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The microbiome and biomarkers for necrotizing enterocolitis: Are we any closer to prediction?

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The past decade has seen 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 preterm morbidity, and unpredictable onset (1, 2). This review focuses on recent advances in NEC biomarker research, including the utilization of the preterm infant gut microbiome patterns, 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 preterm gut microbiome 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 include direct-from-stool amplification and sequencing of the 16S ribosomal RNA subunit (16S rRNA) DNA or whole genome shotgun (WGS) sequencing enable identification of the microbial community

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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).

Compared with term infants, preterm infants have diminished stool microbial diversity, with a reduced number (richness) of taxa present (3, 4). Despite this limited diversity, longitudinal studies in the preterm population 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) (5–10). These limited taxa account for >90% of the taxa present (6). Gram-negative organisms (Gammaproteobacteria class) are proportionally overrepresented in preterm infants, frequently comprising over 50% of taxa, compared with less than 10–20% in term infants (4, 6).

These unique characteristics of the premature infant microbiota bolster the hypothesis introduced by Claud and Walker of an "inappropriate colonization" within the preterm gut rather than a single organism precipitating NEC (11). 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 controls (9, 12, 13), while others report a decrease (4, 14–16). Suppressed maturation of microbial diversity is noted in infants who developed NEC, i.e. gut microbial diversity in controls increases over time as compared with infants with NEC (15, 16). However, because more than 90% of the bacteria within stool samples of cases and controls belong to only four 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, versus changes in the ratios of the four taxa that define diversity in these communities.

What about aberrant community composition and the development of NEC? Longitudinal studies that utilized 16S or WGS sequencing on stool demonstrate a relative increase in Gram-negative bacteria (class Gammaproteobacteria) before the onset of NEC (4, 8–10, 12, 15, 16) (Table III; available at www.jpeds.com) and an associated decrease in anaerobes (classes Clostridia and Negativicutes) (10, 12, 14, 16). These findings were confirmed by a recent meta-analysis that showed that prior to 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 (17). Studies aimed at identifying organisms associated with NEC risk at lower taxonomic levels (i.e. species) have shown greater variation (Table III). Ward et al (4) used a metagenomic sequencing approach that identified two members of the Gammaproteobacteria class, *E. coli* and *Klebsiella* spp. that had the greatest relative

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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 versus the gut community structure play in NEC development. Whether variation in reported species are a function of specific microbial backgrounds, patient populations, or methodological differences in sampling or sequencing requires additional testing and validation across diverse preterm populations.

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 (18), eliciting an inflammatory cascade (19–25), with directed antibiotics being protective (26). 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 (27, 28). The exact role of these metabolites has come under new scrutiny with the effects of butyrate specifically, being dependent on host crypt cell type (29). Given the altered maturational state of the preterm gut and diet, it still remains to be determined if 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, like PCR, 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 utilized volatile organic compounds (VOC). VOCs are carbon-based waste products, excreted in breath, sweat, urine and feces and are detected using 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 (30), including NEC. In a pilot study, four specific esters were absent in all samples up to four days prior to disease onset in stools from infants who developed NEC (N=6) (31). To improve turnaround time, de Meij et al developed a bedside fecal VOC profiling system based on gas sensors and pattern recognition algorithms (32). Fecal VOC profiles discriminated infants who developed NEC (N=13) from those who did not (N=14) 2–3 days prior to onset with 83% sensitivity and 75% specificity (AUC 0.77 ± 21) (32). Because a major source of fecal VOC is the intestinal microbiota, this non-invasive bedside tool offers the potential to identify shifts in microbial community composition and/or host response in real time.

Although utilization of the microbiome as a biomarker for NEC was first introduced a decade ago (33), 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 prior to disease onset offers 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 turnaround 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.

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Non-microbial biomarkers

The ultimate goal of any biomarker is early prediction of an outcome. Both intestinal-specific and non-specific biomarkers have been used in attempts to predict NEC. Among non-specific markers, cytokines have been a common target (Table I). Most studies have examined cytokine alterations at the time of clinical symptomatology identifying alterations in both pro and anti-inflammatory cytokines (34–37). One study of 997 extremely low birth weight infants prospectively collected blood samples from birth and correlated blood cytokine concentrations with development of NEC (38). 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 prior to 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 area under the curve (AUC) of 0.67 (38).

Intestinal fatty acid binding protein (I-FABP) has long been a candidate marker for NEC owing to its gut-specific characteristics. It is present in high concentrations within enterocytes, released into the circulation with injury, and then excreted into the urine (39). However, urinary I-FABP as a single predictive marker of NEC has not been successful. NEC risk identification with urinary I-FABP from one to two days before disease onset to as early as 96 hours of life has been attempted (40–43), 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 have 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 higher at the time of the first clinical signs in infants with proven NEC (N=14) than in those with other conditions (41). Among these, I-FABP alone was associated with NEC severity, a result also reported by other investigators (44–46). Isolated fecal calprotectin concentrations lack specificity for NEC diagnosis with significant overlap of cases with healthy controls (47, 48). Serum amyloid A, 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 (49). However, serum amyloid A has been reported to better differentiate NEC severity, particularly in combination with platelet counts (50). The proinflammatory cytokine IL-8 h also has been correlated with disease severity, discriminating between medical (N=63) and surgical (N=50) NEC (AUC 0.82), and was found to be correlated significantly with 60-day mortality (odds ratio 1.38, CI 1.14–1.67) (51), similar to previous reports for both NEC and sepsis-like syndromes (36). Other recent markers of disease severity have included the "LIT" score based on liver (L)-FABP, I-FABP, and trefoil factor 3 differentiating surgical from non-surgical NEC at the onset of disease (52); the "Totalis" score based on both clinical and serum markers (53); and the complement activation product C5a (54).

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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 (BA) are essential to intestinal fat absorption, but excessive accumulation can be injurious to the intestinal epithelium (55). Hulzebos et al demonstrated that the expected rate of fecal unconjugated BA declines over time and was slower in infants who developed NEC. Five to six days before NEC onset, fecal bile salt levels were significantly higher compared with age matched controls. The slower decay of fecal BS supports a potential role for altered BS metabolism or transport in NEC development (56). This difference in trajectory may be intrinsic to host phenotype, but is also linked to more modifiable factors including the gut microbiota, which has been previously shown to alter the expression of bile salt transporters (57). It may be that microbial dysbiosis may be influencing NEC risk through indirect mechanisms such as this, rather than solely through cell signaling or metabolic byproducts discussed above.

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 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 that give a theoretical functional status of the organism (58). Both proteomics and metabolomics exploit separation techniques prior to mass spectrometry analysis or nuclear magnetic resonance (NMR) for metabolites (59). Depending on the sampling site, metabolites are derived from the human host, diet, or microbiota, adding complexity to source attribution, as bacteria and people share many metabolic pathways (60).

To date, six studies on NEC have used proteomic techniques on blood, urine or buccal samples (61–66). Sampling was typically done at the time of diagnosis (62, 64–66), mainly for differentiation of NEC from sepsis (63, 64, 66) with two studies looking for identification of predictive biomarkers prior to onset (61, 63). Ng et al combined Proapolipoprotein CII and a des-arginine variant of serum amyloid A (SAA) to identify sepsis/NEC cases (62), but the ApoSAA score was a combination marker used only on suspected cases and did not differentiate between the two diseases. However, it did help identify infectious from noninfectious processes and reduced the utilization of antibiotics by 45% in symptomatic preterm infants. Of note, SAA also was found in the most recent proteomic study of serum samples from infants with sepsis and NEC at diagnosis, although inconsistently (63). A proteomic study of urine samples from a multicenter cohort by Sylvester et al (64) identified a panel of seven proteins that distinguished between sepsis (N=17) and NEC (N=85) (AUC 0.98) amedical versus surgical NEC (AUC 0.98). Among the proteins identified, several were within the inflammatory or coagulation cascade. The same group performed another study identifying three urinary coagulation cascade fibrinogen peptides that discriminated between medical and surgical NEC (AUC 0.86).

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When combined with clinical variables, these accurately predicted all infants requiring surgical intervention (65). Furthermore, they recently identified fibrinogen-γ dimers in plasma discriminated NEC (N=40) from sepsis (N=20), with an AUC 0.95. Factor XIII, responsible for fibrin cross-linking, was also significantly lower in NEC versus sepsis (66). The presence of fibrinogen-γ dimers in the urine and serum of infants with NEC is consistent with the prothrombotic coagulation processes that occur as NEC evolves, identifying both a novel potential biomarker and a therapeutic target.

In contrast to these studies of biomarker utility in established NEC, Murgas, Torrazza et al obtained buccal swabs from infants with NEC (N=10) 2–3 weeks before diagnosis and identified a trend (($p<0.08$)) toward a reduction in an IL-1 receptor-antagonist in cases compared with matched preterm controls (N=10) (61). Conversely, Stewart et al utilized serum from preterm infants and found no distinct proteomic profile discriminating NEC (N=6) fourteen (±7) days prior to disease onset (63).

Metabolomics is the study of low molecular weight metabolites found within biologic samples, reflecting the biologic processes that produced them. Similar to the proteomic studies, a variety of metabolites were studied in both urine and stool (13, 15, 63, 67). One limitation to the use of stool is that it is not produced daily in preterm infants, especially in the first weeks after birth, making urinary metabolomics (68) an enticing alternative to detect changes in the gut microbiome. The earliest metabolomics study of NEC by Morrow et al in 2013 did not study stool directly, but coupled stool microbial composition with matched urine samples, comparing 11 cases with 21 controls by NMR (13). They found a significantly higher ratio of alanine to histidine in urine samples from infants with NEC at 4–9 days of age compared with controls and this paralleled a gut microbial dysbiosis. The alanine to histidine ratio was significantly associated with NEC, with a predictive value of 78%, sensitivity of 82% and specificity of 75%. Wilcock et al used serum samples to identify changes in metabolites between seven NEC cases, five preterm and eight term control infants (67). They observed multiple metabolic changes between preterm and term infants and the metabolites were linked to up-regulation of IL-1β. In 2016, Stewart et al published two metabolomics studies on NEC, which evaluated serum and stool samples before NEC onset (15, 63). In the sera, no metabolites discriminated six NEC cases from matching controls (63). In the second analysis, stool from seven NEC cases and 28 matched controls at the day of diagnosis were compared (15). By using pathway analysis, they identified metabolites involved in several pathways including C21-steroid hormone biosynthesis, linoleate, and leukotriene metabolism, as well as prostaglandin formation from arachidonates. The study was expanded to additional time points before and after the onset of NEC and they detected a temporal profile of these metabolites that were significantly different in infants with NEC versus controls. These findings are particularly intriguing, in light of a previous report that showed changes in the gut microbiome of mice after they were exposed to dietary trans-10, cis-12-conjugated linoleic acid (69). A new approach utilized existing metabolomic data from newborn screening and identified multiple acylcarnitines that differed significantly in preterm infants who subsequently developed NEC (70). Because newborn screens are ubiquitous and performed shortly after birth, any sufficiently predictive marker associated with an increased risk of developing NEC would have

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significant diagnostic and prognostic appeal, especially in light of new pathophysiological insights for targeted prevention.

It is important to note that the metabolomic and proteomic studies of NEC reported thus far have often included modest numbers of cases and controls, requiring additional studies to confirm the potential of the identified markers. In addition, it is imperative that future studies maintain a consistent definition for NEC to ease the comparison of data between studies. Nevertheless, these studies offer new hypotheses on metabolic pathways and host-microbiota interactions that could play a role in NEC development. Interestingly, fatty acids have appeared as relevant metabolites in two of the four studies published so far (15, 67), as well as in the newborn screen study by Sylvester et al (70). Lipids are becoming increasingly recognized as playing an important role in gut inflammation and interacting with the microbiota (29, 71–74). Interventions focused on the addition of lipids as dietary supplements or modulating the host response in other gut inflammatory diseases, are especially promising and could potentially be useful in NEC (75–77).

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 above (64, 66). Kim et al 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 (CRP), matrix metalloproteinase-7 (MMp7), and epithelial cell adhesion molecule (EpCAM) (78). The ratio of MMp7/EpCAM 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 two diagnoses, which is not always clear without a laparotomy. Differentiation between the two 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 two 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 (79, 80). Shah et al used a more clinically applicable serum biomarker to distinguish NEC from SIP, called inter-alpha inhibitor proteins (IaIps) (81). 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 (81). An

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earlier study reported that IaIp concentrations were significantly decreased in infants with NEC compared with controls at the time of disease diagnosis (82). Using a prospective nested case control design, 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 ($p<0.0001$, 95% CI 0.84–0.99). However, in the week prior to disease onset, blood concentrations did not differ significantly between groups (81). 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 (83). The reason for this failure of transition into practice is multifactorial, relating to both biomarker and study design (83, 84). A similar pattern of limited success and many failures has emerged in NEC. Sample sizes are often small and diagnostic accuracy is frequently fair using 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.

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Abbreviations

NEC	necrotizing enterocolitis
SIP	spontaneous intestinal perforation
ROC	receiver operator characteristic
AUC	area under the curve
16S rRNA	16S ribosomal RNA
NMR	nuclear magnetic resonance
CRP	C-reactive protein
IL	interleukin
MMp7	matrix metalloproteinase-7
EpCAM	epithelial cell adhesion molecule

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IL-1RA	interleukin1 receptor antagonist
TNFα	tumor necrosis factor alpha
TGF-β	transforming growth factor beta
PAF	platelet-activating factor
I-FABP	intestinal fatty acid binding protein
L-FABP	liver fatty acid binding protein
SAA	serum amyloid A
TTF	trefoil factor 3
"LIT score"	liver (L)-FABP I-FABPand trefoil factor 3
VOC	volatile organic compounds.

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Table 1

Comparison of human biomarkers in literature before and after the Human Microbiome Project

Proposed Mechanism	BEFORE	AFTER
Acute phase reactants/inflammation	CRP (85, 86)	CRP (78, 87–89) IaIps (81)
	Cytokines: IL-2, IL-6, IL-8, IL-1β, TNFα IL-1RA, IL-4, IL-10 (34, 36, 37, 90)	Cytokines: IL-8, TGF-β IL-1RA, IL-1β (38, 51, 61, 67, 91)
	PAF (92, 93)	
Tissue injury/protection	Calprotectin (47)	Calprotectin (94–97)
	I-FABP (44, 98, 99) Serum SAA and apolipoprotein-CII (ApoSAA score) (62) Urine I-FABP, claudin 3, and fecal calprotectin (41)	Combination markers: Urine IFABP, SAA and fecal calprotectin (49) L-FABP, I-FABP, and TFF3 (LIT score) (52) EpCAM/MMp7 ratio (78) Urine protein panel (64, 65) Serum protein panel (63)
	Amino acids arginine and glutamine (100–103)	Non-protein amino acid citrulline (104, 105) Heat shock, angiogenesis, cytoskeleton, metabolism proteins (106)
Lipid metabolism/signaling		C21 steroid, linoleate, leukotriene and prostaglandin metabolism (15) Acylcarnitines (70)
Coagulation/Vascular injury		Fibrinogen-γ dimers (64–66)
Intestinal microbiota	Theoretical (11, 33)	Community structure, reviewed in (107, 108) Metabolic signature Alanine:histidine ratio (13) Volatile organic compounds (31, 32)

Abbreviations: CRP C-reactive protein; IL interleukin; MMp7 matrix metalloproteinase-7; EpCAM epithelial cell adhesion molecule; IaIp inter-alpha inhibitor proteins; IL-1RA interleukin1 receptor antagonist; TNFα tumor necrosis factor; TGF-β transforming growth factor beta; PAF platelet-activating factor; I-FABP intestinal fatty acid binding protein; L-FABP liver fatty acid binding protein; SAA serum amyloid A; TTF trefoil factor 3.

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Table 2

Microbiome and Biomarker Technology

Biomarkers can be derived from presence of specific bacteria or changes in the microbial composition

Technology	*Description*	*Advantages*	*Disadvantages*	*Reviewed Publications*
16S	Sequencing of variable region of ribosomal 16S gene	Provides information on changes in bacterial composition	• Requires some computational capability for interpretation • Can often only identify at the family/ genus level	(*1–8*)
Whole Genome Shotgun	Sequencing of all fragmented bacterial DNA	• Provides information on functional potential and presence of mobile elements • Species level identification • Enhanced detection of bacterial species	Higher computational and sequencing cost than 16S	(*9*)
Proteomics				
Identified protein biomarkers can often be adapted to other more cost efficient detection methods such as ELISA				
ProteinChip	Digested proteins are fingerprinted by MALDI-TOF and compared to protein database	Fast acquisition and identification	Confidence levels are lower	(*10*)
Mass spectrometry	• Digested proteins can be separated by chromatography prior to fragmentation and MS spectra acquisition • Spectra are compared to databases for identification	High sensitivity	Longer acquisition and higher cost	(*11–15*)
Metabolomics				
Identified metabolites can by from host or microbial origin and can help elucidate the role of both in disease development				
NMR	Metabolites in liquid are subjected to ^{1}H nuclear magnetic resonance and resulting spectra are compared to database	• Clear biologics such as serum or urine can be directly assayed • High reproducibility	• Not sensitive and requires large sample volumes • Number of compounds resolved is lower	(*16*)
Mass Spectrometry	• Extracted metabolites are separated by LC and fragmented through collision for MS spectra acquisition • Spectra are compared to databases for identification	Low volume required and very sensitive	• High cost and time consuming • Difficult to interpret in absence of internal standards	(*6, 14, 17*)

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Table 3

Studies using 16S rRNA or metagenomic sequencing to examine relationship of gut microbial community of preterm infants and development of NEC.

Study	Sequencing technology	Number of subjects/ samples without NEC	Number of subjects/ samples with NEC	Comments
Mai, et al. ([1])	16S rRNA gene sequencing	9/18	9/18	Case stools demonstrated an increase in *Proteobacteria*, and a decrease in *Firmicutes* in the week before NEC.
McMurtry, et al. ([2])	16S rRNA gene sequencing	74/74	21/21	Bacterial diversity and relative abundance of Clostridia was significantly lower in NEC specimens compared to controls.
Raveh-Sadka, et al. ([3])	Metagenomic sequencing	5/34	5/21	No clear association between bacterial content as identified by metagenomics and outcome.
Heida, et al. ([4])	16S rRNA gene sequencing of meconium and subsequent stools	22/57	11/30	*Clostridium perfringens* and *Bacteroides dorei* associated with NEC risk, and *Staphylococci* associated with protection.
Torrazza, et al. ([5])	16S rRNA gene sequencing	35/77	18/40	*Klebsiella pneumoniae* during week 1 associated with subsequent development of NEC.
Ward, et al. ([6])	Metagenomic sequencing	89/185	27/60	Specific sequence types of *E. coli* associated with NEC.
Zhou, et al. ([7])	16S rRNA gene sequencing	26/111	10/88	NEC having an association with Clostridia and Gammaproteobacteria, respectively.
Sim, et al. ([8])	16S rRNA gene sequencing	44/369	22/88	*Klebsiella*, *Clostridium* associated with NEC risk.
Stewart, et al. ([9])	16S rRNA gene sequencing	28/520	7/121	*Klebsiella, Escherichia, Staphylococcus* and *Enterococcus* present in all samples, without uniform microbial signature for NEC.
Warner, et al. ([10])	16S rRNA gene sequencing	120/2720	46/866	Gammaproteobacteria associated with risk, and Negativicutes associated with protection; lack of diversity is associated with risk.

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