The Microbiome and Biomarkers for Necrotizing Enterocolitis: Are We Any Closer to Prediction?

Brigida Rusconi, PhD,1,2 Misty Good, MD,2,3 and Barbara B. Warner, MD2,3

The past decade has seen a substantial increase in interest for biomarkers across a spectrum of disease states, fueled by emerging “omics” technologies. Biomarkers hold the promise of early detection and diagnosis, prognostication of disease severity, and new insights into disease mechanisms. Necrotizing enterocolitis (NEC) has been a prime target, with its high mortality, burden of morbidity in infants born preterm, and unpredictable onset.1,2 This review focuses on recent advances in NEC biomarker research, including the use of gut microbiome patterns of infants born preterm, and the application of new proteomic and metabolomic technology. Examination of publications for NEC biomarkers in the decade following the initiation of the Human Microbiome Project demonstrates integration of these new arenas, as well as continued overlap in content (Table I). It remains true, however, that none of these biomarkers has achieved widespread clinical application.

The Microbiome as a Biomarker

To understand the potential role of the gut microbial community as a biomarker for NEC, this review will evaluate the unique characteristics of the gut microbiome in the infant born preterm and the technology used for its study. Until recently, studies of these communities relied on culture or gel-based technology for microbial identification. Current techniques including direct-from-stool amplification and sequencing of the 16S ribosomal RNA subunit DNA or whole-genome shotgun (WGS) sequencing enable identification of the microbial community members and their distribution. WGS sequencing also provides insight into the microbial community’s functional potential, as well as the presence of mobile elements, such as resistance cassettes and virulence factors. Proteomics and metabolomics quantify the functional state at a given time point of both the host and the gut microbiome (Table II; available at www.jpeds.com, for comparison of methodology, benefits, and limitations).

AUC Area under the curve
IaIP Interalpha inhibitor protein
I-FABP Intestinal fatty acid binding protein
IL Interleukin
NEC Necrotizing enterocolitis
ROC Receiver operator characteristic
SAA Serum amyloid A
SIP Spontaneous intestinal perforation
TGF-β Transforming growth factor beta
VOC Volatile organic compounds
WGS Whole-genome shotgun

Compared with infants born at term, infants born preterm have diminished stool microbial diversity, with a reduced number (richness) of taxa present.53,54 Despite this limited diversity, longitudinal studies in the population born preterm have demonstrated a dynamic but choreographed pattern of early intestinal colonization. Initial colonization begins with Gram-positive cocci (within the Bacilli class), soon overtaken by Gram-negative facultative anaerobic organisms (within the Gammaproteobacteria class), counterbalanced by a gradually increasing abundance of anaerobes (within the Clostridia and Negativicutes class).53-56 These limited taxa account for >90% of the taxa present.56 Gram-negative organisms (Gammaproteobacteria class) are overrepresented proportionally in infants born preterm, frequently comprising >50% of taxa, compared with <10%-20% in infants born at term.54,56

These unique characteristics of the microbiota in infants born preterm bolster the hypothesis introduced by Claud and Walker46 of an “inappropriate colonization” within the gut of infants born preterm rather than a single organism precipitating NEC. The advent of high-throughput sequencing has improved our ability to evaluate this hypothesis. When gut microbial diversity is examined in NEC, irrespective of community composition, results are mixed between studies. Some studies find no difference in stool microbial diversity between infants who develop NEC and control infants, whereas others report a decrease.43,54,62,63 Suppressed maturation of microbial diversity is noted in infants who developed NEC, ie, gut microbial diversity in control infants increases over time compared with infants with NEC.43,65 However, because >90% of the bacteria within stool samples of cases and controls belong to only 4 class level taxa, a change in the fractional representation of a single class level taxon will produce a major change in bacterial diversity. As a result, one must be cautious in attributing case or control status to changes in the diversity itself vs changes in the ratios of the 4 taxa that define diversity in these communities.

What about aberrant community composition and the development of NEC? Longitudinal studies that used 16S
riboosomal RNA subunit or WGS sequencing on stool demonstrated a relative increase in Gram-negative bacteria (class Gammaproteobacteria) before the onset of NEC53,34,58-61,63 (Table III; available at www.jpeds.com) and an associated decrease in anaerobes (classes Clostridia and Negativicutes).50-63 These findings were confirmed by a recent meta-analysis that showed that before the onset of clinical NEC, there was a predominance of the Gram-negative phylum Proteobacteria (including the class Gammaproteobacteria) that was offset by a decrease in the relative abundance of the anaerobe containing phyla Firmicutes (including the class Clostridia and Negativicutes) and Bacteroidetes.64 Studies aimed at identifying organisms associated with NEC risk at lower taxonomic levels (ie, species) have shown greater variation (Table III). Ward et al64 used a metagenomic sequencing approach that identified 2 members of the Gammaproteobacteria class, Escherichia coli and Klebsiella spp., that had the greatest relative abundance among infants who developed NEC. Functional genetic subtyping of the E coli strain suggested that uropathogenic E coli lineages presented a risk for NEC and NEC-associated mortality. These intriguing findings again raise the question of what role specific organisms vs the gut community structure play in the development of NEC. Whether variation in reported species is a function of specific microbial backgrounds, patient populations, or methodologic differences in sampling or sequencing requires additional testing and validation across diverse populations of infants born preterm.

Although microbial dysbiosis simply may reflect host risk, mechanistically, several lines of evidence give credence to the role that microbial dysbiosis plays in NEC causation. Gammaproteobacteria elicit similar injury in animal models, mediated through Toll-like receptor 4,65 eliciting an inflammatory cascade,66-72 with directed antibiotics being protective.73 Anaerobic bacteria produce short chain fatty acid byproducts including acetate, butyrate, and propionate, which are biologically active compounds involved in host signaling mechanisms and implicated in maintaining epithelial cell health.74,75 The exact role of these metabolites has come under new scrutiny, with the effects of butyrate specifically being dependent on host crypt cell type.76 Given the altered maturational state of the gut of the infant born preterm and diet, it still remains to be determined whether short chain fatty acids promote or hinder injury.

Importantly, these results offer the potential to include tests of microbial signature into trials of NEC treatment and prevention. Clinically available tools for rapid targeted microbial identification, such as polymerase chain reaction, could be incorporated into study design to stratify risk and provide insight into treatment efficacy. A novel approach to rapid diagnosis for microbial dysbiosis has used volatile organic compounds (VOCs). VOCs are carbon-based waste products, excreted in breath, sweat, urine, and feces, that are detected with the use of gas chromatography and mass spectrometry. Fecal microbial fermentation products are major contributors to VOC and have therefore been applied to a variety of intestinal disorders linked to microbial dysbiosis,77 including NEC. In a pilot study, 4 specific esters were absent in all samples up to 4 days before disease onset in stools from infants who developed NEC (n = 6).51 To improve turn-around time, de Meij et al52 developed a bedside fecal VOC profiling system based on gas sensors and pattern-recognition algorithms. Fecal VOC profiles discriminated infants who developed NEC (n = 13) from those who did not (n = 14) 2-3 days before onset with 83% sensitivity and 75% specificity (area under the curve [AUC] 0.77 ± 21).53 Because a major source of fecal VOC is the intestinal microbiota, this noninvasive bedside tool offers the potential to identify shifts in microbial community composition and/or host response in real time.
Although use of the microbiome as a biomarker for NEC was first introduced a decade ago, we are just now beginning to see its application, manipulating the microbes, their metabolic byproducts, and host responses. The changes in gut microbial community structure before the onset of disease offer potential for prediction. Mechanistic insights into both direct signaling pathways and metabolic byproducts offer new venues for prevention. The advent of new technologies with rapid turn-around times and lower costs can assist in risk stratification. As of yet, there is no specific microbial signal for diagnosis, but proteomic and metabolomic studies of the gut microbial community are emerging.

**Nonmicrobial Biomarkers**

The ultimate goal of any biomarker is early prediction of an outcome. Both intestinal-specific and nonspecific biomarkers have been used in attempts to predict NEC. Among nonspecific markers, cytokines have been a common target. Most studies have examined cytokine alterations at the time of clinical symptomatology, with the authors identifying alterations in both pro- and anti-inflammatory cytokines. In 1 study of 997 infants of extremely low birth weight, the authors prospectively collected blood samples from birth and through the development of NEC. Infants with NEC (n = 108) showed decreased concentrations of transforming growth factor β (TGF-β) and interleukin (IL)-2 and increased IL-8. Of these, only TGF-β discriminated infants who eventually developed NEC from those who did not before the event, beginning on the first day of life. However, the diagnostic accuracy for TGF-β was only “fair” by receiver operator characteristic analysis (ROC), with an AUC of 0.67.

Intestinal fatty acid binding protein (I-FABP) has long been a candidate marker for NEC as the result of its gut-specific characteristics. It is present in high concentrations within enterocytes, released into the circulation with injury, and then excreted into the urine. However, urinary I-FABP as a single predictive marker of NEC has not been successful. NEC risk identification with urinary I-FABP from 1-2 days before onset of disease to as early as 96 hours of life has been attempted but was limited by small numbers of cases and inadequate sensitivity and/or specificity. These excellent indicators of acute inflammation and injury could not be adapted as predictors of subsequent events, such as NEC.

For early diagnosis and prediction of disease severity in infants who had clinical symptoms, serum and urinary I-FABP had better results, particularly when used in combination with other markers. Urinary I-FABP, claudin 3 (a tight junction protein), and fecal calprotectin (a protein originating from neutrophils recruited during inflammation) were all significantly greater at the time of the first clinical signs in infants with proven NEC (n = 14) than in those with other conditions. Among these, I-FABP alone was associated with the severity of NEC, a result also reported by other investigators. Isolated fecal calprotectin concentrations lack specificity for NEC diagnosis, with significant overlap of cases with healthy controls. Serum amyloid A (SAA), another acute-phase reactant, offered no advantage to urinary I-FABP and fecal calprotectin alone in discriminating NEC at first clinical signs from other entities. However, SAA has been reported to better differentiate severity of NEC, particularly in combination with platelet counts. The proinflammatory cytokine IL-8 also has been correlated with severity of disease, with is ability to discriminate between medical (n = 63) and surgical (n = 50) NEC (AUC 0.82), and was found to be correlated significantly with 60-day mortality (OR 1.38, CI 1.14-1.67), similar to previous reports for both NEC and sepsis-like syndromes. Other recent markers of disease severity have included the “LIT” score, based on liver fatty acid binding protein, I-FABP, and trefoil factor 3 differentiating surgical from nonsurgical NEC at the onset of disease, the “Totalis” score, based on both clinical and serum markers, and the complement activation product C5a.

FABP, cytokines, and other acute-phase reactants have an established association with the expected intestinal injury and inflammation evident in NEC. Examination of biomarkers not traditionally linked to injury and inflammation offer the potential for providing novel insights into disease mechanisms. Bile acids are essential to intestinal fat absorption, but excessive accumulation can be injurious to the intestinal epithelium. Hulzebos et al demonstrated that the expected rate of fecal unconjugated bile acid decline over time and was slower in infants who developed NEC. Five to 6 days before the onset of NEC, fecal bile salt levels were significantly greater compared with age-matched controls. The slower decay of fecal bile salt supports a potential role for altered bile salt metabolism or transport in NEC development. This difference in trajectory may be intrinsic to host phenotype but also is linked to more modifiable factors, including the gut microbiota, which has been shown previously to alter the expression of bile salt transporters. It may be that microbial dysbiosis may be influencing the risk of NEC through indirect mechanisms such as this, rather than solely through cell signaling or metabolic byproducts discussed previously.

**Proteomics and Metabolomics Applied to NEC**

NEC is a multifactorial disease, and this is highlighted in the variety of biomarkers reviewed, providing evidence for interactions between diet, environment, gut microbiota, and host response. Proteomics and metabolomics can be used to examine the metabolic readout from these complex interactions. Both techniques are extremely powerful, as they measure the current functional state of all organisms present in the site of sampling and of the host response to this biomass. Proteomic studies deepen our physiological understanding of diseases by detecting modifications and interactions of proteins and peptides, compared with genomics, which give a theoretical functional status of the organism. Both proteomics and metabolomics exploit separation techniques before mass
Metabolomics is the study of low-molecular-weight metabolites found within biologic samples, reflecting the metabolic pathways and host-microbiota interactions that could play a role in the development of NEC. Interestingly, fatty acids have appeared as relevant metabolites in 2 of the 4 studies published so far.\textsuperscript{17,43} as well as in the newborn screen study by Sylvester et al.\textsuperscript{44} Lipids are becoming recognized increasingly as playing an important role in gut inflammation and interacting with the microbiota.\textsuperscript{76,97-100} Interventions focused on the addition of lipids as dietary supplements or modulating the host response in other gut inflammatory diseases are especially promising and potentially could be useful in NEC.\textsuperscript{101-103}

Most importantly, several studies have combined multiple “–omics” approaches to find risk determinants to counteract the sparsity of the data. As for all descriptive “–omics” studies,
follow-up validation studies in animal models are required to determine the therapeutic potential of these newly associated metabolic pathways.

Biomarkers: Disease Discrimination

Using biomarkers to discriminate between disease entities with similar clinical presentations not only aids diagnosis and treatment but also improves the ability to compare across research studies. The ability to differentiate NEC from sepsis has had moderate success, most notably in the proteomic studies described previously.\(^5\)\(^6\) Kim et al\(^5\) developed a new approach to distinguish NEC (n = 10) from sepsis (n = 5) and controls (n = 5) by designing a magnetic multiplexed biosensor system to analyze serum C-reactive protein, matrix metalloproteinase-7, and epithelial cell adhesion molecule. The ratio of matrix metalloproteinase-7/epithelial cell adhesion molecule provided the best discrimination of both NEC to controls and NEC to sepsis. However, from a practical standpoint, a strict differentiation between NEC and sepsis remains challenged by the common co-occurrence of culture-proven bloodstream infections at the time of NEC diagnosis.

Unfortunately, the early clinical features of NEC also often overlap with spontaneous intestinal perforation (SIP), making it difficult to distinguish between the 2 diagnoses, which is not always clear without a laparotomy. Differentiation between the 2 entities is of critical importance for disease categorization in clinical studies and may contribute to the reproducibility and clinical translatability issues that have persisted. Ng et al used genomics to differentiate the 2 entities, reporting both differentially expressed mRNA as well as micro-RNAs in the small bowel tissue of NEC and SIP compared with surgical control tissue.\(^4\)\(^5\) Shah et al\(^4\) used a more clinically applicable serum biomarker to distinguish NEC from SIP called interalpha inhibitor proteins (IaIps). IaIps are serine protease inhibitors that act as negative acute phase reactants, consumed as they protect from damaging proteases that are released into circulation during acute inflammation.\(^7\) An earlier study reported that IaIp concentrations were decreased significantly in infants with NEC compared with controls at the time of disease diagnosis.\(^8\) Using a prospective nested case control design, the authors found that circulating IaIp concentrations at the time of initial presentation were significantly lower in infants with NEC (n = 14) compared with those with SIP (n = 13), who had concentrations indistinguishable from matched controls (n = 26). ROC analysis for NEC yielded an AUC of 0.98 (P < .0001, 95% CI 0.84-0.99). However, in the week before the onset of disease, blood concentrations did not differ significantly between groups.\(^9\) This raises the possibility that there is only a very short interval between organ injury and clinical NEC, such that there would be a low likelihood of a very early in life biomarker.

It is sobering to note that despite the growth in the biomarker literature, few such tests have moved into clinical practice. In cancer for example, <1% of published biomarkers are ever implemented.\(^10\) The reason for this failure of transition into practice is multifactorial, relating to both biomarker and study design.\(^10\)\(^10\) A similar pattern of limited success and many failures has emerged in NEC. Sample sizes are often small, and diagnostic accuracy frequently is fair with the use of ROC analysis. Case definitions vary, and many arise from single institutions, without reproduction, making transferability unclear. Although technology continues to advance, many biomarkers still cannot be used within a timeframe appropriate for clinical care. Unfortunately, no biomarker has been identified thus far that can predict disease risk early enough to provide a targeted prevention strategy. Headway is being made in disease discrimination and prediction of NEC severity, and this body of work has advanced the field and improved our understanding of underlying disease pathology.

References


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The Microbiome and Biomarkers for Necrotizing Enterocolitis: Are We Any Closer to Prediction?
### Table II. Microbiome and biomarker technology

<table>
<thead>
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<th>Technologies</th>
<th>Description</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Authors</th>
<th>Year</th>
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</thead>
<tbody>
<tr>
<td><strong>Biomarkers</strong>&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>16S rRNA</td>
<td>Sequencing of variable region of ribosomal 16S gene</td>
<td>Provides information on changes in bacterial composition</td>
<td>Requires some computational capability for interpretation</td>
<td>Mai et al&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
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<td></td>
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<td>Can only often identify at the family/genus level</td>
<td>McMurtry et al&lt;sup&gt;2&lt;/sup&gt;</td>
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<td></td>
<td></td>
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<td></td>
<td>Enhanced detection of bacterial species</td>
<td>Heida et al&lt;sup&gt;3&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td>Higher computational and sequencing cost than 16S rRNA</td>
<td>Torrazza et al&lt;sup&gt;4&lt;/sup&gt;</td>
<td>2013</td>
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<td></td>
<td>WGS</td>
<td>Sequencing of all fragmented bacterial DNA</td>
<td>Provides information on functional potential and presence of mobile elements</td>
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<td>Sim et al&lt;sup&gt;5&lt;/sup&gt;</td>
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<td>Species level identification</td>
<td>Stewart et al&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>Requires some computational capability</td>
<td>Warner et al&lt;sup&gt;7&lt;/sup&gt;</td>
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<td>Zhou et al&lt;sup&gt;8&lt;/sup&gt;</td>
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<td>Ward et al&lt;sup&gt;9&lt;/sup&gt;</td>
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<td>Ng et al&lt;sup&gt;10&lt;/sup&gt;</td>
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<td>Ward et al&lt;sup&gt;11&lt;/sup&gt;</td>
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<td><strong>Proteomics</strong>&lt;sup&gt;†&lt;/sup&gt;</td>
<td>ProteinChip</td>
<td>Digested proteins are fingerprinted by MALDI-TOF and compared with protein database</td>
<td>Fast acquisition and identification</td>
<td>Confidence levels are lower</td>
<td>Ng et al&lt;sup&gt;12&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Mass spectrometry</td>
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<td>Longer acquisition and greater cost</td>
<td>Murgas Torrazza et al&lt;sup&gt;13&lt;/sup&gt;</td>
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<td>Species level identification</td>
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<td>Spectra are compared with databases for identification</td>
<td>Sylvester et al&lt;sup&gt;15&lt;/sup&gt;</td>
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<td>Tao et al&lt;sup&gt;17&lt;/sup&gt;</td>
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<tr>
<td><strong>Metabolomics</strong>&lt;sup&gt;¶&lt;/sup&gt;</td>
<td>NMR</td>
<td>Metabolites in liquid are subjected to &lt;sup&gt;1&lt;/sup&gt;H NMR and resulting spectra are compared to database</td>
<td></td>
<td>Not sensitive and requires large sample volumes</td>
<td>Morrow et al&lt;sup&gt;18&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>MS</td>
<td>Extracted metabolites are separated by LC and fragmented through collision for MS spectra acquisition</td>
<td></td>
<td>High reproducibility</td>
<td>Stewart et al&lt;sup&gt;19&lt;/sup&gt;</td>
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<td></td>
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<td></td>
<td></td>
<td>Low volume required and very sensitive</td>
<td>Stewart et al&lt;sup&gt;20&lt;/sup&gt;</td>
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<td>Wilcock et al&lt;sup&gt;21&lt;/sup&gt;</td>
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Table III. Studies using 16S rRNA or metagenomic sequencing to examine relationship of gut microbial community of infants born preterm and development of NEC

<table>
<thead>
<tr>
<th>Authors</th>
<th>Year</th>
<th>Sequencing technology</th>
<th>Number of subjects/samples without NEC</th>
<th>Number of subjects/samples with NEC</th>
<th>Comments</th>
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<tr>
<td>Mai et al</td>
<td>2011</td>
<td>16S rRNA gene sequencing</td>
<td>9/18</td>
<td>9/18</td>
<td>Case stools demonstrated an increase in Proteobacteria and a decrease in Firmicutes in the week before NEC.</td>
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<tr>
<td>McMurtry et al</td>
<td>2015</td>
<td>16S rRNA gene sequencing</td>
<td>74/74</td>
<td>21/21</td>
<td>Bacterial diversity and relative abundance of Clostridia was significantly lower in NEC specimens compared with controls.</td>
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<tr>
<td>Raveh-Sadka et al</td>
<td>2015</td>
<td>Metagenomic sequencing</td>
<td>5/34</td>
<td>5/21</td>
<td>No clear association between bacterial content as identified by metagenomics and outcome.</td>
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<tr>
<td>Heida et al</td>
<td>2016</td>
<td>16S rRNA gene sequencing of meconium and subsequent stools</td>
<td>22/57</td>
<td>11/30</td>
<td>Clostridium perfringens and Bacteroides dorei associated with NEC risk and Staphylococci associated with protection.</td>
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<tr>
<td>Torrazza et al</td>
<td>2013</td>
<td>16S rRNA gene sequencing</td>
<td>35/77</td>
<td>18/40</td>
<td>Klebsiella pneumoniae during week 1 associated with subsequent development of NEC.</td>
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<tr>
<td>Ward et al</td>
<td>2016</td>
<td>Metagenomic sequencing</td>
<td>89/185</td>
<td>27/60</td>
<td>Specific sequence types of E.coli associated with NEC.</td>
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<tr>
<td>Zhou et al</td>
<td>2015</td>
<td>16S rRNA gene sequencing</td>
<td>26/111</td>
<td>10/88</td>
<td>NEC having an association with Clostridia and Gammaproteobacteria, respectively.</td>
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<tr>
<td>Sim et al</td>
<td>2015</td>
<td>16S rRNA gene sequencing</td>
<td>44/369</td>
<td>22/88</td>
<td>Klebsiella, Clostridium associated with risk of NEC.</td>
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<tr>
<td>Stewart et al</td>
<td>2016</td>
<td>16S rRNA gene sequencing</td>
<td>28/520</td>
<td>7/121</td>
<td>Klebsiella, Escherichia, Staphylococcus, and Enterococcus present in all samples, without uniform microbial signature for NEC.</td>
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<tr>
<td>Warner et al</td>
<td>2016</td>
<td>16S rRNA gene sequencing</td>
<td>120/2720</td>
<td>46/866</td>
<td>Gammaproteobacteria associated with risk and Negatuvicutes associated with protection; lack of diversity is associated with risk.</td>
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</table>

References 1-10 available as Appendix 2 (available at www.jpeds.com).

Appendix 2


