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

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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).

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).[55-60] 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 Walker[46] 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,[50,59,61] 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.[43,63] 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

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

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Funding by the National Institutes of Health (UH3AI083265 [to B.W.]); Pediatric Gastroenterology Research T32 DK077653-24 and Advanced Postdoc Mobility Fellowship from the Swiss National Science Foundation (P300PA_171556 [to B.R.]); March of Dimes Foundation 5-FY17-79, K08DK101608, and R03DK111473 from the National Institutes of Health (to M.G.); and Children's Discovery Institute of Washington University and the St Louis Children's Hospital (to B.W., M.G., and B.R.). The authors declare no conflicts of interest.

http://dx.doi.org10.1016/j.jpeds.2017.05.075

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

Proposed mechanisms	Before	After
Acute-phase reactants/ inflammation	CRP[3,4]	CRP[5-8]
		Ialps[9]
	Cytokines: IL-2, IL-6, IL-8, IL-1β, TNF-α, IL-1RA, IL-4, IL-10[10-13]	Cytokines: IL-8, TGF-β, IL-1RA, IL-1β[14-18]
	PAF[19,20]	
Tissue injury/protection	Calprotectin[21]	Calprotectin[22-25]
	I-FABP[26-28]	Combination markers:
	Serum SAA and apolipoprotein-CII (apolipoprotein SAA score)[29]	Urine IFABP, SAA, and fecal calprotectin[31]
	Urine I-FABP, claudin 3, and fecal calprotectin[30]	L-FABP, I-FABP, and TTF3 (LIT score)[32]
		EpCAM/MMp7 ratio[5]
		Urine protein panel[33,34]
		Serum protein panel[35]
	Amino acids arginine and glutamine[36-39]	Nonprotein amino acid citrulline[40,41]
		Heat shock, angiogenesis, cytoskeleton, metabolism proteins[42]
Lipid metabolism/signaling		C21 steroid, linoleate, leukotriene, and prostaglandin metabolism[43]
		Acylcarnitines[44]
Coagulation/vascular injury		Fibrinogen-γ dimers[33,34,45]
Intestinal microbiota	Theoretical[46,47]	Community structure, reviewed in Neu and Pammi[48] and Warner and Tarr[49]
		Metabolic signature alanine:histidine ratio[50]
		Volatile organic compounds[51,52]

CRP, C- reactive protein; *EpCAM*, epithelial cell adhesion molecule; *IL-1RA*, interleukin1 receptor antagonist; *L-FABP*, liver fatty acid binding protein; *MMp7*, matrix metalloproteinase-7; *PAF*, platelet-activating factor; *TNF-α*, tumor necrosis factor-α; *TTF3*, trefoil factor 3.

ribosomal RNA subunit or WGS sequencing on stool demonstrate a relative increase in Gram-negative bacteria (class Gammaproteobacteria) before the onset of NEC[43,54,58-61,63] (**Table III**; available at www.jpeds.com) and an associated decrease in anaerobes (classes Clostridia and Negativicutes).[60-63] 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 al[54] 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 turnaround time, de Meij et al[52] 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).[52] 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.