Pediatric RESEARCH

www.nature.com/pr

REVIEW ARTICLE

Intestinal dysbiosis and necrotizing enterocolitis: assessment for causality using Bradford Hill criteria

Jennifer B. Fundora[1], Pallabi Guha[1], Darla R. Shores[1], Mohan Pammi[2] and Akhil Maheshwari[1]

In recent years, several studies have shown that premature infants who develop NEC frequently display enteric dysbiosis with increased Gram-negative bacteria for several days to weeks prior to NEC onset. The importance of these findings, for the possibility of a causal role of these bacteria in NEC pathogenesis, and for potential value of gut dysbiosis as a biomarker of NEC, is well-recognized. In this review, we present current evidence supporting the association between NEC in premature infants and enteric dysbiosis, and its evaluation using the Bradford Hill criteria for causality. To provide an objective appraisal, we developed a novel scoring system for causal inference. Despite important methodological and statistical limitations, there is support for the association from several large studies and a meta-analysis. The association draws strength from strong biological plausibility of a role of Gram-negative bacteria in NEC and from evidence for temporality, that dysbiosis may antedate NEC onset. The weakness of the association is in the low level of consistency across studies, and the lack of specificity of effect. There is a need for an improved definition of dysbiosis, either based on a critical threshold of relative abundances or at higher levels of taxonomic resolution.

Pediatric Research (2020) 87:235–248; https://doi.org/10.1038/s41390-019-0482-9

INTRODUCTION

Premature infants are at risk of developing enteric dysbiosis with a preponderance of Gram-negative bacteria of families *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae* in the class Gammaproteobacteria and the phylum Proteobacteria.[1–4] In recent years, several case–control studies have shown that premature infants who develop NEC beyond 3 weeks of postnatal age frequently show such dysbiosis for several days to weeks leading up to NEC onset.[1–3,5–9] These findings have evoked considerable excitement about the role of these bacteria in NEC pathogenesis, and also for the potential value of enteric dysbiosis as a biomarker for risk-stratification of preterm infants for NEC.

In the following sections, we evaluate the evidence supporting the association between NEC in premature infants and enteric dysbiosis. We collated information from an extensive literature search in the databases PubMed, EMBASE, and Scopus, and applied the Bradford Hill criteria for causality (Table 1).[10] To minimize bias, keywords from PubMed's Medical Subject Heading thesaurus were shortlisted prior to the actual search and combined with text words likely to be used in titles and abstracts. Table 2 provides a glossary of terms frequently used in microbiome studies.

STRENGTH OF THE ASSOCIATION

In the Bradford Hill framework for assessment of causality, strong associations are less likely to be explained by bias or confounding. However, strength is not a requirement because weak associations can also be causal.[11] The role of bacteria in NEC pathogenesis has now been recognized since the 1960s.[12–15] Bacterial overgrowth is a prominent histopathological finding in NEC lesions, and, considering that pneumatosis, the pathognomonic sign of NEC, signifies gaseous products of bacterial fermentation entrapped within the bowel wall, these bacteria are believed to be metabolically active.[16,17] The occurrence of NEC almost always after the 1st postnatal week and never in utero, the difficulty in inducing NEC-like lesions in germ-free animals, the correlation between bacterial invasion within the bowel wall and mortality in surgical NEC, and the protective effect of enteral antibiotics against NEC and NEC-related mortality, underscore the role of bacteria in NEC pathogenesis.[18–20]

NEC cases are known to cluster in time and space, and these mini-outbreaks have long fueled a quest for transmissible infectious triggers of NEC.[21] Cultures of blood and other body fluids from infants with NEC have not consistently implicated a single agent and seem to yield a wide array of microorganisms that are present in the NICU microenvironment and colonize critically-ill preterm infants.[21,22] Nevertheless, Gram-negative bacteria have remained key suspects in NEC pathogenesis, perhaps because the clinical presentation of NEC resembles Gram-negative sepsis, and although positive blood cultures are uncommon during acute NEC, Gram-negative bacteria such as *Klebsiella, Escherichia coli, Enterobacter*, and *Pseudomonas* are frequently identified in the peritoneal fluid from infants with advanced NEC.[23] These agents have also been associated with NEC outbreaks.[24–27]

In the last decade, several studies of the gut microbiome in preterm infants have associated Gammaproteobacteria and its constituent families *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonadaceae*, with increased risk of NEC.[1–3,5–9] Wang et al. analyzed fecal samples from a small sample of preterm infants (10 infants with a diagnosis of NEC and 10 gestational age-matched controls), and showed that the stool microbiome from NEC patients clustered separately from controls and showed low

[1]Department of Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD, USA and [2]Department of Pediatrics, Baylor College of Medicine, Houston, TX, USA
Correspondence: Akhil Maheshwari (akhil@jhmi.edu)

Received: 22 April 2019 Revised: 11 June 2019 Accepted: 18 June 2019
Published online: 25 June 2019

Table 1. Bradford Hill Criteria for Causality

Criteria	Explanation
Strength	Strong associations are less likely to be explained by bias or confounding. Not a requirement because weak associations can be causal.
Consistency	Observed repeatedly by different investigators, in different populations, and with different study designs. Increases confidence for causality, but is not a requirement.
Specificity	Exposure is necessary and sufficient for a specific outcome. Derived from the Koch's postulates, but may not be valid in multifactorial disorders.
Temporality	Exposure precedes the outcome in time; the only required criterion. Prospective studies provide stronger evidence of temporality than retrospective or cross-sectional studies.
Biological gradient	A dose-response relationship between the cause and the outcome. Biological gradients are not a requirement, because some causal relationships have threshold doses or exhibit non-linear relationships to the risk of the outcome.
Plausibility	Known biological explanation for how the exposure might result in or contribute to the outcome
Coherence	Known biological evidence not in conflict with other observations of the outcome.
Experiment	Interventions have predictable effects on the occurrence of the outcome.
Analogous relationships	Existing information on similar cause-effect relationships.
Reversibility	Removal of the exposure reduces/eliminates the outcome.

Table 2. Glossary of terms frequently used in microbiome studies

Term	Explanation
Denaturing gradient gel electrophoresis (DGGE)	A technique used for separating DNA fragments by electrophoresis under increasingly denaturing conditions such as increasing formamide/urea concentrations. The protocol can be optimized for fingerprinting of 200–300 bp fragments of bacterial 16S rRNA genes, and was often used for microbiome studies prior to the advent of sequencing technology.
V1-9 hypervariable regions	16S ribosomal RNA gene encodes for the 30S small subunit of a prokaryotic ribosome and is used in reconstructing phylogenies because of its relatively slow rate of evolution. Bacterial 16S gene contains 9 hypervariable regions (V1–V9), each ranging between 30 and 100 base pairs in length and are involved in the secondary structure of the small ribosomal subunit. Taxonomic studies often utilize sequencing of PCR-amplicons in the V1–3 or the V3–5 regions.
Operational taxonomic unit (OTU)	Operational taxonomic unit; a group of organisms.
Species richness	Conveys the number of species in a sample.
Species evenness	Conveys how equally abundant the species are in a sample.
Alpha diversity	Diversity in one sample; typically summarized by one or more of the following indices: (1) OTU count: richness count for number of OTUs; (2) Shannon-Wiener entropy index: considers richness and evenness with more weight on richness; (3) Simpson concentration index: emphasizes evenness more than the Shannon index; (4) Chao1 index: incorporates abundance data including rare OTUs.
Beta diversity	Diversity between samples; typically summarized by one or more of the following indices: (1) Bray–Curtis dissimilarity index: based on abundance data; range 0 (both samples share same species at same abundances) to 1 (different); (2) Jaccard distance: based on presence/absence of species, not abundance; range 0 (samples share same species) to 1 (no common species); (3) Sørensen–Dice coefficient: similar to Jaccard, but Sørensen distance retains sensitivity in heterogeneous data and gives less weight to outliers; (4) UniFrac, based on sequence distances (phylogenetic tree) and estimates branch length shared between two samples. Unweighted UniFrac is based on sequence distances, not abundance, compared to weighted UniFrac, where branch lengths are weighted by relative abundances (includes both sequence and abundance information)

bacterial diversity with a marked increase in the relative abundance of *Gammaproteobacteria*.[2] Torrazza et al. also noted a distinct pattern of microbial colonization in infants who developed NEC.[28] They observed a higher proportion of Proteobacteria (61%) 2 weeks prior to NEC onset. They also showed that the detection of a novel signature sequence reminiscent of *Klebsiella pneumoniae* during the first postnatal week was associated with later development of NEC.[28] In another study, Morrow et al. noted that the stool microbiome in infants who developed NEC between postnatal days 19–39 days showed more Proteobacteria, specifically *Enterobacteriaceae*.[29] More recently, Warner et al. showed that 28 infants who developed NEC showed increased proportions of Gammaproteobacteria and less Negativicutes, compared to 94 controls.[8] Their statistical models showed fecal Gammaproteobacteria to be a significant predictor of NEC.

CONSISTENCY

There is greater confidence for causality if an exposure is consistently linked with the outcome by different investigators, in different populations, and with different study designs. However, consistency is not a requirement as individual studies can have limitations of methodology, power, and bias.[30] As noted above, the association between Gammaproteobacteria and NEC has been noted in several important studies. However, many

others have not found a consistent link: Millar, de la Cochetiere, Mshvildadze, Mai, Smith, Stewart, Normann, McMurtry, Sim, Stewart, Heida, Barron, Ravi, Romano-Keeler, Brown, Wandro, Itani and others have identified Gram-positive bacteria as the dominant taxa in at least some of their patients with NEC, or found no clear patterns at all.[3,5,6,31–44] A summary of all studies comparing the enteric microbiome of infants with a diagnosis of NEC vs. those who did not develop NEC is provided in Table 3. Clearly, most of these studies included only a few infants in each group, and therefore, Pammi et al. sought to evaluate the evidence by combining 14 eligible reports in meta-analysis.[1] The authors drew attention to the potential fallacies of combining a few small, heterogeneous studies, but cautiously concluded that infants with NEC show a modest, but significantly increased proportion of Proteobacteria, particularly Gammaproteobacteria, and lower proportions of Firmicutes and Bacteroidetes compared to control infants. This increase in Gammaproteobacteria abundance was most evident in infants born at <27 weeks' gestation, and the pattern change was not evident until after the 3rd postnatal week and ~30 weeks' corrected gestational age. They also found lower alpha-diversity (fewer taxa, lower Shannon Diversity Index) in these infants. Methodologically, there were concerns that studies targeting the hypervariable regions V3–V5 in the 16S rRNA bacterial gene reported higher relative abundances of Proteobacteria and decreased abundances of Firmicutes compared to those targeting the V1–V3 regions. Overall, the meta-analysis confirmed the statistical significance of the association between Gammaproteobacteria and NEC, but the pooled data did not show distinct clustering of NEC and control samples by unweighted or weighted UniFrac metrics. These findings suggested that differences in the gut microbiome in infants who developed NEC vs. controls might be relatively modest. Alternatively, these finding may also have resulted from the considerable methodological, clinical, and study heterogeneity of the included studies, indicating a need for further investigation of this association.

SPECIFICITY

The presence of Gammaproteobacteria in the intestinal microbiome is neither specific, because these pathobionts have also been associated with other neonatal diseases; nor necessary, because all cases of NEC do not display enteric dysbiosis with increased Gammaproteobacteria; nor sufficient, because not all infants with dysbiosis develop NEC. Increasing information indicates that intestinal colonization with Gammaproteobacteria may be a normal maturational event during gut microbiome assembly in preterm infants.[45–54] La Rosa et al. evaluated the fecal microbiome of 58 premature infants and showed that the gut microbiota progressed through a choreographed succession of bacterial classes from Bacilli to Gammaproteobacteria to *Clostridia*.[45] They showed that the Gammaproteobacteria abundance peaked between 28 and 34 weeks' post-menstrual age. To investigate the drivers for a possible Gammaproteobacterial bloom in some infants, they also evaluated some of the better-known selection pressures. Antibiotic use was associated with increased proportions of Gammaproteobacteria, but only for infants with ≥26 weeks' gestation. Human milk feedings were associated with increasing proportions of Gammaproteobacteria in the most premature infants. However, these exogenous drivers of gut microbial content did not fundamentally alter the trends in population evolution, only its pace.

In another important study, Gregory et al.[47] showed that infant gut microbiome is influenced by postnatal age, birth weight, gestational age, and nutrition.[47] They also found a relatively ordered succession in bacterial taxa with initial colonization dominated by Bacilli, followed by Gammaproteobacteria, and finally *Clostridia* and *Bifidobacteria*. They found an important effect of diet, where infants fed mother's own milk had greater initial diversity in their microbiome that was most strongly influenced by the presence of a variety of phylotypes that include lower levels of *Bacillales* and *Lactobacillales*, in favor of *Clostridia*, and *Enterobacteriales* as early as 26 weeks of adjusted gestational age.

We have recently investigated the clinical antecedents of increased fecal abundance of Gammaproteobacteria in premature infants.[55] In this study, we enrolled 45 premature infants born with a birth weight ≤1500 g and analyzed their fecal microbiome first at an early time-point within the first 2 weeks and then serially during the 3rd and 4th postnatal weeks. Our goal was to identify the clinical characteristics of preterm infants who developed enteral dysbiosis, which in turn, could inform future efforts to direct microbiome screening in a clinical setting. We hypothesized that most premature infants begin with few Gammaproteobacteria in their stool and acquire these bacteria from the hospital microenvironment or from human interaction[50,56–58] as a function of postnatal age. Consistent with this hypothesis, we found that the overall proportion of fecal Gammaproteobacteria increased with postnatal age. Interestingly, about half of the infants in our cohort started with a low relative abundance of Gammaproteobacteria (<10%) in early stool samples and gained these bacteria over time. However, a second subgroup within our cohort started with very high relative abundances of Gammaproteobacteria (>90%). This dichotomy in gut microbiome assembly was novel, and in linear mixed models, the high Gammaproteobacteria abundance in our 2nd cluster was associated with vaginal birth, indicating possible vertical, mother-to-infant transmission. During the 3rd and the 4th weeks, these two subgroups began to resemble each other and showed comparable alpha-diversity and Gammaproteobacteria abundance. Overall, a large proportion of infants in our cohort showed a Gammaproteobacteria abundance >50% – 45.5% at ≤2 weeks, 64.3% in the 3rd week, and 79.5% in the 4th week. Our cohort was typical for a regional referral NICU in the United States, without an unusually high exposure to factors typically identified with dysbiosis in premature infants.[48,59–62] For instance, 42 of our 45 infants (93.3%) were receiving either mother's own or donor human milk even in the 4th postnatal week, and did not receive substantial amounts of infant formula. None received acid-blocking drugs. A majority were exposed to antibiotics during evaluation for early-onset sepsis, but the duration was not exceptionally prolonged in most (2.8 ± 2.3 days). Our findings suggest that enteric colonization with Gammaproteobacteria may not be an unusual event in VLBW infants, and considering that NEC occurs only in a minority of these infants, suggests that the association between Gammproteobacteria are neither necessary nor sufficient for NEC pathogenesis. The possibility remains that Gammaproteobacteria may constitute a contributory factor in a larger, multifactorial schematic. These findings also call for cautious interpretation of data from small cohorts for a relatively rare outcome, NEC. Gammaproteobacteria colonization and the incidence of NEC seem to peak during the same post-menstrual epoch (31 ± 3 weeks). There is a need for careful estimation of sample size to study this disorder, and the need to interrogate an abundance of specimens prior to the event to refute the possibility that this association is not merely an alpha error or an artifact of confounding.

TEMPORALITY

The detection of increased fecal Gammaproteobacteria prior to NEC is exciting for its potential value as a biomarker for risk-stratification and its potential clinical/therapeutic implications.[11] As outlined in Table 2, a number of studies indicate that premature infants who develop NEC beyond 3 weeks of postnatal age may display such dysbiosis for several days to weeks leading up to NEC onset.[1–3,5–9] Pammi et al. also showed in their meta-analysis that infants who developed NEC showed a consistent rise in Proteobacteria abundance with decreased Firmicutes and

Table 3. Studies of gut microbiome in preterm infants with a diagnosis of NEC vs. controls

Study	Setting and time period	Participants/study design	Methods	Alpha diversity metrics	Beta diversity metrics	Microbial profiles
Millar et al.[3]	Place: 3 hospitals in the United Kingdom. Study period: Sept 1991–Jan 1992	10 cases (24–34 weeks' gestation); stool samples available from 9 infants at −9 to +7 days after NEC onset. One infant had intestinal tissue at post-mortem, 14 days after onset. 22 controls. Stool samples every week for a mean of 5.3 weeks	1. Conventional cultures 2. PCR-DGGE (denaturing gradient gel electrophoresis)	Not clearly reported	Uncultured organism types by PCR-DGGE similar in cases and controls.	7 infants had DGGE sequences similar to *Streptococcus salivarius*. All these infants have been supplemented with *Lactobacillus rhamnosus GG*. The 193 bp fragment from V3 region of the 16S rDNA was insufficient to construct phylogenetic trees. Band related *Klebsiella oxytoca* on day 14 in one control infant. 500 bp amplification allowed better identification of species.
De la Cochetiere et al.[34]	Place: Nantes, France, single center Study period: not clear.	3 cases (mean ± SD 28.5 ± 2.1 weeks' gestation; birth weight 880 ± 170 g). 9 matched controls. Stool samples every week.	1. Conventional cultures 2. PCR-DGGE (denaturing gradient gel electrophoresis)	Not clearly reported	A band corresponding to *Clostridium perfringens* in 3 NEC cases before NEC onset but not in controls.	*Clostridium perfringens* in 3 NEC cases seen in early stool samples before NEC onset.
Wang et al.[2]	Place: Chicago, IL, USA, single center Study period: not clear.	10 cases (25–32 weeks' gestation). Stool obtained on postnatal days 4–49 days; 1 sample collected −3 days before NEC, others after NEC. 10 controls matched for gestational age and age at onset of NEC. Stool samples every week.	1. Terminal restriction fragment length polymorphism (T-RFLP) length polymorphism 2. Sequencing of random clones and compared to online libraries to find the nearest matched species.	Compared to controls, NEC cases had lower absolute richness (12.8 ± 7.3 vs 25.2 ± 9.8, $p < 0.05$) and Shannon's diversity (1.13 vs. 1.88, $p = 0.035$)	Stool samples from patients with NEC clustered separately from controls	Stool from NEC patients showed increased abundance of Gammaproteobacteria and a decrease in other bacterial genera. Controls showed 4 phyla: *Proteobacteria, Firmicutes, Bacteroidetes* and *Fusobacteria, whereas* NEC infants had only 2: *Proteobacteria* and *Firmicutes*.
Mshvildadze et al.[5]	Place: Gainesville, Florida, USA, single center. Study period: not clear.	4 cases of NEC and 2 infants with systemic signs of inflammation. GA 23–32 weeks. Stool obtained at birth and weekly thereafter. 6 controls matched for birth weight, gestational age, and postnatal day of stool collection.	1. 16 S rDNA amplification and 454 pyrosequencing. 2. DGGE profiling of stool samples	No comparison of diversity between cases and controls	Cases and controls did not cluster separately on weighted UniFrac analysis and principal coordinate analysis	At the genus level, higher abundance of *Enterococcus* seen in cases. Citrobacter-like sequences seen in 3 out of 4 NEC cases. *Klebsiella* and OTUs with closest match to *Enterobacteriaceae* associated with controls.
Mai et al.[6]	Place: 3 hospitals affiliated to the University of Florida, USA. Study period: not clear.	9 cases (23–30 weeks' gestation, birth weight 570–1269 g). Fecal samples obtained at birth and then weekly. Samples 1 week before NEC diagnosis and within 72 h of NEC were analyzed. 9 controls matched for gestation, birth weight, birth center, date of birth, and predominant enteral nutrient (breast milk vs. formula). Stool microbiome	1. 16 S rRNA amplification and 454 pyrosequencing. 2. DGGE analyses of the V6–V8 region was used for initial quality control.	Cases and controls had similar Chao-1 diversity profiles	NEC and control samples clustered separately 1 week before NEC onset on UniFrac unweighted analysis.	Cases and controls showed different microbiota profiles at −7 days but not at −3 days before NEC onset. 34% increase in *Proteobacteria* and 32% decrease in *Firmicutes* in NEC cases between −7 and −3 days. NEC cases frequently showed unique bacterial OTUs belonging to *Enterobacteriaceae*.

Table 3 continued

Study	Setting and time period	Participants/study design	Methods	Alpha diversity metrics	Beta diversity metrics	Microbial profiles
		of cases and controls compared at equivalent time-points				
Smith et al.[32]	Place: single center, in Copenhagen, Denmark. Study period: September 2006–January 2009.	21 cases (gestation mean 26.2 weeks (range 23.7–28.7). Stool obtained at 0–5 days, day 10, and day 30. 142 controls matched for gestation and postnatal day of stool collection.	1.Conventional cultures 2.PCR-DGGE (denaturing gradient gel electrophoresis)	Not reported	Cases and controls showed similar PCR-DGGE profiles.	Stool cultures from NEC cases dominated by Gram-positive bacteria, whereas the controls showed a mixed flora of Gram-positive and Gram-negative bacteria.
Stewart et al.[36]	Place: single center, in Newcastle, United Kingdom. Study period: not clear	38 preterm infants, median 27 weeks. GA (range 23–31 6/7 wk) and Bwt 895 g (range 520–1850 g) contributed to cultures. Only 27 infants contributed to PCR- DGGE. 1. NEC developed in 8 infants and all but one contributed to PCR-DGGE	1. Conventional cultures 2.PCR-DGGE (denaturing gradient gel electrophoresis) using eubacterial PCR primers targeting the V3 region of the 16 S rDNA gene	Not reported	DGGE profiling showed significant differences in NEC infants compared to controls.	Cultures showed that infants who developed NEC were more likely to be colonized CONS (45 vs. 30%) and less *Enterococcus faecalis* (31 vs. 57%) compared to controls.
Normann[37]	Place: single center, in Uppsala, Sweden. Study period: June 2009–June 2010.	10 cases (gestation mean 23.5, range 22–25.5 weeks; birth weight mean 582 g (487–965) g. 10 controls matched by sex, gestation, and mode of delivery. Stool obtained weekly for 7 weeks or until NEC onset.		The Shannon diversity index did not reveal any differences between cases and controls.	No significant differences in microbial communities were detected between cases and controls.	A high relative abundance of *Bacillales* and *Enterobacteriaceae* was detected in early time points in NEC (not statistically significant). Healthy infants had microbiota dominated by *Enterococcus*.
Torraza et al.[28]	Place: 3 hospitals affiliated to the University of Florida, USA. Study period: not clear.	18 cases (gestation 23–30 weeks, birth weight 570–1269 g). Stool obtained at birth and then weekly. Samples from −2 weeks, −1 week before NEC onset, and closest to NEC diagnosis were analyzed. 35 controls matched for post-menstrual age, birth weight, birth center, and date of birth. Stool microbiome of cases and controls analyzed at equivalent time-points.	1. 16 S rRNA amplification and 454 pyrosequencing. 2. DGGE analyses of the V6–V8 region was used for initial quality control.	Cases and controls showed similar Chao-1 diversity at −2 weeks, −1 week, and during the week of NEC onset.	Cases and controls clustered separately on UniFrac analyses for beta diversity at −2 weeks before NEC onset but not later.	Cases showed higher proportion of *Proteobacteria* −2 week before and *Actinobacteria* at −1 week before NEC onset, and lower *Bifidobacteria* and *Bacteriodetes* than controls. In the first stool samples, a novel sequence closest to *K. pneumoniae* strongly associated with NEC.
Morrow et al.[29]	Place: 2 level III neonatal intensive care units in Cincinnati, OH, USA. Study period: October 2009–August 2010.	11 cases (gestation mean ±SD 25.5±(1.8) weeks. and Bwt 791 g (212) in mean (SD). Controls: 21 controls, GA 25.9 (1.9) wks. and Bwt 839 (187) in mean (SD). The	1. 16 S rRNA amplification and 454 pyrosequencing targeting the V3–V5 region. 2. Urine metabolome was assessed by NMR (nuclear	In the samples obtained between days 4–9, Chao-1 diversity index and Simpson diversity index showed a lower trend (not statistically	UniFrac analyses revealed 2 NEC cluster distinct from controls. During days 10–16 one NEC cluster dispersed but the other cluster	Two types of intestinal dysbiosis were found associated with NEC. In the first type, in 4 NEC cases (onset 7–21 days), in days 4–9, stool microbiota was dominated by *Firmicutes*. In the remaining 7 NEC

Table 3 continued

Study	Setting and time period	Participants/study design	Methods	Alpha diversity metrics	Beta diversity metrics	Microbial profiles
		infant stool microbiome was analyzed in 2 time periods, days 4–9 and 10 to 16 and two sample collections in each period.	magnetic resonance) analysis.	significant) compared to controls. The lower trend continued after day 9 in cases.	was still together. A high urine alanine/histidine ratio was associated with intestinal microbial dysbiosis and predicted overall NEC (predictive value 78%).	cases (onset 19–39 days), in days 10–16, stool microbiota was dominated by *Proteobacteria*, specifically *Enterobacteriaceae*. All NEC cases lacked *Propionibacterium*.
Zhou et al.[7]	Place: single center NICU at Brigham and Womens Hospital, Boston, Massechusetts, USA. Study period: not clear	12 cases (gestation mean 27.8, range 24–31 weeks; birth weight 1048 g, range 940–1860 g. 26 controls matched for gestation and chronological age.	1. 16 S rRNA amplification and 454 pyrosequencing targeting the V3–V5 region.	NEC cases had lower Shannon diversity index than controls.	Early onset NEC (≤22 days onset) segregated from controls at genus levels during the 2nd week. No segregation noted in the late-onset NEC (>22 days).	In early onset NEC, close to the disease onset, abundances of *Clostridium sensu stricto* higher than controls. In late-onset NEC, the relative abundance of *Escherichia/Shigella*showed a temporal increase starting at −6 days prior to NEC onset. Cases of late-onset NEC showed higher *Cronobacter*abundance than controls at 1–3 days prior to NEC onset.
McMurtry et al.[31]	Place: A part of a multi-center study and IRB approved at Louisiana State University Health Sciences center, Touro Infirmary, East Jefferson General Hospital and Children's Hospital of New Orleans, USA. Study period: 2007–2011	21 cases (gestation mean 27.8, range 24–31 weeks; birth weight 1048 g, range 940–1860 g. 74 controls matched for chronological age, gestation, and birth weight. From NEC cases, stools from −1 to −5 days prior to NEC onset were included.	16S rRNA amplification and 454 pyrosequencing targeting the V3–V5 region.	Cases showed lower Chao-1 richness and Shannon's diversity than controls. These indices were lower in cases with lethal NEC than those with mild disease.	UniFrac analyses for beta diversity showed no distinct clustering of cases and controls.	Cases had lower relative abundance of *Actinobacteria* and *Clostridia* than controls. No differences in the *Bacilli* or Gammaproteobacteria. As the severity of NEC increased, the prevalence and relative abundance of *Clostridia* decreased along with Chao1 and Shannon's diversity indices.
Sim et al.[33]	Place: Imperial College healthcare National Health Service Trust NICUs (St. Mary's Hospital and Queen Charlotte's and Chelsea Hospitals), London, United Kingdom. Study period: Jan 2010–Dec 2012	12 cases (gestation mean 27 (interquartile range 25.5–28.3 weeks; birth weight 845 g (685–899 g). 36 controls matched for gestation, birth weight, mode of delivery, admission hospital, and antibiotic use. 8 stool samples collected −2 weeks prior to NEC onset were included.	16 S rRNA amplification and 454 pyrosequencing targeting the V3–V5 region.	Not reported	Not reported	Cases showed increased abundance of *Clostridia or Klebsiella* in the days prior to NEC onset.
Warner et al.[8]	Place: St Louis Children's Hospital, USA, between 7 July 2009, and 16 Sept 2013 Secondary cohorts: Kosair Children's Hospital and Children's Hospital at Oklahoma University between 12 Sept 2011 and 25 May 2013	46 cases with birth weight <1500 g. 120 controls matched for gestation, birth weight, and time period.	16S rRNA amplification of the V3–V5 region using the Riche 454 platform	Cases showed lower Shannon diversity index than controls. Difference related to a maturational increase in microbial diversity in controls, but not in cases.	Not reported	Cases showed higher relative abundance of Gammaproteobacteria and relative paucity of strict anaerobic bacteria (especially *Negativicutes*) prior to NEC onset

Table 3 continued

Study	Setting and time period	Participants/study design	Methods	Alpha diversity metrics	Beta diversity metrics	Microbial profiles
Romano-Keeler et al.[35]	Place: Monroe Carell Jr. Children's Hospital at Vanderbilt.	12 cases with surgical NEC (gestation mean 29 weeks, range 25–33 weeks; birth weight 1274 g, range 440–2101 g; postnatal age 17 days (range 5–46 days). 14 controls were surgical patients without NEC with comparable gestation, birth weight, and postnatal age. Intestinal tissue and corresponding fecal samples were collected; eligible if intestinal resection performed <180 days of age.	Amplification and sequencing of the V1–V3 hypervariable region of the bacterial 16S rRNA gene extracted from intestinal tissue and corresponding fecal samples.	NEC tissue showed lower microbial richness or diversity than controls, and a trend towards lower alpha-diversity. Stool samples from NEC cases also showed lower microbial richness (observed OTU counts, Chao1) and alpha diversity than controls.	Cases and controls clustered separately on principal coordinates analysis (Adonis PerMANOVA $p = 0.003$).	Fecal and tissue microbial communities were different. NEC microbiome showed lower diversity, with higher abundances of Staphylococcus and Clostridium_sensu_stricto. No differences in fecal abundance of Clostridium_sensu_stricto, but Staphylococcus was more abundant during NEC. Compared to controls, NEC tissue samples were more likely to be dominated by a single genus such as Staphylococcus, Clostridium, Escherichia, or Bacteroides.
Wandro et al.[38]	Place: Children's Hospital of Orange County, CA. Study period: 2011–2014	21 healthy controls, 8 late-onset sepsis, 3 NEC. Birth weight 620–1570 g. Fecal samples were collected between postnatal days 7 and 75.	1. 16S rRNA gene sequencing; 2. Metabolomics by gas chromatography-mass spectrometry in fecal samples	Cases and controls showed similar Shannon diversity.	Not reported	Bacterial abundances lower in patients who developed NEC compared to controls but specific differences in taxa not reported.
Itani[39]	Place: 3 Lebanese NICUs: the Hotel-Dieu de France Hospital, the Bellevue Hospital and the Saint Charles Hospital. Study period: January 2013 and March 2015	11 cases (gestation 27–35 weeks). 11 controls matched for gestational age, postnatal age, birth weight, birth center, date of birth, and predominant enteral feeding (breast milk or formula).	Fecal samples collected before NEC diagnosis, at NEC diagnosis, and after NEC diagnosis. Microbiota analyzed by culture, quantitative PCR (qPCR) and temperature temporal gel electrophoresis (TTGE).	NEC cases showed mean 5.9 (range 1–10) major bands on TTGE vs. mean 6.7 (2–11) in controls. No major bands common to all NEC cases. Bands corresponding to *E. coli* and Staphylococcus epidermidis were seen in 1 case each.	No clustering between cases or controls; high inter-individual variability.	Quantitative PCR showed cases to have a higher bacterial load of *Staphylococcus*, and lower *Enterococcus* ($p = 0.039$) and Lactobacillus ($p = 0.048$) than controls. All infants colonized by *Enterobacteriaceae* at high levels.
Heida[44]	Place: tertiary NICU in The Netherlands. Study period: October 2012 to February 2014	11 cases (gestation ≤30 weeks and birth weight ≤1000 g. 22 controls included infants born at GA ≤ 32 weeks and small for gestational age with birth weight ≤ 1200 g, neonates born with cardiovascular defects with plausible reduction in splanchnic perfusion, and neonates antenatally exposed to indomethacin tocolysis. Patients with congenital intestinal disorders excluded.	Analyzed meconium, stool collected twice a week, and last 2 stool samples prior to NEC onset. 16S rRNA genes (V3–V4 region) analyzed on a MiSeq sequencer.	No difference in alpha diversity between NEC and controls	Differences between cases and controls described, but statistical significance was unclear.	NEC cases showed significantly higher abundance of *Clostridium perfringens* (8.4%) and *Bacteroides dorei* (0.9%) in meconium than controls (0.1% and 0.2%; $p < 0.001$). In post-meconium samples, the abundance of *Staphylococcus* was negatively associated with NEC; *Clostridium perfringens* continued to be more prevalent in NEC cases.

Table 3 continued

Study	Setting and time period	Participants/study design	Methods	Alpha diversity metrics	Beta diversity metrics	Microbial profiles
Barron[40]	Place: St. Louis Children's Hospital NICU. Study period: July 2009 to September 2013	30 cases (birth weight <1500 g), grouped for medical NEC, surgical NEC, and NEC *totalis*.	16S rRNA pyrosequencing	No difference in alpha diversity between NEC and controls	Not reported	No difference in gut microbiome between infants with medical NEC, surgical NEC, and NEC totalis in a 4-week period prior to NEC onset. Gammaproteobacteria remained the predominant class at all time points.
Brown[41]	Place: Magee Women's hospital, Pittsburgh. Study period- not clear	14 cases and 21 controls. Stool collected in 3 months after birth. 87 stool samples.	Metagenomic sequencing and metaproteomics	NEC cases with lower Shannon diversity than controls	Not reported separately for cases and controls	Microbiota correlated with infant, antibiotic administration, and NEC diagnosis. Bacterial communities clustered into 7 primary types, which varied within and between subjects over time. No species or community consistently associated with NEC. Microbial proteomes correlated with community composition.
Stewart et al.[43]	Place: NICU of the Royal Victoria Infirmary, Newcastle upon Tyne, United Kingdom. Study period: not clear	7 infants with NEC and 28 controls matched for gestation, birth weight, and delivery mode.	16S rRNA gene sequencing. Metabolomic profiling performed on 6 NEC and 10 matched controls	Not reported separately comparing NEC and controls.	Not reported	A core community of *Klebsiella*, *Escherichia*, *Staphyloccocus*, and *Enterococcus* was present in all samples. Gut microbiota profiles grouped into 6 distinct clusters, termed preterm gut community types (PGCTs). Each PGCT reflected dominance by the core taxa, except PGCT 6, which had high diversity and was dominant in Bifidobacteria. PGCTs 1–5 were observed in cases prior to NEC diagnosis, but PGCT 6 was seen only in controls. NEC infants had significantly more PGCT transitions (or microbiome instability) prior to diagnosis.
Lindberg et al.[9]	Place: single level IV neonatal intensive care unit (NICU) located in Hartford, CT, USA, Study period: September 2013 to September 2015	7 cases (gestational ages < 30 weeks), 72 control infants matched to NEC cases by gestation, birth weight, mode of delivery, sex, and predominant enteral nutrition. Fecal samples were collected prospectively. Mean gestation of all infants was 25.2 weeks (range, 23–27 weeks), and the mean birth weight was 680 g (range, 485–1026 g).	16 S rRNA gene sequencing was used to compare the composition and diversity of microbiota in samples collected from five NEC infants and five matched controls.	No difference in Simpson diversity index between cases and controls	Principal coordinate analysis showed NEC cases clustered toward vector regions corresponding to Proteobacteria, unlike controls that clustered towards Firmicutes.	Low diversity in all preterm infants; antibiotic exposure further reduced diversity among both NEC cases and controls. NEC cases showed greater abundance of Proteobacteria and class Gammaproteobacteria. Control infants demonstrated a greater abundance of Firmicutes.

Table 3 continued

Study	Setting and time period	Participants/study design	Methods	Alpha diversity metrics	Beta diversity metrics	Microbial profiles
Ravi[42]	Place: Beth Israel Hospital in Boston, MA ($n = 24$); Comer Children's Hospital at University of Chicago ($n = 29$); and NorthShore University Health System Hospital in Evanston, IL ($n = 9$). Study period: not clear.	23 cases and 39 controls	16S rRNA amplicon sequencing, shot-gun metagenome sequencing, and quantitative PCR. The study focused on mobile genetic elements in the microbiota	No difference in alpha diversity between NEC and controls.	Not reported	No major differences in the microbiome structure taking into account the adjusted gestational age between all infants (including NEC-positive and NEC-negative infants). Cases had higher proportions of Enterobacteriaceae (59%) than controls (44%). An OTU that mapped to enteropathogenic *E. coli* revealed the strongest association with NEC. Major differences noted between cases and controls in the plasmid signature genes.

Bacteroidetes, as a function of post-menstrual age.[1] Infants who did not develop NEC showed lower abundances of Proteobacteria and higher abundances of Firmicutes.

BIOLOGICAL GRADIENT

There are no data to suggest that the risk of NEC increases proportionate to the relative abundance of Gammaproteobacteria in the preterm gut microbiome. We recently investigated the relationship between fecal Gammaproteobacteria and fecal calprotectin (FC), which is derived from mucosal leukocytes and is a useful marker of mucosal inflammation.[63,64] In our cohort, Gammaproteobacteria abundance did not affect FC expression. Instead, we found FC to be associated specifically with the presence of *Klebsiella*, and even more strongly, with a single amplicon-sequence variant within this genus. *Klebsiella* abundance >83% predicted FC > 280 µg/g stool, which have been associated with mucosal inflammation and NEC.[65]

Our observation that FC correlated with a specific bacterial genus and not the entire class of Gammaproteobacteria suggest that Gammaproteobacteria may be too diverse a group to consistently exert a net inflammatory effect, and perhaps a need for defining dysbiosis at higher levels of taxonomic resolution. There is also a need to confirm whether the dominance of *Klebsiella* in the preterm gut microbiome in our cohort was specific to our center. The inflammatory effects of *Klebsiella* in the intestine are plausible, considering the presence of potent virulence factors such as cell wall components and enterotoxins.[66,67] *Klebsiella* are recognized intestinal pathogens of preterm and term neonates, having been identified in diarrhea, ecchymotic colitis, bacteremia during NEC and short-bowel syndrome, and even in NEC outbreaks.[24–26,68,69] The correlation between fecal *Klebsiella* and elevated FC has been previously noted in infantile colic.[70] Early colonization with *Klebsiella* has been noted in other preterm cohorts,[8,28] but the possibility of finding distinct inflammation-driving pathobiont(s) at other centers cannot be excluded.

PLAUSIBILITY

Gammaproteobacteria serve an important purpose in the normal newborn. *Enterobacteriaceae* normally reside in the gut at low levels, localized in close proximity to the mucosa as these bacteria can tolerate relatively high levels of oxygen that diffuses across from the epithelium. In the newborn intestine, *Enterobacteriaceae* deplete this oxygen and render the microenvironment suitable for colonization of strict anaerobes, such as *Bacteroides*, *Clostridium*, and *Bifidobacterium*.[71] During early infancy, breast milk allows oligosaccharide fermenters such as *Bifidobacterium* to thrive. Subsequent weaning and introduction of solid foods rich in polysaccharides not digestible by host enzymes lead to the expansion of polysaccharide fermenters *Bacteroides, Clostridium, Ruminococcus*, and simultaneously a decrease in *Bifidobacterium* and Enterobacteriaceae.[72,73]

In preterm infants, this normal, seemingly innocuous colonization with Gammaproteobacteria can plausibly turn deleterious. The premature intestine displays heightened sensitivity to Gram-negative bacteria and their products, due to high levels of expression of the Toll-like receptor (TLR)-4, the cognate pathogen recognition receptor for lipopolysaccharides (LPS) and lipid A expressed by coliform bacteria; downstream signaling mediators such as the myeloid differentiation primary response gene 88 (MyD88), the interleukin (IL)-1 receptor-associated kinase 1 (IRAK1), the tumor necrosis factor receptor-associated factor 6 (TRAF6); and the transcriptional regulator nuclear factor kappa B 1 (NF-κB$_1$).[74–78] Consistent with these observations, a range of NF-κB-dependent cytokines are increased during NEC, including the tumor necrosis factor (TNF), IL-1, IL-6, IL-8/CXC-motif ligand

(CXCL)-8, CXCL1, CXCL2, CC-motif ligand (CCL)-2, CCL3, CCL5, endothelin 1, and the vascular endothelial growth factor.[16,32–34]

The preterm intestine shows a paucity of normal anti-inflammatory adaptations, which further accentuate its pro-inflammatory bias. In the adult intestine, macrophages display a unique functional dichotomy, where these cells are profoundly anergic to LPS and other bacterial products and yet display avid phagocytic and bacteriocidal properties.[79,80] These adaptations of the resident macrophages promote the normal absence of inflammation in the intestine despite close physical proximity to luminal bacteria, and is mediated by transforming growth factor-beta (TGF-β), particularly the isoform TGF-β_2, present in the local extracellular matrix.[81] The preterm intestine is developmentally deficient in TGF-β_2, and this deficiency is further accentuated during NEC due to epigenetic modifications in the TGF-β_2 nucleosome.[82] During NEC, the newly recruited macrophage precursors also display a high degree of resistance to TGF-β-mediated non-inflammatory differentiation because of increased expression of Smad7.[83] Smad7 blocks TGF-β signaling in NEC macrophages by competing with the activating Smads, and sensitizes these cells to LPS through transactivation of IκB kinase-β gene expression and augmentation of NF-κB signaling.[83] The midgestation intestine is also deficient in several negative regulators of TLR4-NFκB signaling, including single Ig interleukin-1-related receptor (SIGIRR), IRAK-M, tumor necrosis factor-alpha-induced protein 3 (TNFAIP3), Toll-interacting protein (TOLLIP), and inhibitor of κB (IκB).[84]

In the preterm intestine, luminal Gammaproteobacteria are more likely to interact with the mucosa because of a developmental paucity of physical and immunological barriers. The mucus layer contains low amounts of the protective mucin 2,[85] which is further compromised during NEC.[86] There are fewer Paneth cells, and lower expression of antibacterial proteins such as lysozyme and α-defensins.[87] The deficiency of secretory IgA (sIgA) is also well known. The appearance of sIgA in mucosal secretions is delayed and increases slowly as a function of post-menstrual age.[88–91] IgA responses are dominated by monomeric sIgA[92,93] and the IgA1 sub-class,[94] and the antibodies show low antigen affinity, polyreactivity, and autoreactivity.[95,96] In addition, the immunoglobulin heavy chains have short complementarity-determining regions,[97] which markedly lowers the potential antibody diversity available to premature neonates.[97]

COHERENCE

The biological explanations for the association between Gammaproteobacteria and NEC are generally coherent. The two elements that need further scrutiny are the observed lack of any correlation between Gammaproteobacteria and FC, which is a widely accepted marker of mucosal inflammation, and the possibility that high Gammaproteobacteria abundance may not be uncommon in premature infants.[55,64]

EXPERIMENT

In the Bradford Hill framework, a causal inference is supported when interventions (treatments or risk-factor modifications) show a predictable effect on the outcome.[11] Several studies show that prolonged exposure to antibiotics, particularly aminoglycosides, can shift the preterm gut microbiome towards decreased alpha diversity and increased Gammaproteobacteria abundance. Fouhy et al. followed the microbiome of 9 infants who received parenteral antibiotic treatment with ampicillin and gentamicin starting within 48 hours of birth.[98] Samples collected 4 and 8 weeks later showed significantly higher proportions of Proteobacteria and reduced alpha diversity compared to controls. In another study, Greenwood et al. showed that infants who received 5–7 days of empiric antibiotics during the 1st week showed increased relative abundance of *Enterobacter* and lower bacterial diversity in the 2nd and 3rd postnatal weeks.[59] The effects of antibiotic exposure are not consistent across studies, and a clear effect was not detected in the studies by La Rosa et al. who showed that antibiotics merely influenced the pace, but not the sequence of the patterned colonization in the preterm gut microbiome.[45] Torrazza et al. also could not correlate antibiotic usage to specific changes in microbiota.[28] Pammi et al. examined the effects of antibiotics in their meta-analysis,[1] and showed similar effects of antibiotics in both cases and controls with increased relative abundances of *Proteobacteria*, and decreased abundances of *Firmicutes*, *Actinobacteria*, and *Bacteroidetes*. At the genus level, antibiotic exposure increased the relative abundances of *Klebsiella*, unclassified *Enterobacteriaceae*, *Proteus*, *Paenibacillus*, *Epulopiscium*, and *Pseudomonas*.

Prolonged empirical antibiotic treatment may increase the risk of NEC in premature infants. Cotten et al. analyzed data from 5693 extremely low birth weight (ELBW) infants admitted to the 19 neonatal research network (NRN) centers.[99] The median antibiotic therapy duration was 5 days (range: 1–36 days); 2147 infants (53%) received prolonged (>5 days) empirical therapy (center range: 27–85%) and these infants had increased odds of NEC or death. Similar findings have been reported by Alexander et al., Esmaeilizand et al., Abdel Ghany and Ali, Kuppala et al., and Cantey et al.; some of these studies have used a composite outcome of NEC or late-onset sepsis.[100–104] Consistent with these observations, Weintraub et al. noted an association between perinatal exposure to ampicillin and NEC.[105] These findings are of interest, but need to be interpreted cautiously. In a recent study, the NRN centers re-examined empiric antibiotic use in 5730 ELBW infants.[106] The proportion of infants receiving prolonged early antibiotics varied from 30 to 69% among centers and declined from 49% in 2008 to 35% in 2014. However, prolonged early antibiotic treatment was no longer associated with NEC.

Drugs used to suppress gastric acid production such as histamine (H)-2 receptor blockers have also been examined for potential effects on the preterm gut microbiome and NEC. Gupta et al. compared stool microbiome in 25 preterm infants who received H2 blocker treatment from postnatal days 3–58 vs. 51 controls had not received such treatment.[107] The H2 blocker-treated infants showed decreased alpha diversity and a shift towards an increased abundance of Proteobacteria. Guillet et al. examined data from 787 preterm infants from the NRN centers and found antecedent H2-blocker use to be associated with NEC.[108] More et al. evaluated this issue in meta-analysis ($n =$ 11,346) and found a significant association between H2 blocker use and NEC (odds ratio 1.78, 95% confidence interval 1.4, 2.27, $p < 0.00001$).[109]

ANALOGOUS RELATIONSHIPS

Gammproteobacterial blooms can be seen during diverse inflammatory conditions of the gastrointestinal tract, including inflammatory bowel disease (IBD), obesity, colorectal cancer, celiac disease, and primary sclerosing cholangitis.[110] Intestinal colonization with Proteobacteria has received considerable investigative attention in Crohn's disease.[111] Similar to NEC, a specific organism has not been causally linked with IBD, but abnormalities in the intestinal microbiome are considered part of the underlying pathogenesis. In Crohn's disease, increased relative abundance of *Klebsiella* has been linked to an aberrant inflammatory and T-helper cell response.[112] Interestingly, a contrarian view is now emerging on Gammaproteobacterial blooms in IBD, where this dysbiosis is believed to be a consequence rather than a cause of inflammation. The selection pressures implicated in these Gammaproteobacterial blooms in the inflamed gut include dietary changes, altered redox potential, mucin utilization, available of metal cofactors, decreased production of antimicrobial peptides, and horizontal gene transfer.[113]

Table 4. Bradford Hill Causality Score

Criterion	Score	Explanation
Strength	4	Effect size >5 in a large, well-designed clinical studies or meta-analysis
	3	Low but significant difference in well-designed clinical studies or meta-analysis
	2	Effect size >5 in smaller studies or in secondary outcomes
	1	Differences seen in smaller studies, in secondary outcomes, or important trends
	0	No difference
Consistency	4	Highly consistent results across nearly all studies
	3	Consistent results in >75% studies
	2	Consistent results in 50–75% studies
	1	Consistent results in a few studies
	0	No consistency
Specificity	4	Exposure is specific, necessary, and sufficient with high frequency of outcome in exposed population
	3	Exposure is specific, necessary, and sufficient, but with low frequency of outcome in exposed population
	2	Exposure is not specific to the study outcome, but is necessary and sufficient with high frequency of outcome in exposed population
	1	Exposure is not specific to the study outcome, but is necessary and sufficient with a low frequency of outcome in exposed population
	0	Exposure is not specific, necessary, or sufficient to cause the outcome in exposed population
Temporality	4	Exposure always precedes outcome
	3	Exposure precedes outcome in most instances
	2	Exposure precedes outcome in some instances
	1	Exposure precedes outcome in few instances
	0	No clear evidence for temporality
Biological gradient	4	High level of confidence for a dose-response effect
	3	Some confidence for a dose-response effect
	2	High level of confidence for a dose-response effect in particular settings
	1	Some confidence for a dose-response effect in particular settings
	0	No evidence for a dose-response effect
Plausibility	4	Highly plausible explanations
	3	Some confidence in scientific explanation
	2	Modest confidence in scientific explanation
	1	Low confidence in scientific explanation; speculations based on correlative data
	0	No plausible explanations
Coherence	4	Highly coherent explanations
	3	Modest coherence in explanations
	2	Low coherence, explanations valid for specific subsets of patients
	1	Low coherence, explanations valid for specific stages of disease
	0	Major discrepancy in existing evidence
Experiment	4	Interventions show strong evidence of an effect; studies evaluated for design, quality, consistency, directness, and reporting bias
	3	Interventions show evidence of an effect; studies evaluated for design, quality, consistency, directness, and reporting bias
	2	Interventions show weak evidence of an effect; studies evaluated for design, quality, consistency, directness, and reporting bias
	1	Interventions show poor/inconsistent evidence of an effect; studies evaluated for design, quality, consistency, directness, and reporting bias
	0	No evidence of an effect
Analogy	4	Conclusions can be extrapolated with high levels of confidence
	3	Conclusions can be extrapolated with some confidence
	2	Conclusions can be extrapolated with modest confidence
	1	Conclusions may be cautiously extrapolated
	0	No analogous conditions, or conclusions cannot be extrapolated
Reversibility	4	Interventions show strong evidence of an effect; studies evaluated for design, quality, consistency, directness, and reporting bias
	3	Interventions show evidence of an effect; studies evaluated for design, quality, consistency, directness, and reporting bias
	2	Interventions show weak evidence of an effect; studies evaluated for design, quality, consistency, directness, and reporting bias

Table 4 continued

Criterion	Score	Explanation
	1	Interventions show poor or inconsistent evidence of an effect; studies evaluated for design, quality, consistency, directness, and reporting bias
	0	No evidence of an effect

Table 5. Conclusions

Criteria	Summary of findings	Bradford Hill Causality Score
Strength	Studies with larger number of subjects, and a meta-analysis show a difference	3
Consistency	Modest, with many studies failing to show a difference	2
Specificity	Low level support; only a minority of infants with dysbiosis develop NEC	0
Temporality	Observations that dysbiosis antedates NEC are supportive	3
Biological gradient	No support	0
Plausibility	High level of support from preclinical data	4
Coherence	High frequency of dysbiosis in VLBW infants; lack of correlation between Gammaproteobacteria abundance and FC lower the level of support	2
Experiment	Supportive observational data on exposure to antibiotics and H2 blockers	2
Analogy	Supporting data from IBD	3
Reversibility	No data	No data

REVERSIBILITY

There are no data yet to show that the correction of the enteric dysbiosis by fecal transplant, specific antibiotics, or other interventions can reduce the risk of NEC.

CONCLUSIONS

In above sections, we have presented a detailed appraisal of current evidence supporting an association between Gammaproteobacterial blooms in the intestine and NEC. We looked for, but did not find an objective assessment scale for the Bradford Hill criteria. Therefore, we developed a 5-point scale to systemically evaluate the evidence for each of the 10 Bradford Hill criteria for causal inference (Table 4), and then applied this new assessment metric to the association of enteric dysbiosis and NEC (Table 5). Despite important methodological and statistical limitations, there is support for the association from the larger studies and a meta-analysis.[114] The evidence for temporality, that dysbiosis antedated NEC onset, adds strength to a possible causal inference. The role of Gram-negative bacteria in NEC pathogenesis is highly plausible, and is supported by a considerable amount of preclinical evidence. Corroborating observational data on the effects of prolonged exposure to antibiotics and H2-blockers are also supportive.

The weakness in this association is the low level of consistency across studies, and the lack of specificity. Although a large proportion of premature infants may develop an abnormal gut microbiome dominated by Proteobacteria, NEC is still seen only in a minority of these infants. Furthermore, the lack of any correlation between Proteobacteria/Gammaproteobacteria abundance and FC also calls for a better-informed definition of dysbiosis, either by relative abundance or at higher levels of taxonomic resolution. These unresolved concerns indicate that a need for further work before enteric dysbiosis can be causally tied to NEC pathogenesis.

Finally, we need to consider the possibility that the Gammaproteobacterial bloom antedating NEC could be a consequence, not the cause, of mucosal inflammation. This alternative view finds support in evidence that perinatal inflammation arising from chorioamnionitis, prior culture-positive sepsis, or infections such as cytomegalovirus or herpes simplex virus, may predispose to NEC.[21,115] In our own cohort,[64] the absence of correlation between Gammaproteobacteria abundance and FC may also be consistent with this possibility.

ACKNOWLEDGEMENTS

NIH awards HL124078 and HL133022 (to A.M.).

AUTHOR CONTRIBUTIONS

J.B.F., P.G., D.R.S., M.P., and A.M. reviewed the literature and contributed to the manuscript. A.M. developed the Bradford Hill Causality Score. All the authors reviewed and approved the manuscript.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES

1. Pammi, M. et al. Intestinal dysbiosis in preterm infants preceding necrotizing enterocolitis: a systematic review and meta-analysis. *Microbiome* **5**, 31 (2017).
2. Wang, Y. et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME J.* **3**, 944–954 (2009).
3. Millar, M. R. et al. Application of 16S rRNA gene PCR to study bowel flora of preterm infants with and without necrotizing enterocolitis. *J. Clin. Microbiol.* **34**, 2506–2510 (1996).
4. Groer, M. W. et al. Development of the preterm infant gut microbiome: a research priority. *Microbiome* **2**, 38 (2014).
5. Mshvildadze, M. et al. Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. *J. Pediatr.* **156**, 20–25 (2010).
6. Mai, V. et al. Fecal microbiota in premature infants prior to necrotizing enterocolitis. *PLoS ONE* **6**, e20647 (2011).
7. Zhou, Y. et al. Longitudinal analysis of the premature infant intestinal microbiome prior to necrotizing enterocolitis: a case-control study. *PLoS ONE* **10**, e0118632 (2015).
8. Warner B. B., et al. Gut bacteria dysbiosis and necrotising enterocolitis in very low birthweight infants: a prospective case-control study. *Lancet* **387**, 1928–36 (2016).

9. Lindberg T. P., et al. Preterm infant gut microbial patterns related to the development of necrotizing enterocolitis. *J. Matern. Fetal Neonatal Med.* **9**, 1–10 (2018).
10. Hill, A. B. The environment and disease: association or causation? *Proc. R Soc. Med.* **58**, 295–300 (1965).
11. Fedak, K. M., Bernal, A., Capshaw, Z. A. & Gross, S. Applying the Bradford Hill criteria in the 21st century: how data integration has changed causal inference in molecular epidemiology. *Emerg. Themes Epidemiol.* **12**, 14 (2015).
12. Polin, R. A. et al. Necrotizing enterocolitis in term infants. *J. Pediatr.* **89**, 460–462 (1976).
13. Ballance, W. A., Dahms, B. B., Shenker, N. & Kliegman, R. M. Pathology of neonatal necrotizing enterocolitis: a ten-year experience. *J. Pediatr.* **117**, S6–S13 (1990).
14. Tait, R. A. & Kealy, W. F. Neonatal necrotising enterocolitis. *J. Clin. Pathol.* **32**, 1090–1099 (1979).
15. Berdon, W. E. et al. Necrotizing enterocolitis in the premature infant. *Radiology* **83**, 879–887 (1964).
16. Remon, J. I. et al. Depth of bacterial invasion in resected intestinal tissue predicts mortality in surgical necrotizing enterocolitis. *J. Perinatol.* **35**, 755–762 (2015).
17. Pear, B. L. Pneumatosis intestinalis: a review. *Radiology* **207**, 13–19 (1998).
18. Bury R. G., Tudehope D. 2001 Enteral antibiotics for preventing necrotizing enterocolitis in low birthweight or preterm infants. *Cochrane Database Syst. Rev.* **CD000405**.
19. Maheshwari, A. Immunologic and hematological abnormalities in necrotizing enterocolitis. *Clin. Perinatol.* **42**, 567–585 (2015).
20. Gephart, S. M. et al. Changing the paradigm of defining, detecting, and diagnosing NEC: Perspectives on Bell's stages and biomarkers for NEC. *Semin. Pediatr. Surg.* **27**, 3–10 (2018).
21. Coggins, S. A., Wynn, J. L. & Weitkamp, J. H. Infectious causes of necrotizing enterocolitis. *Clin. Perinatol.* **42**, 133–154 (2015), ix.
22. Nanthakumar, N. N., Fusunyan, R. D., Sanderson, I. & Walker, W. A. Inflammation in the developing human intestine: a possible pathophysiologic contribution to necrotizing enterocolitis. *Proc. Natl Acad. Sci. USA* **97**, 6043–6048 (2000).
23. Mollitt, D. L., Tepas, J. J. 3rd & Talbert, J. L. The microbiology of neonatal peritonitis. *Arch. Surg.* **123**, 176–179 (1988).
24. Boccia, D., Stolfi, I., Lana, S. & Moro, M. L. Nosocomial necrotising enterocolitis outbreaks: epidemiology and control measures. *Eur. J. Pediatr.* **160**, 385–391 (2001).
25. Gregersen, N. et al. Klebsiella pneumoniae with extended spectrum beta-lactamase activity associated with a necrotizing enterocolitis outbreak. *Pediatr. Infect. Dis. J.* **18**, 963–967 (1999).
26. Hill, H. R., Hunt, C. E. & Matsen, J. M. Nosocomial colonization with Klebsiella, type 26, in a neonatal intensive-care unit associated with an outbreak of sepsis, meningitis, and necrotizing enterocolitis. *J. Pediatr.* **85**, 415–419 (1974).
27. van Acker, J. et al. Outbreak of necrotizing enterocolitis associated with Enterobacter sakazakii in powdered milk formula. *J. Clin. Microbiol.* **39**, 293–297 (2001).
28. Torrazza, R. M. et al. Intestinal microbial ecology and environmental factors affecting necrotizing enterocolitis. *PLoS ONE* **8**, e83304 (2013).
29. Morrow, A. L. et al. Early microbial and metabolomic signatures predict later onset of necrotizing enterocolitis in preterm infants. *Microbiome* **1**, 13 (2013).
30. Hofler, M. The Bradford Hill considerations on causality: a counterfactual perspective. *Emerg. Themes Epidemiol.* **2**, 11 (2005).
31. McMurtry, V. E. et al. Bacterial diversity and Clostridia abundance decrease with increasing severity of necrotizing enterocolitis. *Microbiome* **3**, 11 (2015).
32. Smith, B. et al. Investigation of the early intestinal microflora in premature infants with/without necrotizing enterocolitis using two different methods. *Pediatr. Res.* **71**, 115–120 (2012).
33. Sim, K. et al. Dysbiosis anticipating necrotizing enterocolitis in very premature infants. *Clin. Infect. Dis.* **60**, 389–397 (2015).
34. de la Cochetiere, M. F. et al. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. *Pediatr Res* **56**, 366–370 (2004).
35. Romano-Keeler, J. et al. Distinct mucosal microbial communities in infants with surgical necrotizing enterocolitis correlate with age and antibiotic exposure. *PLoS One* **13**, e0206366 (2018).
36. Stewart, C. J. et al. The preterm gut microbiota: changes associated with necrotizing enterocolitis and infection. *Acta Paediatr* **101**, 1121–1127 (2012).
37. Normann, E., Fahlen, A., Engstrand, L. & Lilja, H. E. Intestinal microbial profiles in extremely preterm infants with and without necrotizing enterocolitis. *Acta Paediatr* **102**, 129–136 (2013).
38. Wandro S., et al. The Microbiome and Metabolome of Preterm Infant Stool Are Personalized and Not Driven by Health Outcomes, Including Necrotizing Enterocolitis and Late-Onset Sepsis. mSphere 3 (2018).
39. Itani, T. et al. Preterm infants with necrotising enterocolitis demonstrate an unbalanced gut microbiota. *Acta Paediatr* **107**, 40–47 (2018).
40. Barron, L. K. et al. Independence of gut bacterial content and neonatal necrotizing enterocolitis severity. *J Pediatr Surg* **52**, 993–998 (2017).
41. Brown, C. T. et al. Hospitalized Premature Infants Are Colonized by Related Bacterial Strains with Distinct Proteomic Profiles. *MBio* **9**, e00441–18 (2018).
42. Ravi, A. et al. Association of the gut microbiota mobilome with hospital location and birth weight in preterm infants. *Pediatr Res* **82**, 829–838 (2017).
43. Stewart, C. J. et al. Temporal bacterial and metabolic development of the preterm gut reveals specific signatures in health and disease. Microbiome **4**, 67 (2016).
44. Heida, F. H. et al. A Necrotizing Enterocolitis-Associated Gut Microbiota Is Present in the Meconium: Results of a Prospective Study. Clin Infect Dis **62**, 863–870 (2016).
45. La Rosa, P. S. et al. Patterned progression of bacterial populations in the premature infant gut. *Proc Natl Acad Sci U S A* **111**, 12522–12527 (2014).
46. Itani, T. et al. Establishment and development of the intestinal microbiota of preterm infants in a Lebanese tertiary hospital. *Anaerobe* **43**, 4–14 (2017).
47. Gregory, K. E. et al. Influence of maternal breast milk ingestion on acquisition of the intestinal microbiome in preterm infants. *Microbiome* **4**, 68 (2016).
48. Cong, X. et al. Gut microbiome developmental patterns in early life of preterm infants: impacts of feeding and gender. *PLoS ONE* **11**, e0152751 (2016).
49. Parm, U., Metsvaht, T., Ilmoja, M. L. & Lutsar, I. Gut colonization by aerobic microorganisms is associated with route and type of nutrition in premature neonates. *Nutr. Res.* **35**, 496–503 (2015).
50. Raveh-Sadka T., et al. Gut bacteria are rarely shared by co-hospitalized premature infants, regardless of necrotizing enterocolitis development. *Elife* **4**, e05477 (2015).
51. Arboleya, S. et al. Intestinal microbiota development in preterm neonates and effect of perinatal antibiotics. *J. Pediatr.* **166**, 538–544 (2015).
52. Arboleya, S. et al. Establishment and development of intestinal microbiota in preterm neonates. *FEMS Microbiol. Ecol.* **79**, 763–772 (2012).
53. Mai, V. et al. Distortions in development of intestinal microbiota associated with late onset sepsis in preterm infants. *PLoS ONE* **8**, e52876 (2013).
54. Schwiertz, A. et al. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. *Pediatr. Res.* **54**, 393–399 (2003).
55. Ho, T. B. T. et al. Dichotomous development of the gut microbiome in preterm infants. *Microbiome* **6**, 157 (2018).
56. Gibson, M. K. et al. Developmental dynamics of the preterm infant gut microbiota and antibiotic resistome. *Nat. Microbiol.* **1**, 16024 (2016).
57. Morowitz, M. J. et al. Strain-resolved community genomic analysis of gut microbial colonization in a premature infant. *Proc. Natl Acad. Sci. USA* **108**, 1128–1133 (2011).
58. Brooks, B. et al. Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants. *Microbiome* **2**, 1 (2014).
59. Greenwood, C. et al. Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of Enterobacter. *J. Pediatr.* **165**, 23–29 (2014).
60. Aagaard, K. et al. The placenta harbors a unique microbiome. *Sci. Transl. Med.* **6**, 237ra265 (2014).
61. Palmer, C., Bik, E. M., DiGiulio, D. B., Relman, D. A. & Brown, P. O. Development of the human infant intestinal microbiota. *PLoS Biol.* **5**, e177 (2007).
62. Dominguez-Bello, M. G. et al. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proc. Natl Acad. Sci. USA* **107**, 11971–11975 (2010).
63. MacQueen, B. C. et al. Elevated fecal calprotectin levels during necrotizing enterocolitis are associated with activated neutrophils extruding neutrophil extracellular traps. *J. Perinatol.* **36**, 862–869 (2016).
64. Ho T. B. T., et al. Enteric dysbiosis and fecal calprotectin expression in premature infants. *Pediatr. Res.* **85**, 361–368 2018. in press.
65. Zhang, M., Zhang, X. & Zhang, J. Diagnostic value of fecal calprotectin in preterm infants with necrotizing enterocolitis. *Clin. Lab* **62**, 863–869 (2016).
66. Lu, M. C. et al. Colibactin contributes to the hypervirulence of pks(+) K1 CC23 Klebsiella pneumoniae in mouse meningitis infections. *Front. Cell. Infect. Microbiol.* **7**, 103 (2017).
67. Straus, D. C., Lonon, M. K., Woods, D. E. & Garner, C. W. Production of an extracellular toxic complex by various strains of Pseudomonas cepacia. *J. Med. Microbiol.* **30**, 17–22 (1989).
68. Stone, H. H., Kolb, L. D. & Geheber, C. E. Bacteriologic considerations in perforated necrotizing enterocolitis. *South Med. J.* **72**, 1540–1544 (1979).
69. Canioni, D. et al. Histopathology and microbiology of isolated rectal bleeding in neonates: the so-called 'ecchymotic colitis'. *Histopathology* **30**, 472–477 (1997).

70. Rhoads, J. M. et al. Altered fecal microflora and increased fecal calprotectin in infants with colic. *J. Pediatr.* **155**, 823–828 e821 (2009).
71. Arrieta, M. C., Stiemsma, L. T., Amenyogbe, N., Brown, E. M. & Finlay, B. The intestinal microbiome in early life: health and disease. *Front. Immunol.* **5**, 427 (2014).
72. Koenig, J. E. et al. Succession of microbial consortia in the developing infant gut microbiome. *Proc. Natl Acad. Sci. USA* **108**(Suppl 1), 4578–4585 (2011).
73. Fallani, M. et al. Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. *Microbiology* **157**, 1385–1392 (2011).
74. MohanKumar, K. et al. Trinitrobenzene sulfonic acid-induced intestinal injury in neonatal mice activates transcriptional networks similar to those seen in human necrotizing enterocolitis. *Pediatr. Res.* **81**, 99–112 (2016).
75. MohanKumar, K. et al. Cytokines and growth factors in the developing intestine and during necrotizing enterocolitis. *Semin. Perinatol.* **41**, 52–60 (2016).
76. Good, M. et al. Amniotic fluid inhibits Toll-like receptor 4 signaling in the fetal and neonatal intestinal epithelium. *Proc. Natl Acad. Sci. USA* **109**, 11330–11335 (2012).
77. Hackam, D. J., Upperman, J. S., Grishin, A. & Ford, H. R. Disordered enterocyte signaling and intestinal barrier dysfunction in the pathogenesis of necrotizing enterocolitis. *Semin. Pediatr. Surg.* **14**, 49–57 (2005).
78. Leaphart, C. L. et al. A critical role for TLR4 in the pathogenesis of necrotizing enterocolitis by modulating intestinal injury and repair. *J. Immunol.* **179**, 4808–4820 (2007).
79. Smythies, L. E. et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J. Clin. Invest.* **115**, 66–75 (2005).
80. Maheshwari, A. et al. Cytomegalovirus blocks intestinal stroma-induced down-regulation of macrophage HIV-1 infection. *J. Leukoc. Biol.* **80**, 1111–1117 (2006).
81. Maheshwari, A. et al. TGF-β2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. *Gastroenterology* **140**, 242–253 (2011).
82. Namachivayam, K. et al. Smad7 inhibits autocrine expression of TGF-beta2 in intestinal epithelial cells in baboon necrotizing enterocolitis. *Am. J. Physiol. Gastrointest. Liver Physiol.* **304**, G167–G180 (2013).
83. MohanKumar, K. et al. Smad7 interrupts TGF-β signaling in intestinal macrophages and promotes inflammatory activation of these cells during necrotizing enterocolitis. *Pediatr. Res.* **79**, 951–961 (2016).
84. Nanthakumar, N. et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS ONE* **6**, e17776 (2011).
85. Montgomery, R. K., Mulberg, A. E. & Grand, R. J. Development of the human gastrointestinal tract: twenty years of progress. *Gastroenterology* **116**, 702–731 (1999).
86. Martin, N. A. et al. Active transport of bile acids decreases mucin 2 in neonatal ileum: implications for development of necrotizing enterocolitis. *PLoS ONE* **6**, e27191 (2011).
87. Zhang, C. et al. Paneth cell ablation in the presence of Klebsiella pneumoniae induces necrotizing enterocolitis (NEC)-like injury in the small intestine of immature mice. *Dis. Model Mech.* **5**, 522–532 (2012).
88. Haworth, J. C. & Dilling, L. Concentration of gamma-A-globulin in serum, saliva, and nasopharyngeal secretions of infants and children. *J. Lab Clin. Med.* **67**, 922–933 (1966).
89. Brandtzaeg, P., Nilssen, D. E., Rognum, T. O. & Thrane, P. S. Ontogeny of the mucosal immune system and IgA deficiency. *Gastroenterol. Clin. North Am.* **20**, 397–439 (1991).
90. Gleeson, M. et al. Ontogeny of the secretory immune system in man. *Aust. N. Z. J. Med.* **12**, 255–258 (1982).
91. Mellander, L., Carlsson, B. & Hanson, L. A. Appearance of secretory IgM and IgA antibodies to Escherichia coli in saliva during early infancy and childhood. *J. Pediatr.* **104**, 564–568 (1984).
92. Cripps, A. W., Gleeson, M. & Clancy, R. L. Ontogeny of the mucosal immune response in children. *Adv. Exp. Med. Biol.* **310**, 87–92 (1991).
93. Weemaes, C. et al. Development of immunoglobulin A in infancy and childhood. *Scand. J. Immunol.* **58**, 642–648 (2003).
94. Fitzsimmons, S. P. et al. Immunoglobulin A subclasses in infants' saliva and in saliva and milk from their mothers. *J. Pediatr.* **124**, 566–573 (1994).
95. Bhat, N. M. et al. The ontogeny and functional characteristics of human B-1 (CD5 + B) cells. *Int. Immunol.* **4**, 243–252 (1992).
96. Chen, Z. J. et al. Polyreactive antigen-binding B cells are the predominant cell type in the newborn B cell repertoire. *Eur. J. Immunol.* **28**, 989–994 (1998).
97. Bauer, K. et al. Diversification of Ig heavy chain genes in human preterm neonates prematurely exposed to environmental antigens. *J. Immunol.* **169**, 1349–1356 (2002).
98. Fouhy, F. et al. High-throughput sequencing reveals the incomplete, short-term recovery of infant gut microbiota following parenteral antibiotic treatment with ampicillin and gentamicin. *Antimicrob. Agents Chemother.* **56**, 5811–5820 (2012).
99. Cotten, C. M. et al. Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. *Pediatrics* **123**, 58–66 (2009).
100. Alexander, V. N., Northrup, V. & Bizzarro, M. J. Antibiotic exposure in the newborn intensive care unit and the risk of necrotizing enterocolitis. *J. Pediatr.* **159**, 392–397 (2011).
101. Esmaeilizand, R. et al. Antibiotic exposure and development of necrotizing enterocolitis in very preterm neonates. *Paediatr. Child Health* **23**, e56–e61 (2018).
102. Abdel Ghany, E. A. & Ali, A. A. Empirical antibiotic treatment and the risk of necrotizing enterocolitis and death in very low birth weight neonates. *Ann. Saudi Med.* **32**, 521–526 (2012).
103. Kuppala, V. S., Meinzen-Derr, J., Morrow, A. L. & Schibler, K. R. Prolonged initial empirical antibiotic treatment is associated with adverse outcomes in premature infants. *J. Pediatr.* **159**, 720–725 (2011).
104. Cantey, J. B., Pyle, A. K., Wozniak, P. S., Hynan, L. S. & Sanchez, P. J. Early antibiotic exposure and adverse outcomes in preterm, very low birth weight infants. *J. Pediatr.* **203**, 62–67 (2018).
105. Weintraub, A. S. et al. Antenatal antibiotic exposure in preterm infants with necrotizing enterocolitis. *J. Perinatol.* **32**, 705–709 (2012).
106. Greenberg R. G., et al., Eunice Kennedy Shriver National Institute of Child H, Human Development Neonatal Research N Prolonged duration of early antibiotic therapy in extremely premature infants. *Pediatr. Res.* **85**, 994–1000 (2019).
107. Gupta, R. W. et al. Histamine-2 receptor blockers alter the fecal microbiota in premature infants. *J. Pediatr. Gastroenterol. Nutr.* **56**, 397–400 (2013).
108. Guillet, R. et al. Association of H2-blocker therapy and higher incidence of necrotizing enterocolitis in very low birth weight infants. *Pediatrics* **117**, e137–e142 (2006).
109. More, K., Athalye-Jape, G., Rao, S. & Patole, S. Association of inhibitors of gastric acid secretion and higher incidence of necrotizing enterocolitis in preterm very low-birth-weight infants. *Am. J. Perinatol.* **30**, 849–856 (2013).
110. Winter, S. E. & Baumler, A. J. Dysbiosis in the inflamed intestine: chance favors the prepared microbe. *Gut Microbes* **5**, 71–73 (2014).
111. Atarashi, K. et al. Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science* **358**, 359–365 (2017).
112. Larmonier, C. B., Shehab, K. W., Ghishan, F. K. & Kiela, P. R. T lymphocyte dynamics in inflammatory bowel diseases: role of the microbiome. *Biomed. Res. Int.* **2015**, 504638 (2015).
113. Zeng, M. Y., Inohara, N. & Nunez, G. Mechanisms of inflammation-driven bacterial dysbiosis in the gut. *Mucosal Immunol.* **10**, 18–26 (2017).
114. Friede, T., Rover, C., Wandel, S. & Neuenschwander, B. Meta-analysis of few small studies in orphan diseases. *Res. Synth. Methods* **8**, 79–91 (2017).
115. Been, J. V., Lievense, S., Zimmermann, L. J., Kramer, B. W. & Wolfs, T. G. Chorioamnionitis as a risk factor for necrotizing enterocolitis: a systematic review and meta-analysis. *J. Pediatr.* **162**, 236–242 e232 (2013).