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Journal

Seminars in Perinatology, 47(1)

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Publication Date

2023-02-01

DOI

10.1016/j.semperi.2022.151693

Peer reviewed

HHS Public Access

Author manuscript

Semin Perinatol. Author manuscript; available in PMC 2024 February 01.

Published in final edited form as:

Semin Perinatol. 2023 February ; 47(1): 151693. doi:10.1016/j.semperi.2022.151693.

Biomarkers of Necrotizing enterocolitis in the Era of Machine Learning and Omics

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Abstract

Necrotizing enterocolitis (NEC) continues to be a major cause of morbidity and mortality in preterm infants. Despite decades of research in NEC, no reliable biomarkers can accurately diagnose NEC or predict patient prognosis. The recent emergence of multi-omics could potentially shift NEC biomarker discovery, particularly when evaluated using systems biology techniques. Furthermore, the use of machine learning and artificial intelligence in analyzing this 'big data' could enable novel interpretations of NEC subtypes, disease progression, and potential therapeutic targets, allowing for integration with personalized medicine approaches. In this review, we evaluate studies using omics technologies and machine learning in the diagnosis of NEC. Future implications and challenges inherent to the field are also discussed.

Introduction

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal pathology affecting approximately 7% of very low birth weight (VLBW) infants[1]. Despite decades of research, morbidity and mortality are unacceptably high[2], due, in part, to a lack of understanding of disease pathogenesis. In addition, identification of biomarkers to aid in early diagnosis and treatment, thus far, has not been successful. Clinically, diagnosis of NEC is challenging. Early signs and symptoms are non-specific and difficult to distinguish from those of similar pathologies[2, 3]. Moreover, early radiologic features and pneumatosis intestinalis

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Disclosure:
The authors report no conflicts of interest.

are neither sensitive nor specific in diagnosing NEC[10,11.] Thus, a specific biomarker or panel of biomarkers capable of identifying infants with early NEC is critical in improving patient outcomes[4, 5]. The recent introduction of high-throughput multi-omics technologies could potentially shift NEC biomarker discovery into a new era[6]. Multiple informational layers can now be explored using molecular platforms measuring and characterizing the behavior of both diseased and healthy cells or tissue, potentially leading to discovery of predictive, diagnostic, or prognostic biomarkers of NEC[7]. Unfortunately, methodological inconsistencies among studies, disagreement on conventions for data reporting and analysis, and the sheer magnitude of data produced are significant drawbacks to these studies. To overcome these challenges, machine learning and artificial intelligence could be exploited to integrate omics data with NEC clinical features, phenotypes of progression, and predicted therapeutic targets, resulting in clinically meaningful information. In this review, NEC studies utilizing omics technologies and machine learning are evaluated. Future directions and challenges facing clinical implementation of this new information are also discussed.

Genomics and Single Nucleotide Polymorphisms (SNPs)

The human genome plays a critical role in cellular programming and tissue architecture, supplying virtually all instructions needed for organismsal function and survival. Variations in the genome, the totality of genes and their interactions within an organism, among individuals has been implicated in several disease etiologies, including cancer, autoimmune diseases, and inflammatory conditions[8–10]. Increasingly, evidence suggests premature infant susceptibility to specific diseases may also, in part, be dictated by genomic variants. In particular, single nucleotide polymorphisms (SNPs) have been implicated in the pathogenesis of NEC[11], either individually through *a priori* hypothesis or significantly associated with disease development as a cluster via genome-wide association studies (GWAS).

SNPs are naturally occurring nucleotide substitutions appearing approximately every 100 to 300 base pairs and are, by far, the most common type of genomic variant[12]. The majority of SNPs have not been associated with disease pathogenesis, but some appear to increase susceptibility to, or severity of, certain diseases. For example, the rs17810546 SNP alters the role of interleukin-12 A (IL-12A) in T-cell regulation and has been associated with increased susceptibility to celiac disease (CD)[13, 14]. In NEC, SNPs in tripartite motif containing-21 (TRIM21), a receptor for antigen-antibody comples, and inflammatory cytokines from 184 premature (< 32 weeks) infants were assessed for association with incidence and outcomes. An IL-6 SNP (rs1800795) was associated with increased incidence and severity of NEC. The transforming growth factor beta-1 (TGFβ-1) SNP, rs2241712, resulted in a reduction in NEC-associated intestinal perforation but increased mortality, while the TRIM21 SNP, rs660, was also associated with increased incidence of perforation[15]. Furthermore, a single-center study evaluated the potential relationship between SNPs of dual specificity phosphatases (DUSPs), genes suppressing the proinflammatory mitogen-activated protein kinase (MAPK) pathway, and susceptibility to NEC. Thirty-one SNPs in 9 DUSP genes were examined, and infants with the rs704074 variant were at significantly lower risk of NEC development, including surgical NEC[16]. Together, these preliminary studies suggest a patient's genomics may dictate their disease susceptibility and clinical course in NEC.

Additional candidate genes have been investigated for biologically relevant SNPs in the development of NEC, including single immunoglobulin and toll-interleukin 1 receptor (SIGIRR), mannose-binding lectin (MBL), Autophagy-related 16-Like 1 (ATG16L1), and vascular endothelial growth factor (VEGF).

Single Immunoglobulin and Toll-Interleukin 1 Receptor

SIGIRR is an inhibitor of proinflammatory toll-like receptor (TLR) signaling[17], an important initiator of Gram-negative-associated inflammation in NEC infants[18]. In preterm NEC infants, SIGIRR variants are enriched compared with controls, and these variants are functional, resulting in excessive intestinal inflammation[19]. For example, transgenic mice that express a stop variant of SIGIRR, p.Y168X, suffer from increased intestinal inflammation through a mechanism involving signal transducer and activator of transcription 3 (STAT3)-dependent microRNA expression, an important example of host genetics regulating intestinal adaptation to luminal microbes or antigens[20].

Mannose-Binding Lectin

MBL is an acute phase reactant with an integral role in complement activation and innate immunity[21]. Upon exposure to the repeating polysaccharide pattern of specific microorganism surfaces, MBL either initiates the complement cascade or directly opsonizes the microorganisms[22]. SNPs in MBL contributing to low or absent levels of the protein have been associated with both neonatal sepsis and pneumonia[22], as well as infant prematurity[23]. Prencipe *et al.* analyzed genotypes correlated with high serum MBL levels: infants with gain-of-function variants resulting in high serum MBL levels were characterized by a higher incidence and severity of NEC[24], corroborating high levels of MBL in the intestine of preterm NEC infants[25]. However, Dogan *et al.* demonstrated the reverse relationship[26], while Koroglu *et al.* found no correlation between MBL genotype and NEC incidence or mortality[27]. Together, these studies suggest that MBL both facilitates bacterial destruction and promotes pathologic host immune responses, however, the counterregulatory measures of MBL activity remain poorly studied in the setting of NEC.

Autophagy-related 16-Like 1 (ATG16L1)

Induction of autophagy is associated with worse injury in experimental NEC, while diminished autophagy decreases NEC.[38] Sampath et al.[14] evaluated the key autophagy gene, ATG16L1, and found that a common ATG16L1 variant (Thr300Ala) conferred protection against NEC. This variant, which increases sensitization of ATG16L1 to caspase-3-mediated degradation, results in diminished autophagy and is thus consistent with prior studies showing protective role of deceased autophagy against NEC. An independent replication cohort (260 infants, 23 with NEC) also showed a trend towards decreased NEC among infants who were homozygous for the loss of function variant allele, providing further evidence for the importance of this gene in NEC.[14] Further studies are needed to evaluate the role of this and other autophagy-related genes in NEC.

Vascular Endothelial Growth Factor

Though the pathogenesis of NEC is incompletely understood, evidence suggests vascular compromise and and necrosis are critical events[28]. Mesenteric vessels are of smaller caliber and are more vulnerable in infants compared with adults, and arterioles in NEC infants are significantly smaller than those of infants without-NEC[29, 30]. Levels of VEGF, important in angiogenesis and vasculogenesis, are low in NEC infants[28]. A VEGF SNP (VEGF-2578) resulting in low VEGF levels increases susceptibility to NEC[31], while two variants of VEGFA (rs699947 and rs833061), a heparin-binding gene critical in angiogenesis, increase the risk for NEC[32]. These studies highlight the importantance of angiogenesis, likely via growth factors such as VEGF, and their protective role in NEC.

Transcriptomics

Transcriptomics is a relatively young field, with most large-scale transcriptomic data collected in just the past decade[6, 33, 34]. Recently, large-scale RNA sequencing (RNA-seq) of human intestinal tissue has provided important descriptions of differentially expressed genes (DEGs) in NEC, showing some similarity to those expressed in certain chronic inflammatory conditions, such as Crohn's disease[35]. While obtaining tissue samples of premature infants with NEC is not always feasible, transcriptomic analyses of experimental murine and porcine NEC models have also yielded important information on disease pathogenesis. Transcriptomic analysis of formula-fed compared with maternal-fed murine pup intestines revealed decreased transcription of structural integrity genes, including vinculin and desmin, with increased transcription of host defense and immunity genes[36], a pattern present in transcriptomic studies of formula-fed and breastfed human infants[37]. In porcine subclinical NEC models, transcriptomics has indicated downregulation of genes related to innate and adaptive immunity, rendering piglets immunosuppressed and susceptible to conditions such as sepsis[38].

Several miRNAs have been identified and correlated to specific cellular disease processes, such as apoptosis, proliferation, or differentiation, and are now considered biomarkers. Recently, multiple miRNAs have been suggested as potential biomarkers for NEC. Specifically, intestinal miRNA-429/200a/b and miRNA-141/200c clusters have been demonstrated to be poorly expressed in NEC, resulting in high expression of these miRNA target genes, including VEGFA, E-selectin (SELE), kinase insert domain receptor (KDR), fms-like tyrosine kinase 1 (FLT1), and hepatocyte growth factor (HGF)[39]. miRNAs are also being considered for potential therapeutic modalities in NEC prevention since they can exert highly targeted effects on downstream protein translation. In a rat NEC model, pups were injected with miR-21, previously found to play an important role in apoptosis inhibition[40]. Compared with control, pups treated with miRNA-21 suffered reduced necrosis of the small bowel, demonstrating miR-21 could be protective against small intestinal NEC pathology[41].

Further transcriptional regulation is provided by circRNAs and lncRNAs, with the former often functioning similarly to that of miRNA. CircRNAs are long-lived, single-stranded RNAs with a variety of regulatory roles in transcription and protein intereaction[42]. In neonatal rat pups, NEC induction resulted in 9 upregulated and 44 downregulated circRNAs compared to control, with several of these circRNAs now implicated in development or

progression of NEC through their interactions with miRNA[43]. LncRNAs are noncoding sequences of at least 200 nucleotides interacting with mRNA to regulate gene expression.[44] When comparing lncRNA expression in NEC lesions with that of healthy surgical margins, several lncRNA DEGs were associated with the inflammatory response, and lncRNA-mRNA network interaction analysis indicated several of the identified lncRNAs may regulate pathogenesis of NEC[45]. The complex interactions between miRNA, circRNA, and lncRNA culminate in the proteome, which is the next level of regulation and possible aberrancies involved in NEC pathogenesis (further described in the next section). Although transcriptional regulation of genes through expression of miRNA, circRNA, and lncRNA in NEC is a fairly new area of study, preliminary reports are promising for biomarker discovery but will require further validation in clinical and animal studies.

Proteomics

Proteomic interrogation of pathways involved in NEC allows for high-throughput discovery of proteins for use as potential drug targets or biomarkers[46]. Recent studies have attempted to identify NEC biomarkers from proteomic analysis of animal model tissues, or when available, from surgically resected intestinal tissue, plasma or serum, stool, and buccal mucosal swabs. Jiang *et al.* compared proteomic signatures of intestinal skip lesions with adjacent healthy margins, and identified 30 small intestinal and 23 colonic proteins with differential expression. Heat shock proteins (HSPs) were a unique discovery, and the proteomic signatures between small intestine and colon indicated differential NEC progression in the two tissues[47]. Chatziioannou *et al.* attempted to distinguish between late-onset sepsis (LOS) and NEC, conditions with early, overlapping clinical signs, using serum proteomics. A panel of proteins consisting of LCAT (lecithin-cholesterol acyltransferase), APOA4 (apolipoprotein A4), and APOC1 (apolipoprotein C1) showed high diagnostic potential in distinguishing between LOS and NEC, with a receiver operating characteristic (ROC) area under the curve (AUC) of nearly one[48]. A second, case-controlled study compared proteomic signatures of LOS and NEC preterm infants, finding 8 proteins (C-reactive protein, macrophage migration inhibitory factor, TGF-β-induced protein ig-h3, *etc.*) associated with NEC and 4 proteins (haptoglobin, transthyretin, *etc.*) associated with LOS[49].

A multicenter prospective study analyzed urine samples in infants with suspected NEC or sepsis versus controls. Urine was collected at the time of disease suspicion while the definitive diagnosis of NEC could not yet be confirmed. A panel of 7 biomarkers (alpha-2-macroglobulin-like protein 1, cluster of differentiation protein 14, cystatin 3, fibrinogen alpha chain, pigment epithelium-derived factor, retinol binding protein 4, and vasolin) was found to correlate with presence or severity.[50] Recently, there has been increased interest in stool proteomics, specifically calprotectin, as a diagnostic marker for NEC. Calprotectin, an antimicrobial protein excreted into the intestinal lumen by neutrophils during inflammation, is a known and sensitive marker for the differentiation of inflammatory bowel disease (IBD) from irritable bowel disease (IBS)[51]. Qu *et al.* performed a meta-analysis to evaluate the potential of fecal calprotectin as a biomarker for NEC. With the inclusion of 10 studies in the analysis, fecal calprotectin levels were significantly associated with NEC diagnosis, especially in preterm infants[52].

Lastly, Murgas Torrazza *et al.* analyzed buccal swabs of premature (< 32 weeks), very low birth weight infants (< 1,250g) and age- and birth weight-matched controls at 2 and 3 weeks postnatally. Twenty-one proteins were found to be altered in association with NEC, with functions ranging from inflammation to metabolism. Three (IL-1RA [IL-1 receptor antagonist], Prdx1 [peroxiredoxin-1], and A1AT [isoform 1 of α-antitrypsin]) of the 21 proteins were further characterized by western blot in an attempt to determine any temporal relationship with NEC development. IL-1RA was decreased in NEC patients 2–3 weeks before the development of NEC, while A1AT was significantly lower 1 week before diagnosis. No significant temporal correlation to NEC diagnosis was elucidated with Prdx1[53]. In summary, studies suggest a single biomarker is unlikely to distinguish NEC from either LOS or controls, but a panel of proteins holds greater promise.

Metabolomics at the Host-Microbiota Interface

Metabolomics refers to the study of all metabolic substrates and byproducts of cellular processes, largely low-molecular-weight products of protein expression[54]. While transcriptomics and proteomics capture transcription and translation of active genes, the complementary study of metabolomics goes a step beyond by characterizing the cellular processes actively occurring in that tissue[54, 55]. Evaluation of the metabolome is essential as it reveals differential biochemical processes occurring in healthy compared with diseased tissues, and recent data suggests favorable manipulation of the metabolic profile of a diseased tissue can have profound implications on host immune response and inflammation[55]. Metabolomics is also useful at the luminal interface of host gut tissue and intestinal flora, where the interaction can provide a glimpse into bacterial biochemical processes and, thus, an additional readout of NEC-associated microbiome alterations. Similar to proteomics, metabolomics analyzes a variety of samples with either nuclear magnetic resonance (NMR) or mass spectrometry[56].

Recent studies have been conducted utilizing metabolomics to identify individual or panels of biomarkers for diagnosis or progression of NEC. Wilcock *et al.* evaluated serum samples from preterm infants, both early postnatally and once fully fed. Sixteen metabolites differed comparing fully fed samples between NEC and control infants, 7 of which were related to IL-1β upregulation. However, a relatively small sample size precluded the authors from confidently suggesting metabolites as biomarkers[57]. Another study utilizing blood was conducted by Sinclair *et al.* Preterm infant dried blood spot samples from day of life (DOL) 1, 7, 28, and 42 were analyzed from circulating metabolites. As early as DOL 1, alanine, phenylalanine, free carnitine, C16, arginine, and the ratios of C14:1/C16 and citrulline/phenylalanine were correlated with NEC development[58]. In a pilot study, Picaud *et al.* examined urine NMR profiles of preterm infants and controls over the first 2 months of life. While most metabolites correlated with postnatal age, suberate and lactate correlated with age only in preterm NEC infants or controls with significant food intolerance. In addition, myo-inositol was significantly lower in the urine of late-onset NEC infants and babies with significant feeding intolerance, but not infants with early-onset NEC[59]. An additional pilot study of untargeted NMR and targeted liquid chromatography-tandem mass spectrometry discovered 25 amino and organic acid, carbohydrate, and vitamin metabolites in association with NEC. While no single metabolite was diagnostic, metabolite combinations were highly

correlated to the development of the disease[60]. Finally, a recent study compared broad range metabolomics of stool samples from premature infants with NEC and age-matched controls. Metabolites of the pregnenolone, carnitine, and sphingolipid pathways were overrepresented in network analyses comparing the two infant groups. Machine learning was only able to provide 73% accuracy when using these potential biomarkers to predict NEC development, but hierarchical clustering identified a potentially distinct subset of NEC cases[61]. These studies serve as examples for the promise of using metabolites for diagnostic benefit in identifying patients susceptible to NEC.

While NEC pathogenesis is highly multifactorial, an accepted component of the etiology is microbiome alterations leading to intestinal dysbiosis. Studying alterations to the microbiome in conjunction with metabolomics can provide further insight into the pathogenesis and progression of NEC. A case-control cohort study of stool samples from children with suspected mild NEC were evaluated for differences in the microbiome and metabolome. Between 10 and 20 DOL, NEC infants had a higher abundance of *Streptococcus*, and between 20 and 30 DOL, a higher abundance of *Staphylococcus*. The metabolome in the second month of life differed the most with control, characterized by increased glycosaminoglycan breakdown, lysosome activity, and seleno-compound and thiamine metabolism[62]. In a separate, multicenter study, 64 NEC and 81 preterm infant control stool samples were analyzed for microbial colonization and metabolomics influenced by the host-microbiota interface. NEC samples were characterized by a loss of biodiversity, primarily from commensal or beneficial taxa such as *Bifidobacterium* and *Akkermansia*. *Escherichia coli* and *Enterococcus faecalis* were the predominant taxa in NEC infants when compared with control. Interestingly, *Clostridium* species were found in abundance in NEC infants when its presence in healthy neonates is rarely noted. Furthermore, there was an overall increase in the metabolite – lactate. High lactate is likely a result of increased lactate dehydrogenase (LDH) in preterm infants who developed NEC compared to controls, suggesting the possibility of lactate as an early biomarker for NEC[63].

Machine Learning in the Healthcare Setting

Machine learning (ML) has been a component of the computer science and artificial intelligence (AI) realm since 1959[64]. Arthur Samuel officially coined the term ML to refer to the program developed to play checkers without explicitly programming the move set, but instead using AI[64]. In the 1980's, the idea that ML may be capable of improving healthcare by recognizing disease patterns was proposed, although the computational power and algorithms available at the time were insufficient to do so effectively[64, 65]. Within the last five to ten years, substantial evolution of computer technology and ML capabilities has occurred, allowing for ML prediction of diseases such as cancer or sepsis via biomarker analysis, optimization of features, and improvement of treatment and management strategies[64, 65]. While ML and AI are currently utilized for common diseases, recent studies worked to adapt these technologies for use with rarer diseases, such as NEC[3, 65–72], specifically for better diagnosis and predicted development[72–75]. The two major classes of ML classifiers are supervised (inductive) and unsupervised[64, 65]. Supervised ML involves utilizing training data (a subset of ~80% of the total data pool) with known outputs/labels of interest into an algorithm[64, 65]. The algorithm then creates a model making

predictions on desired outputs based on training data. This model is subsequently corrected based on the true outcome of those predictions[64, 65]. The tailored model is then evaluated using the novel test set data (other 20% of the data from the total pool) to determine the accuracy, sensitivity, and specificity of the model[64, 65]. Supervised ML is particularly useful for classification or regression, with ideal applications in disease prognosis or treatment outcome modeling, where features of electronic medical records (EMRs) can be used for additional inputs[64, 65]. On the other hand, unsupervised ML involves providing unlabeled data to the algorithm and allowing it to find patterns or structures that are not apparent to the human eye[64, 65]. Unsupervised ML can be further subdivided into associations, where co-occurrences are identified between two different diseases, clustering, grouping of similar features (*e.g.*, sick versus healthy), or dimensionality reduction, data transformation through feature optimization[64, 65]. Unsupervised ML can be used to optimize features used for supervised ML inputs[64, 65]. Additionally, deep learning, with both supervised and unsupervised aspects, has recently become a popular way to perform ML on extremely large datasets, such as omics[64, 65]. In supervised ML, deep learning can be used to classify images, such as radiographs, which are too complex for standard ML models to process. In unsupervised ML, deep learning can be used to lower image dimensionality in order to simplify their use in standard ML models.

Integrated Use of EMRs and Omics Data for Early Diagnosis and Prognosis of Disease

EMRs are valuable for use in ML because they provide readily available information, easily converted ino ML inputs, that is easily processed by ML algorithms. Studies in adult and neonatal sepsis have demonstrated ML models can use EMR data to predict sepsis before clinical onset, identify patients experiencing future shock or poor outcomes, and exceed the sensitivity and specificity of clinicians in identifying the disease[76–78]. Using EMR data, Ji *et al.* published two ML models with differing outputs[79]. One model classified NEC versus non-NEC, while the other model determined low, intermediate, or high NEC severity[79]. Together, these two models could identify patients with the highest risk for severe NEC and guide clinical and surgical management of the disease[79]. Irles *et al.* and Lure *et al.* conducted retrospective studies using ML algorithms to distinguish between NEC and spontaneous intestinal perforation (SIP)[80], while Lueschow *et al.* published a retrospective analysis using different machine learning algorithms to evaluate current definitions of NEC[74] using a cohort of patients with NEC, SIP, or general gastrointestinal concern[73]. From these studies, subsets of features from EMRs were identified that were important for the ML model decision-making process, helping to guide future ML model or NEC definition development[73, 80]. In addition to EMRs, omics data inputs can improve both the performance of ML models and precision medicine techniques[81]. For example, Lin *et al.* used ML to evaluate longitudinal stool microbiota samples from preterm infants at risk for NEC, providing predictions of NEC development approximately 8 days before actual onset[82].

However, as with any technology, investigators and clinicians should be aware of potential pitfalls and limitations when using EMRs and omics data as ML inputs. While EMR

data contains a wealth of information, there are often temporal gaps in the data. ML models experience difficulty in navigating data gaps, requiring biostatisticians and clinicians to work together on the best way to address existing shortcomings. Due to differences among provider and hospital care protocols, the strategies and information provided by EMRs may vary and carry a level of subjectivity, limiting the generalizability of ML models to other sites. The addition of both EMR and omics data together into ML models substantially increases the complexity of the required model, resulting in a more difficult model to interpret clinically[66]. Additionally, ML models can develop biases which may not be obvious when they are overly complex[66, 83]. Further, specific to use with NEC, the lack of a standardized and universal diagnostic definition limits the predictive potential of these models, resulting in variable outputs depending on the model definition of NEC[72, 73, 84, 85]. While these potential limitations are noteworthy, collaboration among researchers, biostatisticians, and clinicians can ensure any limitations are overcome, and effective and generalizable ML models are developed. Future studies on the use of ML with omics data will facilitate an improved understanding of NEC pathogenesis and potential disease-modifying interventions.

Conclusions and Future Directions

The intersection of large-scale omics data and bioinformatic processing holds great promise for NEC biomarker discovery. In addition to predicting NEC susceptibility, these biomarkers could guide disease stratification, distinguish NEC from disease with similar early symptomatology, and predict response to treatment. As the field progresses, several factors will be important in the utility and global application of omics data. The small number of multi-omics datasets in the field of NEC, lack of standardized protocols, and disagreements on NEC diagnostic definitions will hinder broad dissemination of these approaches. To overcome these limitations, establishing an open-access biorepository of both healthy and NEC samples for data analysis, ideally collected in a standardized fashion and iteratively mined as more sophisticated and standardized libraries are developed, is essential[86]. Integration of multi omics with clinical data could provide a more precise overview of the biological processes underlying NEC susceptibility, development, and progression. Finally, an imperative need exists for validating bioinformatic data on a biological level using traditional molecular analysis of RNA, protein, and metabolite expression with animal models and patient samples. However, advances in transcriptomics, proteomics, and metabolomics, as well as machine learning and artificial intelligence, continue to pave the way for discovery of novel NEC biomarkers.

Acknowledgments

From the University of Oklahoma Health Sciences Center, University of Iowa, and University of California Davis, supported in part by P20GM134973 (H.C.), Presbyterian Health Foundation (PHF) grant (K.B.), DK125415 (NIDDK, S.M.).

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