsiella* abundance and genes encoding fimbrae and secondary metabolites associated with NEC risk

Gopalakrishna et al., 2019 [33], WGS	10 of 23	98	High abundance of *Enterobacteriaceae* (particularly IgA-unbound Enterobacteriaceae) and reduced anaerobes associated with NEC risk