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Recent advances in our understanding of NEC pathogenesis, diagnosis, and treatment

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Recent advances in our understanding of NEC pathogenesis, diagnosis, and treatment

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Editorial: Recent advances in our understanding of NEC pathogenesis, diagnosis, and treatment

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Editorial on the Research Topic
Recent advances in our understanding of NEC pathogenesis, diagnosis, and treatment

Necrotizing enterocolitis is a leading cause of death among premature infants, and despite research spanning over six decades, the pathogenesis is still not completely understood. The onset of NEC can be rapid and unpredictable, with clinical signs such as abdominal distension and bloody stools, progressing to fulminant bowel necrosis and death within hours. Even though the clinical and pathological descriptions of NEC were first described many decades ago, the management options have not progressed significantly and continue to be supportive care, such as cessation of feedings, intravenous fluids, antibiotic administration, and, in some cases, surgical bowel resection. Although treatment options for NEC remain limited, one effective preventative strategy is the administration of human milk. Recent advances in identifying the precise nutrients in human milk shed light on its bioactive components and their impact on the intestine. In recent years, several studies have highlighted the benefit of using prebiotics and probiotics as additional preventative options for NEC. Clinical studies focused on diagnostic tools such as using serum biomarkers or big data and artificial intelligence may pave the way for earlier detection to minimize disease progression, avoid the negative impact on other organ systems, and improve the poor neurodevelopmental outcomes associated with NEC. The primary objectives for this topic were to focus on recent advances in our understanding of NEC pathogenesis, new diagnostic strategies such as biomarkers and artificial intelligence, and explore new therapeutic options for treating this devastating disease.

This editorial highlights recent developments in the underlying pathogenesis of NEC, including the use of animal models, bench-to-bedside approaches, and machine-learning approaches for diagnostic purposes. This series of publications comprises state-of-the-art reviews, meta-analyses, and original research.

Singh et al. discuss bench-to-bedside approaches to understanding NEC pathogenesis, including a summary of the immunological status of infants with NEC and several defense mechanisms that become impaired in prematurity and NEC. The article by Klinke et al. describes the function of neutrophil extracellular traps (NETs) in necrotizing enterocolitis. NETs are released by neutrophils after contact with pathogens, and studies

have shown that NET release is seen in mice and infants with NEC. This review discusses the various roles that NETs play in NEC, and specifically, that excessive NET formation can lead to hyperinflammation, contributing to disease pathogenesis. The manuscript by Bautista et al. is a state-of-the-art review describing the *in vivo* models of NEC. This review focuses on the descriptions of the different animal models, the phenotypic considerations, the strengths and weaknesses of each model, and how they recapitulate the human disease *in vivo*.

Sami et al. describe the role of human milk nutrients in preventing NEC. Preterm infants represent the most fragile population susceptible to developing NEC. Shortly after birth, their intestines face a series of challenges, including ongoing maturation, dietary demands driven by high nutritional needs, and the establishment of their gut microbial communities. Human milk is instrumental in shaping the gut microbiome, and this article summarizes the components of human milk, including lactoferrin, human milk oligosaccharides, dietary amino acids, vitamins, trace elements, and the interactions of these nutrients on the gut microbiota in NEC.

A review article of the current probiotic therapies for NEC by Sajankila et al. and a meta-analysis evaluating probiotics to prevent NEC in premature infants by Zhou et al. are helpful updates on this important aspect of preventing NEC. The review article discusses the various probiotic formulations, including single-strain formulations vs. multiple-strain formulations. In the meta-analysis, which included 27 randomized controlled trials with several different treatment interventions, they found that *Lactobacillus rhamnosus GG* and bovine lactoferrin can significantly reduce NEC incidence in preterm infants. While some questions are yet to be answered in terms of optimal probiotic combination and dosage, and there are concerns about sepsis related to non-medical grade probiotic use in premature infants, Sajankila et al. provided hope for the future with a discussion about the next generation of "designer probiotics," which will need detailed study and evaluation.

Bethell et al. focus on recent advances surrounding NEC diagnosis, imaging modalities, and a discussion on the surgical approach to NEC. The state-of-the-art review on machine learning and artificial intelligence in NEC by McElroy and Lueschow explores using machine learning methods as a biomarker for NEC diagnosis, including using stool microbiome data and clinical demographics to predict infants at the highest risk for NEC. The limitations and pitfalls of our current use of machine learning and artificial intelligence should not dissuade us from utilizing these powerful tools for earlier diagnosis of NEC and improving outcomes.

Manohar et al. review the impact of the gut-brain axis on the long-term complications of NEC. NEC is associated with impaired long-term neurodevelopmental outcomes, including a higher incidence of cerebral palsy and cognitive deficits. The authors discuss the ways in which neurodevelopmental impairment is assessed, including cognitive developmental tests, as well as magnetic resonance imaging, and regions of the brain affected by NEC. This review discusses how the gut-brain axis plays a role in the neurodevelopmental impairment seen in NEC and how the microbiome, the innate immune system, and various neurotransmitters play a role in the pathogenesis of NEC-related neurodevelopmental impairment.

Early randomized controlled trials in the 1970s–1990s demonstrated that prophylactic antibiotics decreased the risk of NEC. However, more recent retrospective studies suggest prolonged antibiotic exposure is associated with increased risk for NEC. Cuna et al. discuss the use of early antibiotics and the risk of NEC in premature infants and mouse models of the disease, highlighting the mechanistic work evaluating the effects of early and prolonged antibiotic exposure in neonatal mice and piglets on the gut microbiome and intestinal immunity.

Finally, Mackay et al. report a pilot study using an untargeted aptamer-based proteomics assay as a biomarker discovery for NEC. They found ten serum proteins that could differentiate infants with NEC compared to controls with high sensitivity on a small sample volume. We look forward to further detailed study in this area.

This research topic has inspired significant discussions in the field of NEC research. Although more studies are desperately needed in this field, it is exciting that new developments are on the horizon.

Author contributions

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We would like to take this opportunity to thank all the authors for their valuable contributions to this important research topic. It is also important to salute the selfless contribution of parents who enroll their infants in research studies on NEC and help the scientific community improve care and outcomes for future generations.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Necrotizing enterocolitis: Bench to bedside approaches and advancing our understanding of disease pathogenesis

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Necrotizing enterocolitis (NEC) is a devastating, multifactorial disease mainly affecting the intestine of premature infants. Recent discoveries have significantly enhanced our understanding of risk factors, as well as, cellular and genetic mechanisms of this complex disease. Despite these advancements, no essential, single risk factor, nor the mechanism by which each risk factor affects NEC has been elucidated. Nonetheless, recent research indicates that maternal factors, antibiotic exposure, feeding, hypoxia, and altered gut microbiota pose a threat to the underdeveloped immunity of preterm infants. Here we review predisposing factors, status of unwarranted immune responses, and microbial pathogenesis in NEC based on currently available scientific evidence. We additionally discuss novel techniques and models used to study NEC and how this research translates from the bench to the bedside into potential treatment strategies.

KEYWORDS

intestinal development, neonates, prematurity, necrotizing enterocolitis, intestinal epithelium

Introduction

Necrotizing enterocolitis (NEC) is a gastrointestinal disease that commonly affects preterm infants and is a major cause of morbidity and mortality in neonatal intensive care units (NICUs). Despite the advancements made in providing neonatal intensive care in recent years, NEC remains a devastating disease in NICUs. Approximately 7%–8% of premature infants in the NICU are diagnosed with NEC, with mortality rates approaching 20%–30% (1, 2). Of those that survive, many suffer from detrimental long-term effects on the intestines, growth, and neurodevelopment (3, 4).

NEC is characterized by inflammation and necrosis in the intestines, and often presents with a distended abdomen and blood in the stool (5, 6). Currently, NEC is treated with either a medical or surgical approach. The medical approach for the milder stages of NEC, consists of cessation of feedings, stomach decompression, antibiotics, frequent monitoring, and supportive care. Surgery is required if the infant experiences gangrene or intestinal perforation, and this treatment approach carries a

higher rate of mortality (7). These treatment approaches have not changed in several decades and novel approaches to prevent or treat NEC are desperately needed.

Research into identifying the etiology of NEC has revealed that the most prominent risk factor is infant prematurity (8, 9). Approximately 9 of 10 infants diagnosed with NEC are born premature (gestational ages 22–37 weeks), with the most severe cases typically manifesting in very low birth weight (VLBW) preterm infants with a birth weight of <1,500 grams. Although cases of NEC have been observed in full-term infants, VLBW infants maintain the highest chances of contracting and succumbing to NEC (10).

This increased occurrence and fatality in premature infants has been attributed in part to their underdeveloped innate and adaptive immune systems, as well as decreased diversity of the gut microbiome compared to those of full-term infants (11, 12). Research suggests that intestinal immaturity and undeveloped immunity of preterm infants allows pathogens to bypass the epithelial cell layer to induce inflammation (13). One of the ways to decrease NEC incidence is to provide maternal breast milk to infants. Human milk oligosaccharides (HMOs) and immunoglobins (Ig), such as immunoglobulin A (IgA), are present in breast milk and have been shown to protect against NEC (14, 15). The components in breast milk help prevent the onset of NEC and shift the infant's gut microbial composition, which in turn bolsters the immune response (16). While we have some idea of the factors that contribute to and the factors that protect against the disease, the specific mechanisms that lead to the pathogenesis of NEC are not fully understood.

In this review, we examine factors that may contribute to NEC and associated pathogenesis, including the role that the mucosal immune response and the microbiome play in disease. Furthermore, we outline the various *in vitro* and *in vivo* NEC models used to demonstrate these findings and explore how these conclusions can lead to the development of preventative measures and treatments designed for NEC.

Factors that may contribute to NEC

Although the etiology of NEC has yet to be completely elucidated, there are a multitude of factors, before and after birth, that can predispose infants to NEC. Maternal health status can provide substantial insight into an infant's risk of contracting NEC. According to a review of NEC risk factors in infants, variables such as maternal age, intrapartum antibiotics, incomplete steroid exposure, and maternal high neutrophil to lymphocyte ratio (NLR) are significant prognostic factors (9). Several observational studies have examined these factors in detail. A retrospective case control study with 97 matched pairs of infants showed a significantly higher odds ratio for antenatal ampicillin exposure for infants who later developed NEC than control infants (17).

Considering antenatal steroid exposure, it has been established that this treatment reduces morbidities and improves overall neonatal survival. However, an incomplete course of antenatal steroids or no steroid exposure has been associated with higher rates of more severe NEC (18). In a separate retrospective cohort study, an elevated maternal NLR (indicative of systemic inflammation) was significantly associated with the development of NEC (19). It is critical to note that blood NLR is a key diagnostic and prognostic indicator for disease states such as diabetes, obesity, hypertension, and heart disease, which are marked by inflammation. As such, the positive association between elevated maternal NLR and NEC suggests a possible relationship between NEC and placental vascular dysfunction caused by these disease states.

Preeclampsia, a serious complication of pregnancy, is also associated with an increased risk of NEC in preterm infants. Although the pathogenesis of preeclampsia remains unclear, it is theorized that the placental ischemia, abnormal hemostasis, leukocyte activation, and dysregulated nitric oxide metabolism associated with preeclampsia seem to be core components that may contribute to NEC development in preterm infants (20). Overall, preeclampsia reduces placental perfusion, which can lead to fetoplacental hypoxia and the pathogenesis of intrauterine growth restriction (IUGR). Both IUGR and reduced placental support, as indicated by abnormal patterns in antenatal umbilical dopplers, can impose increased risks for later NEC development (20, 21). Additionally, maternal diabetes mellitus (DM) poses a significant risk of NEC to infants, as determined by a retrospective study of low birthweight infants born to mothers with and without DM (21, 22).

Birth route may also provide insight into an infant's risk of developing NEC due to the impact that birth route has on the infant microbiome. However, the effects of Cesarean section (C-section) on the risk of NEC development are highly contested. A recent retrospective review discovered that delivery by C-section (and the presence of an umbilical arterial catheter) is associated with a decreased risk of NEC, possibly due to a decreased stress burden on the neonate during the C-section birthing process as compared to vaginal birth (23). A secondary analysis of data from a randomized controlled trial found no significant association between C-section in extreme preterm delivery and the onset of NEC (24). In contrast, another national case-control study established a positive association between C-section and the risk of NEC (25). Thus, there is conflicting data describing the relationship between C-sections and NEC incidence in neonates. Such disparities in data further indicate that NEC is a multifactorial condition and additional studies are required to delineate the maternal conditions that may predispose an infant to the disease.

Infant prematurity, characterized by both low birth weight and gestational age, is one of the most important risk factors for the development of NEC. Several studies have established that infants with a lower gestational age have a greater risk of developing NEC, along with higher mortality and surgical need (26, 27). Another retrospective study reported a higher NEC incidence in preterm infants that are small for gestational age (SGA) (28). While NEC pathogenesis in SGA neonates has not been completely explained, it has been proposed that gastrointestinal (GI) tract ischemia can contribute to NEC pathogenesis in preterm infants. Immature development of the GI tract can prime a "leaky" gut barrier susceptible to bacterial translocation due to incomplete formation of tight junctions, impaired peristalsis, and a thin mucus layer (29). The reduced structural integrity of the gut barrier can further decrease the uptake of essential nutrients for growth, exacerbating the effects of NEC.

Different types of infant nutrition can impact the pathogenesis of NEC. The nutritional requirements of preterm infants usually cannot be sustained solely with breast milk or standard formula—bovine and human-milk-based fortifiers are often needed to provide additional proteins, fats, vitamins, and minerals for adequate growth and development. However, some studies suggest that bovine milk-based infant formulas are positively associated with a higher risk of NEC, reviewed in (30). Although the exact link between bovine milk-based standardized formulas and NEC pathogenesis is not clear, one theory suggests that in the absence of the protective factors found in breast milk, infants receiving formula are at an increased incidence of NEC. This may render the gut more susceptible to the overgrowth of pathogenic microbes, such as the family of Gram-negative Enterobacteriaceae, and the initiation of widespread pro-inflammatory responses to bacterial translocation across the gut barrier (31). In contrast, the administration of maternal breast milk has been conclusively established to decrease NEC incidence (32). It has been long-established that human milk is the ideal source of nutrition for both premature and full-term infants. Several studies have demonstrated that there is a clear benefit to maternal human milk or donor human milk in the absence of maternal milk, reviewed in (33). Premature infants who received human milk have a demonstrably lower incidence of NEC than those who did not (34).

Intestinal dysbiosis, or the imbalance of a healthy gut microbial composition, has also been implicated as a predisposing factor to NEC. It is known that the gut microbiome of preterm infants has considerably reduced bacterial diversity and increased vulnerability to pathogens as compared to full-term infants (35). Additionally, there is a positive association between early antibiotic use and NEC onset, which supports the intestinal dysbiosis hypothesis (36). There have also been reports of immune dysregulation in conjunction with intestinal dysbiosis, particularly concerning heightened toll-like receptor 4 (TLR4) signaling and downstream inflammatory responses (37). Taken together, the pathogenesis of NEC is multifactorial and complex, rendering the root pathophysiology of NEC largely unanswered.

Immunological status of infants with NEC

Immature intestinal immune defense is among several factors associated with the high morbidity and mortality rates of NEC. Alteration of key innate and adaptive immune responses leads to dysfunction in intestinal barrier thus resulting in an increased inflammatory response (**Figure 1**) (38–40). The onset of NEC has been linked to low birth weight and gestational age, so while all infants have immature innate immunity, premature infants are also born with undeveloped adaptive immune systems. To make up for this weakened immunity, the transfer of maternal milk components, including secretory IgA (sIgA), as well as placental immunoglobulin G (IgG), provide protection to newborns until their own adaptive immune defenses can develop (15). In formula-fed premature infants, the levels of transferred maternal immune defenses are significantly reduced, potentially increasing their susceptibility of developing NEC (41).

In this section, we summarize the current scientific evidence of the innate and adaptive immune responses in infants with NEC. Specifically, we discuss how NEC pathogenesis relates to the vertical transfer of immunity from mother to child, alteration in physical barriers, and immunity guarding the gastrointestinal tract.

Maternal antibody transfer

Newborns do not cultivate a fully mature immune system until a few years after birth (42). To compensate, maternal IgG and IgA antibodies are donated from the placenta and maternal breast milk (if provided) to protect against pathogens and the development of NEC (15). Maternal IgG transfer to the fetus across the syncytiotrophoblast depends on the IgG-FcRn (neonatal Fc receptor) interaction. The expression of IgG-FcRn begins during the first trimester (12 weeks) of pregnancy and continues to rise until between 17 and 41 weeks gestation. The majority of placental IgG transfer occurs after 28 weeks of gestation. IgG levels reach 50% maternal concentration between 28 and 33 weeks gestation and will rise above maternal levels by 20%–30% at term (43, 44). It is possible that low IgG levels in preterm infants may predispose these infants to develop NEC.

In addition to the transfer of maternal IgG, transfer of maternal IgA through breast milk, also protects infants from

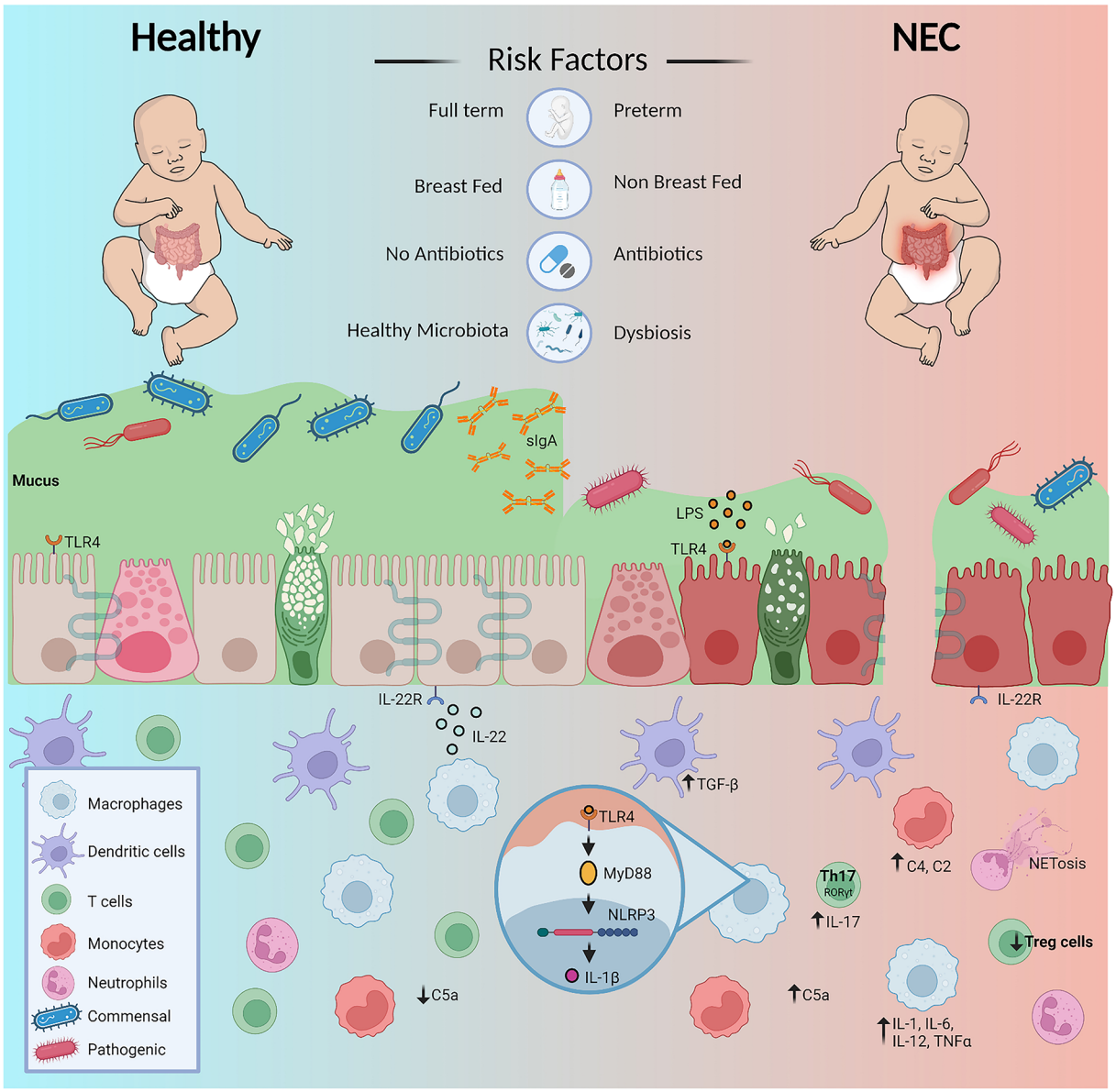

FIGURE 1
Diagrammatic overview of factors predispose premature infants to NEC and dysregulation of immunity contributing to the diseased state. Figure created with Biorender.com and affinity designer.

NEC. Originating from IgA+ plasma cells in the gut and educated by gut microbiota, IgA in the intestine can bind to pathogens and aid in their clearance. The ability of bacteria to bind to IgA was negatively correlated to NEC development, and the reduced stool bacterial diversity known to precede NEC was associated with a higher amount of unbound *Enterobacteriaceae* (15). Taken together, this data suggests that the absence of sIgA creates higher susceptibility to infections as well as delayed gut microbiota maturation which leads to gastrointestinal inflammatory diseases such as NEC.

Breast milk components

As the primary source of nutrition, breast milk ensures proper growth and development for newborns. Human milk is composed of micro and macronutrients, bioactive components, growth factors, antibodies, and HMOs (45). HMOs, in particular, play an important role in shaping microbiome composition and modulating neonatal immunity. HMOs act as natural prebiotics, functioning as soluble decoy receptors or antiadhesives to block the adhesion of pathogens to epithelium. They also enhance commensal growth and limit pathogen

growth (46). HMOs are non-digestible sugars, composed of five basic monosaccharide units: glucose, fucose, d-galactose, N-acetylglucosamine, and sialic acid (47, 48). These monosaccharide units are joined by glycosidic linkage to generate a variety of HMOs with different functions. HMOs are indigestible in the human upper digestive tract and remain intact while in the colon. Colonic microbes secrete enzymes to utilize these HMOs as nutrition (49, 50). Many of the commensals that degrade HMOs for fuel are members of the Bifidobacterium genus, mostly beneficial bacteria for infant health. Specific examples are *B. longum* and *B. breve* that are usually prominent in the digestive tract of breastfed infants.

In addition, Bacteroides species possess an excellent capacity to metabolize dietary polysaccharides to host-derived mucus-associated glycans. A study by Sodhi and colleagues has shown that HMOs 2′-fucosyllactose (2′-FL) and 6′- sialyllactose (6′- SL) can reduce NEC severity through TLR4 inhibition (51). 2′-FL also suppresses lipopolysaccharide (LPS) induced inflammation during *Escherichia coli* (*E. coli*) invasion of intestinal epithelial cells (52). Similarly, Masi et al. found significantly lower disialyllacto-N-tetraose (DSLNT) in the maternal milk given to infants prior to NEC development (53). Furthermore, authors reported that low DSLNT in milk was also associated with a significantly lower relative abundance of Bifidobacterium sp. and higher *Enterobacter cloacae* in the stool of infants prior to NEC (53). Fractions of HMOs were also shown to decrease mucus penetrability and bacterial attachment by enhancing the expression of Mucin 2 (MUC2) in a mouse model of NEC (54).

Other milk factors such as casein, a highly glycosylated breast milk protein, promotes intestinal defenses by increasing goblet cell numbers, enhancing *Muc2* expression, and Paneth cell activity (55, 56). Additional factors found in breast milk include lactoferrin and lysozymes that possess antipathogenic properties. Enteral supplementation of lactoferrin has been shown to decrease the likelihood of late-onset bacterial and fungal sepsis in preterm infants, but meta-analysis has shown there was no significant decrease in NEC in infants who were received lactoferrin (16). Breastmilk platelet activating factor-acetyl hydrolase (PAF-AH) potentially protects preterm newborns from NEC (57). Similarly, interleukin-10 (IL-10) found in breast milk has been found protective against developing NEC in premature infants (58). In addition to IL-10, maternal transforming growth factor beta (TGF-β) provides protection by helping to increase IgA locally in the gut (59). Growth factors found in breast milk, such as insulin-like growth factor (IGF) and epidermal growth factor (EGF), support intestinal health and may protect against the development of NEC (60–65).

First line defense of the intestinal barrier

Mucus is one of the first lines of intestinal host defense. Mucus is produced by goblet cells, which are found in the crypts of Lieberkühn. The colonic mucus layer is divided into two layers, an outer, penetrable layer, and an inner, impenetrable layer. This contrasts with the mucus in the small intestine (SI) which is single layered and penetrable by bacteria. A protective layer of mucus keeps bacteria in the SI away from the intestinal epithelium by antimicrobial proteins (AMPs) secreted by Paneth cells (66). Studies have found defective and a significantly lower number of goblet and Paneth cells in the SI of infants with NEC compared to NEC (67). Using HT29-MTX-E12, a mucus secreting cell line, Hall and colleagues reported that breast milk significantly lowered the adherence and internalization of NEC-associated pathogenic *E. coli* into the mucus compared to infant formula, suggesting that breast milk enhances mucus integrity (68). *Clostridium difficile (C. difficile),* a known gut pathogen, also influences mucus production and composition (69).

Antimicrobial peptides (AMPs), such as defensins, including human β-defensin-3 (hBD3), cathelicidins, C-type lectin receptors (CLRs), regenerating islet-derived protein 3, and intestinal enzymes such as phospholipase A2-IIA (PLA2) and lysozyme are expressed in the gut epithelium and provide protection for the intestinal mucosa from pathogenic bacteria either by killing pathogens or by inhibiting their growth (70, 71). In addition, AMPs are involved in the immune response and shaping the microbiome (72). Using an experimental rat NEC model, Underwood and colleagues found increased intestinal mRNA expression of the AMPs *lysozyme*, secretory *PLA2*, and *pancreatic-associated proteins* 1 and 3 in rats with NEC compared to either dam-fed or formula-fed rats supplemented with the probiotic bacteria *Bifidobacterium bifidum* (*B. bifidum*), suggesting that AMP induction is a mucosal response to gut inflammation in NEC (73). Another study evaluated the defensin hBD3, a small cationic antimicrobial peptide that can exert multiple protective properties on the gut. Using an animal NEC model, Sheng et al., showed that hBD3 administration decreased the incidence of NEC, reduced NEC severity (decreased pro-inflammatory cytokines, intact intestinal barrier), and increased the survival rate of the animals (74). Collectively, these studies suggest a protective role for mucus and associated AMPs in neonatal mucosal defense and intestinal barrier function in NEC.

Complement proteins and NEC

During infection, complement proteins assist in the phagocytosis of invading pathogens by opsonization, generating inflammatory responses, and altering the activity of B and T lymphocytes (75, 76). Three different pathways—lectin, alternative, and classical—activate the complement cascade. Previous studies have reported defective complement protein activity in preterm infants (77, 78). More specifically,

one study reported low complement component 3 (C3) and complement component 9 (C9), intermediates of complement pathways, in preterm infants (79, 80). C5a, a cleavage product of complement component 5 (C5), is a potent chemoattractant, anaphylatoxin, and intermediary in both the conventional and non-canonical complement pathways. C5a was reported to be substantially expressed in NEC cases and could be partially responsible for inflammation in NEC. Due to its multifaceted nature, C5a is being studied for its utility as a clinical marker for the diagnosis of neonates with NEC in conjunction with radiographic evidence of disease (81). In addition, MBL-associated serine protease-2 (MASP-2), an enzyme associated with C2 and C4 cleavage and activity, is detected in higher concentrations in the cord blood of premature children who are susceptible to NEC and is linked to a threefold increased risk of developing NEC (82, 83).

Toll-like receptors and innate immune cells in NEC

Drosophila Toll was discovered as a receptor for dorso-ventral patterning during development and was later identified as a participant in immunity against fungal infections (84). Consequently, several other homologues of Toll, named Toll-like receptors (TLRs) were discovered in mammals. TLRs sense pathogen-associated molecular pattern molecules (PAMPs) and danger-associated molecular patterns (DAMPs) through their N-terminal extracellular leucine-rich repeats (LRRs) and elicit innate immunological responses, including the production and release of inflammatory cytokines (85). So far, 10 different types of TLRs have been identified in humans and 12 in mice. TLR1, TLR5, TLR6, and TLR10 are membrane receptors that may detect extracellular ligands while TLR3, TLR7, TLR8, and TLR9 work on subcellular structures. For example, TLR9 is found on endosomes and recognizes nucleic acids derived from pathogens and self-damaged cells (85, 86). TLR2 and TLR4 are expressed on the cell membrane as well as on subcellular structures.

TLR4 is a receptor for LPS, a component of Gram-negative bacteria's outer membrane that is critical for the NEC pathogenesis (87). TLR9 binds to and is activated by unmethylated cytosine-guanine oligodeoxynucleotides (CpG ODNs) in bacterial genomes, and acts as antagonist of TLR4. Activation of TLR4 in newborn mouse epithelial cells by LPS results in undesired activation of the NF-κB pathway that leads to damage of the intestinal mucosa through production of pro-inflammatory cytokines, which is one of the hallmarks of NEC (87). Several studies have shed light on the association of TLR4 with NEC (41). Recently, Liu and coworkers have shown both TLR4 and necro apoptotic protein upregulation in both NEC patients with NEC and animal NEC models (88). Egan et al., highlighted the role of TLR4 in recruiting the inflammatory $CD4^+$ Th17 cells into the intestinal mucosa *via* activation of cognate chemokine ligand 25 (CCL25) in NEC (89). In an another study, Colliou et al., found a commensal *Propionibacterium* bacterial strain named UF1 that can reduce intestinal inflammation through the reduction of Th17 cell expansion in the gut of a mouse NEC model (90). TLR4 activation significantly inhibits the β-catenin signaling that is important for enterocyte proliferation in the ileum of newborn mice, which further leads to apoptosis and can lead to NEC (91). Studies have shown that activation of TLR9 can decrease experimental NEC severity, and that TLR9 activation can inhibit TLR4 signaling *via* IL-1R-associated kinase M (92, 93). In addition to TLR9, NOD2 reduces NEC severity *via* suppressing TLR4 and genetic variants in NOD2 are associated with NEC development (94, 95).

Monocytes and macrophages

Originating from myeloid cell lineage monocytes, macrophages (Mϕ) act as a frontline guard of innate immunity against invading pathogens. Monocytes and Mϕ have several weapons in their arsenal to tackle incoming threats. By recognizing molecular patterns *via* toll-like receptors (TLRs), nucleotide-binding oligomerization domain-containing proteins (NOD2), and C-type lectin receptors (CLRs,) these cells either actively engage in phagocytosis or secrete various cytokines and chemokines to alert and recruit other immune cells (96). Classical monocytes ($CD14^+CD16^-$), intermediate monocytes ($CD14^+CD16^+$), and non-classical monocytes ($CD14^{dim}CD16^+$) are the three subsets of human monocytes. In mice, monocytes are grouped based on expression levels of lymphocyte antigen 6 complex ($Ly6C^+$ and $Ly6C^-$) on their cell surface (97).

Several studies have suggested that tissue infiltration and enrichment of monocyte-derived Mϕ occur during inflammation in NEC (98–100). Intestine monocyte-derived Mϕ are nonproliferative, short lived and terminally differentiated, rendering their continuous replacement necessary for homeostasis. A study by Managlia et al., revealed the significance of nuclear factor kappa B (NF-κB)-driven monocyte activation, recruitment, and differentiation in neonatal intestines in NEC (99). They concluded that NF-κB-mediated activation and differentiation of $Ly6c^+$ monocytes into Mϕ and their recruitment into the intestine are critical for NEC development and disease progression. Olaloye and colleagues have identified a novel subtype of inflammatory $CD16^+CD163^+$ monocytes/Mϕ associated with infants with NEC (100). In infants with NEC, peripheral monocyte counts drop due to their recruitment to the damaged intestine (101). Following recruitment, monocytes undergo differentiation to form pro-inflammatory M1-type Mϕ (102). Monocyte-derived M1 Mϕ in humans and in animal models have been linked to

the severity of NEC (102, 103). Interferon regulatory factor 5 (IRF5), a factor crucial for M1 Mϕ polarization is highly expressed in infants with NEC compared to controls. Specifically, IRF5 deficiency significantly reduced M1 polarization, inflammation, and intestinal injury in experimental NEC (103). Inflammation and intestinal cell damage caused by M1 Mϕ is linked with their high level of pro-inflammatory cytokines such as IL-1, IL-6, IL-12, chemokines (Ccl4, Ccl5), and tumor necrosis factor (TNF) production. By inhibiting M1 and promoting M2 polarization of Mϕ, heparin-binding epidermal growth factor (HB-EGF) has also been found to protect against experimental NEC (102).

Neutrophils

As one of the most abundant immune cells (nearly 70% of total leukocytes) in human blood, neutrophils are among the first responders in the fight against potential pathogens or tissue damage/injury. Neutrophils eliminate pathogens either by recruiting a wide variety of immune cells through the secretion of cytokines, chemokines, and leukotrienes or by causing direct damage to tissue or pathogens by releasing lytic proteases and neutrophil extra cellular trap (NETs) (104). In addition to their well-documented protective role, neutrophils are also able to cause significant tissue damage through the release of reactive oxygen species (ROS) in intestinal inflammation (105).

Early neutropenia has been associated with higher odds of developing NEC (106). Interestingly, neutrophils in preterm newborns have altered immunological functions, including impaired phagocytosis. Another study by Zindl and colleagues revealed the protective role of IL-22-producing neutrophils in experimental colitis by increasing AMP production and promoting mucosal repair (107). In the context of NEC, a recent study from Mihi et al., demonstrated a protective role of IL-22 treatment in attenuating intestinal injury and enhancing epithelial proliferation in experimental NEC (108). This study also found that there was a lack of IL-22 production in preterm infants or developing mice, suggesting that immunomodulatory treatments may help protect premature infants from the intestinal inflammation seen in NEC.

As specialized antigen presenting cells (APCs), dendritic cells (DCs) serve as critical link between innate and adaptive immunity. In intestine, DCs are present in Peyer's patches, mesenteric lymph nodes (MLNs), and the colonic lamina propria to provide protection against invading pathogens. To date, several studies have highlighted the protective role of DCs in regulating the gut inflammation; however, studies investigating the role of DCs in NEC is limited. In one study, which utilizes *Cronobacter sakazakii* (*C. sakazakii*) to induce NEC in mice, Emami and colleagues have reported higher DC recruitment in mouse gut. They found that DC recruitment to the gut accelerated the destruction of the intestinal epithelium and promoted NEC onset with increased TGF-β production (109). *C. sakazakii* also induced pyroptosis in the intestinal epithelium and promoted NEC by induction of IL-1β and Gasdermin D (GSDMD) through TLR4/MyD88 mediated activation of the nucleotide-binding oligomerization domain (NLRP3) inflammasome (110). Another study by Nolan and colleagues investigated the role of aryl hydrocarbon receptor (AhR) signaling in DCs during experimental NEC, as this signaling pathway helps regulate intestinal immunity and homeostasis. They found that a lack of AhR signaling in DCs increased NEC-mediated intestinal inflammation, and that this effect was associated with an increase in a specific subset of macrophages in the small intestinal lamina propria (111).

Trained immunity and NEC

Adult human intestine is made of a single layer of epithelium, covering an area of 32 m^2 (112). The intestinal epithelium is important for digesting food and absorbing nutrients, but it is also the largest entry port for pathogens. To provide protection against these pathogens, "as a guard of port", complex and tightly controlled innate and acquired immunity are required. Among the many different types of immune cells involved in this protection are intraepithelial lymphocytes (IELs). IELs are positioned between intestinal epithelial cells and constantly patrol the epithelial barrier (113). IEL subsets, composed of antigen-experienced T cells, are in direct contact with enterocytes and antigens in the gut lumen. These cells are classified based on the expression of T cell receptor-γδ $(TCR\gamma\delta)^+$ and $TCR\alpha\beta^+$ (114). Approximately 60% of small intestinal IELs are TCR^+ cells. γδ IEL play a crucial role in mucosal defense by regulating the production of IgA, clearing and repairing damaged epithelium, increasing production of TGF-β cytokines and by decreasing IFN-γ and TNF-α in response to stress and infection (115). The protective role of IELs is also evident in TCRγδ-deficient mice, as these mice have defective gut epithelial morphology and impaired IgA production (116). When compared to non-NEC controls, surgical NEC patients with NEC had a lower number of γδ IELs in the ileum (116). Researchers have shown that subsets of IELs are dependent on AhR activation for their survival (117). However, a recent study did not find any involvement of IELs in AhR activation-mediated protection against NEC, indicating that the protective role of IELs against NEC is not AhR-mediated (118).

In addition to IELs, infants with NEC also have altered functions of some subsets of $CD4^+$ T cells, Th17, and regulatory T (Treg) cells (89, 119–121). Th17 cells are strongly implicated in intestinal inflammation and are linked with the pathogenesis of NEC. In infants with NEC, Pang and colleagues found a lower percentage of Foxp3-expressing Tregs with several functional defects, including the inability to

block IL-17 expression (121). In NEC tissue, Th17 cells appear to cause intestinal damage that is reduced by IL-17 receptor inhibition by STAT3 activation (122). Additionally, retinoic acid-induced polarization of $CD4^+$ T cells towards Treg from Th17 resulted in reduced NEC severity (123). Furthermore, Zhao et al. reported an increased percentage of $ROR\gamma t^+$ cells (inflammatory Th17 and type 3 innate lymphoid cell populations) in the intestinal lamina of mice and humans with NEC compared to those without NEC (84). Studies have also demonstrated a significant decrease in lamina propria associated Treg cells in surgical NEC specimens (85, 86, 89). In addition, a Treg/Th17 imbalance leads to the excessive proinflammatory response preceding tissue injury and necrosis associated with NEC development (122).

Intestinal microbiome and NEC

Although the direct association between the microbiota and the pathogenesis of NEC is not well understood, mounting evidence suggests a link between early gut microbiota dysbiosis and NEC (87, 88, 90). Probiotic supplementation to premature neonates has been shown in some studies to decrease the incidence or severity of NEC, further establishing the relationship between NEC and microbiota (91–94).

Early microbiota composition and its diversity in the gut of newborn infants is mainly influenced by delivery mode, antibiotic exposure, human milk feeding, and time spent in the NICU. Vaginally born infants not only develop stronger immunity but also are predominantly colonized by beneficial microbes such as *Lactobacillus* sp. present in mother's vaginal microbiota (95). Members of *Lactobacillus* are well known to prevent pathogen colonization by lowering the pH or by secreting inhibitory compounds (124, 125). The microbiota of infants born *via* C-section resemble the mothers' skin microbiota in early life and lack members of *Bacteroides* species that are present mostly in vaginally-delivered infants (126).

In addition to delivery mode, feeding also affects microbiome composition and diversity. Formula-fed newborns have lower overall bacterial diversity, lesser beneficial bacterial number, and a higher number of pathogenic bacteria like *Clostridium* sp. compared to breast-fed infants (127). *Clostridium* sp. and their secreted toxins can be associated with NEC severity (128, 129). Time spent in the NICU with lifesaving machines attached to preterm infants including, ventilators, and incubators, have also been shown to harbor pathogenic bacteria including members of *Streptococcus*, *Klebsiella*, *Staphylococcus*, *Neisseria*, and *Enterobacteriaceae* communities (130–133). Members of the phyla Firmicutes, such as coagulase-negative staphylococci (CoNS) and Proteobacteria are implicated in NEC pathogenesis, however, many of their members are also found in healthy infants (134). Higher bacterial relative abundance from the class Gammaproteobacteria, namely *C. sakazakii*, *Klebsiella* sp., *E. coli*, and those from the phylum Proteobacteria are also present in the feces of infants who develop NEC (135). In addition to bacteria, viral presence is also associated with NEC. Stool analysis from 51 infants with NEC and 39 controls demonstrated that the presence of adenovirus and Epstein-Barr virus are associated with NEC severity (136). In another recent study, stool samples obtained from 9 infants with NEC infants and 14 controls matched for weight and gestational age, showed reduced viral beta diversity over the 10 days before NEC onset. This study also identified that viral NEC-associated contigs belonging to *Myoviridae*, *Podoviridae* and *Siphoviridae* are associated with the time period 0–10 d post NEC onset (137).

Models for studying NEC

In vivo

With the high prevalence of NEC, the need for effective *in vivo* models has become more important in recent years. Due to the aggressive nature of the disease and the scarcity of available human specimens, performing experiments with human samples is difficult and multi-center studies are typically needed (138). As a result, animal models are commonly used to study NEC by inducing inflammation that mimics the intestinal damage seen in human infants.

While the conditions of *in vivo* experimental NEC models are generally based on similar underlying principles, several different animals have been used to study NEC (**Figure 2**). The rat's intestinal development is similar to a human premature infant, making it an excellent model for investigating preventative measures and therapeutics for NEC (139). Early studies using a rat model concluded that the gut microbiota and the absence of breast milk are significant factors in NEC pathogenesis (140, 141). Further, several laboratories have used hypoxia, LPS, and hypothermia at different time points in a day for several days to help induce NEC in laboratory settings (142). Due to their affordability, preterm survivability, and resistance to typical stressors used to develop the disease, rat models are a desirable option when investigating NEC but rats are not ideal for research at the genomic level. Their slower development and challenges with culturing embryonic stem cells in rats makes it difficult to generate transgenic lines compared to mice (143, 144). These shortcomings necessitated the creation of other types of animal NEC models.

Although their small size makes them technically challenging to work with, mice are the preferred model for genomic studies as it is far easier to create transgenic colonies. Another appealing feature of the mouse model is its experimental flexibility, with some models successfully inducing NEC by beginning the gavage feed at postnatal day 4 while others begin at postnatal day 7 (145, 146). However, mice delivered more than one day prior to the

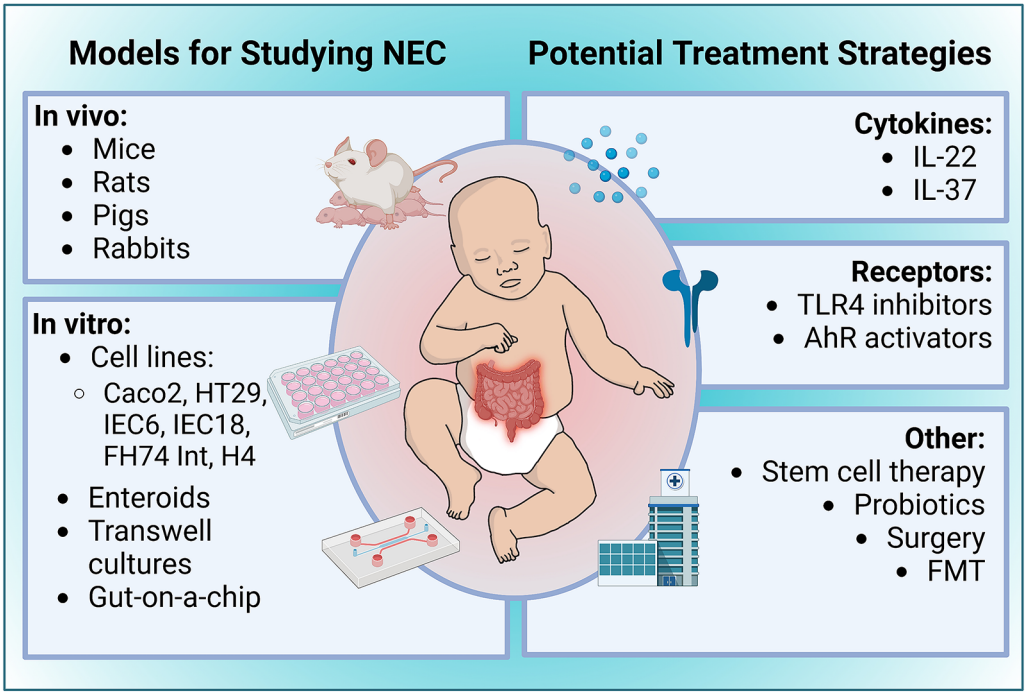

FIGURE 2
Overview of experimental models of NEC and potential treatment strategies. Figure created with Biorender.com.

determined due date have a 100% mortality rate (147). Because of this low viability, it is extremely difficult to use a preterm mouse model for studies that require animals to be delivered *via* cesarean section.

Pigs share many features of anatomy and physiology with humans, rendering them one of the more popular choices when exploring NEC pathogenesis. Additionally, the piglet's larger size affords the ability to study preterm neonates (148, 149). Piglets are a good model for testing preclinical drugs, effects of various diet formulations, and pathological manifestation on NEC (150). While it is true that hypoxia and hypothermic stress induces histological changes that resembled NEC in piglet models, the inflammation induced by this model is not always contained within the lower gastrointestinal tract, with some instances reported of inflammation spreading to the stomach and jejunum (139, 150–152).

Rabbit NEC models are infrequently used but have been used to study the effects of NEC that extend past the gut. Non-human primate models, although rare and expensive, have also been used as an experimental NEC models due to the homology to humans in both anatomy and at the genomic level (139).

In vitro

In vivo animal models allow for limited NEC modeling as the cellular genetics, drug metabolism, immunology, gut microbiomes, and HMOs can differ significantly from humans. *In vitro* intestinal models used to study NEC are briefly summarized in this section and have been covered extensively elsewhere (153–156).

Different *in vitro* models such as the human epithelial cell line Caco2, colon adenocarcinoma derived cell line HT-29, IEC-6 and IEC-18 derived from rat SI, and most importantly, fetus derived FHs 74-Int and H4 cells are frequently used in *in vitro* NEC studies (153). These cell lines are optimized and phenotypically mimic different regions of the gut including ileum, duodenum, jejunum, and colon, each requiring specific culturing conditions.

Recent scientific advancements in culturing human intestinal organoids (enteroids) also called "mini guts", allow investigators to recapitulate the intestinal cell morphology that is crucial for studying the molecular mechanisms of NEC. Enteroids derived from $LGR5^{+}$ progenitor cells of the SI and colon, allow for the study of barrier function, gut inflammation, cell proliferation, drug responses, and intestinal microbial interactions characteristic of NEC (157). Further advancements of *in vitro* models led to the development of a "gut-on-a-chip", a method which cultures intestinal cells to mimic the microenvironment of the intestine (158, 159). The gut-on-a-chip model provides a suitable environment to culture different human cell types including epithelial, endothelial, and immune cells with gut microbes together in a controlled environment, to explore gut physiology and inflammatory changes seen in NEC, and can

also be used as a pharmacological platform to test potential drug treatments (160).

Though, these *in vitro* models excellently resemble human intestine, several key criteria are considered in cell culture model design. **Table 1** compares common different models and devices, specifically summarizing whether the models are static or microfluidic, *in vitro* or *ex vivo,* cell differentiation, cell polarity (apical out or basal out), nutrient absorption, drug metabolism, crypt villus formation, mechanical stimulation or peristalsis, oxygen gradient modulation, measure trans epithelial electrical resistance (TEER), co-culture with endothelial, vascular, and immune cells, and co-culture with gut microbes.

Static vs. Microfluidic models

Static models are standard tissue culture models which include "NEC-on-a-dish" 2D, 3D organoid and transwell culture models (175). Additionally, synthetic scaffolds, and *ex vivo* tissue (Ussing chambers) are used to measure live tissue (167, 168). Static models use growth factors to differentiate intestinal epithelial cells (IECs) and organoids, derived from $LGR5^+$ progenitor cells, into diverse functional intestinal cells (163). Static models are generally less time consuming, less expensive, and more accessible, but are relatively limited to the degree of differentiation, co-culture, and microbiome interactions. Typically, in static models, microbiome interactions are limited to between 1 and 24 h based on the model due to rapid microbial overgrowth in static conditions.

Gut-on-a-chip microfluidic devices use soft lithography to layer polydimethylsiloxane (PDMS) or micromilling to produce luminal and vascular channels separated by a porous membrane (reviewed in (176). Short term *ex vivo* microfluidic devices can evaluate live tissue conditions under constant flow (169, 170). The luminal flow in a microfluidic model enhances differentiation and 3D villus and crypt-villus like topography where adjacent air channels are regulated to mimic peristalsis through mechanical stimulation, thus providing a major advantage over static models. The NEC microbiome and HMO interactions, drug metabolism, and tissue integrity assays can be measured within the microfluidic chip system (177, 178). A major advantage of the microfluidic flow is that it reduces the static overgrowth of microbes, in turn reducing the limitations on the microbial co-culturing time to more than 7 days, depending on the specifics of the model. Gut-on-a-chip models can additionally be cultured under oxygen gradient modulation. Intestinal disease pathology is increased by lower oxygen gradients which induce Hif1-α signaling (179). Oxygen gradients under aerobic, hypoxic, and anaerobic culturing conditions have also been applied to resemble microbial intestinal environments under inflammatory conditions (176).

Treatments for NEC

The several known risk factors of NEC discussed in this review provide promising treatment targets for NEC (**Figure 2**). One such treatment is IL-22, a cytokine belonging to the IL-10 family that is involved in epithelial cell regeneration, maintenance of gut barrier integrity, and tempering intestinal inflammation by mediating the microbiome (180). Given the observations of the versatile roles that IL-22 plays in gastrointestinal physiological processes and pathologies, especially as a stabilizer of intestinal homeostasis, there is a strong foundation to investigate the role of IL-22 in the context of NEC pathogenesis. As mentioned above, a recent study by Mihi et al., showed that neonatal mice and humans lack intestinal IL-22 production during NEC and supplemental administration of IL-22 attenuated experimental NEC severity, decreased intestinal inflammation, and enhanced intestinal epithelial repair (108). Additionally, IL-22 administration induced the expression of antimicrobial genes such as *Reg3γ* and fucosyltransferase 2 (*Fut2*). The AMP Reg3γ has been shown to protect the intestinal mucosa against pathogenic infections by limiting their expansions. Given this protective role of IL-22 in the experimental murine model of NEC, it is imperative that IL-22 administration be further investigated as a therapeutic for infants with NEC (108).

Another study by Cho et al., highlighted the importance of another cytokine, IL-37 in attenuating the inflammation in NEC (181). The study found that transgenic IL-37 pups were completely protected from inflammation caused by IL-1β, IL-6, TNF, and IL-17F compared to wild-type mice. In addition, IL-37 treatment restored the expression of cytokines *Il4*, *Il13*, and *Il33* to baseline levels. Further, authors found that IL-37-mediated protection against NEC is largely achieved through modulation of the TLR repertoire (reducing TLR4 expression and inducing TLR5, TLR7, TLR9, and TLR13), and prevention of NEC-induced dysregulation of adaptive immunity (181).

Another promising treatment modality is the use of TLR4 inhibitors to mediate intestinal injury propagated by NEC. Hackam and colleagues have published several studies indicating that expression of TLR4 and members of its gene family render the premature intestine more susceptible to inflammation. Therefore, exploring TLR4 modulation or inhibition as a model for NEC treatment may be valuable. Lien et al., and Tidswell et al., noted the synthetic inhibitor eritoran tetrasodium (E5564) bound well to TLR4 (182, 183). Based on the structure of this inhibitor, an *in silico* search and screening of small molecule libraries conducted by Hackam and colleagues pinpointed a family of TLR4 inhibitors that reduces intestinal inflammation in experimental NEC (184, 185). Particularly, the compound

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TABLE 1 Characteristics and limitations of *in vitro* static and microfluidic devices for NEC disease modeling (● = yes, ○ = no).

Model	Description	Nutrient absorption	Co-culture	Differentiation	Drug metabolism	Microbiome	Crypt-villus axis	Oxygen gradient	Mechanical stimulation	Fluid flow	NEC modeling	TEER	Model Advantages	Model Disadvantages	References
Static															
Transwell	A 2D dual chamber well separated by a porous membrane allowing for compartmented cell culture media, cells, and drugs.	●	●	○	○	●	○	○	○	○	●	●	Simple multi-well culture model, can be modified for differentiation, endothelial co-culture, and immune cell migration.	Microbiome interactions are limited due to static culture (<24 h). Rapidly becomes overgrown.	(161, 162)
Organoid	An expanded 3D-spherical cell culture from intestinal LGR5$^+$ stem cells (enteroids). Organoids are differentiated to resemble intestinal epithelial tissue in a 3D matrix.	●	○	●	●	●	●	○	○	○	●	○	Can be expanded and differentiated with apical or basal polarity in ECM. Suitable for assays. Can form villus-like structures.	Cannot be co-cultured with endothelial cells. Static culture becomes easily overgrown by microbes (<1 h).	(163–166)
Ex vivo	Functional live tissues with complex cellular components that replicate *in vivo* environments.	●	○	●	●	●	●	●	○	○	●	●	Complex differentiated tissue most similar to *in vivo* tissue.	Limited by tissue availability. Static microbial culture (<3 h).	(167)
Scaffold	An artificial intestine that mimics native intestinal architecture. Stem cells are seeded onto the scaffold and differentiated to form villus-like structures.	○	○	●	○	○	●	○	○	○	○	○	Provides a structured scaffold for crypt-villus formation. Enhanced metabolic enzymatic activity relative to 2D-cultures or chips without scaffolding.	Cannot be co-cultured with endothelial cells. Not suitable for microbiome co-culture. No basal permeability.	(168)
Microfluidic															
Ex vivo	Live functional intestinal tissue section enclosed in a microfluidics chamber.	●	●	●	●	●	●	○	●	●	●	●	Live functional tissue is subject to microfluidic flow where tissue is differentiated with crypt-villus structures and supportive endothelial tissues.	Requires fresh tissue. Very short time frame (<3 h) for tissue viability.	(169, 170)

(*continued*)

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TABLE 1 Continued

Model	Description	Nutrient absorption	Co-culture	Differentiation	Drug metabolism	Microbiome	Crypt-villus axis	Oxygen gradient	Mechanical stimulation	Fluid flow	NEC modeling	TEER	Model Advantages	Model Disadvantages	References
Multichannel	A PDMS microchannel system (HuMix) with 3 co-laminar fluidic channels. An epithelial, medium perfusion and a microbial culture channel. The microbe channel is separated from the epithelial layer by a nanoporous membrane (0.5–1 mm).	●	●	○	●	●	●	●	○	●	○	○	Designed for TEER measurements and oxygen gradients across multiple channels. Allows for a membrane separated microbial and epithelial chamber to reflect microbial/cell signaling in a healthy gut.	Bacteria separated from the epithelial cells. No mucus layer interaction. Intentionally not designed for direct bacterial interaction and bacterial movement across the epithelial barrier required for NEC studies.	(171)
Gut-on-a-Chip	A PDMS dual microchannel system designed for specific gastrointestinal tissues. Gut-on-a-chip microfluidics are designed for 3D differentiated tissue.	●	●	●	●	●	●	●	●	●	●	●	Intestinal epithelial cells or organoids are cultured under peristalsis as a differentiated layer on an ECM scaffold. Continuous flow allows for extended culture (>7 days) and increased differentiation. Allows for co-culture with endothelial cells and a NEC microbiome. Can be cultured under different oxygen conditions.	Requires a high operating cost/chip and dedicated equipment. Requires enteroids. Experiments have a longer turn-over time and may require >7 days to allow for confluence and differentiation. PDMS may absorb small molecules.	(154, 172–174)

C17H27NO9 (C34), a 2-acetamidopyranoside, significantly reduced NEC incidence in animal models and decreased TLR4 signaling *ex vivo* in resected ileum from infants with NEC (185). Indeed, these findings indicate C34 and its analogs are lead compounds for TLR4 inhibition that can provide therapeutic value and improve clinical treatments for NEC. In a recent study Lu et al., showed that activation of AhR either by its ligand indole-3-carbinol or by breast milk components prevented experimental NEC through inhibition of TLR4 signaling (118).

Stem cell therapy is another treatment option currently being explored because of anti-inflammatory properties with a focus on bone marrow-derived mesenchymal stem cells (BM-MSCs). Several studies have demonstrated that BM-MSCs extracted from mice, rats, and humans significantly reduce both NEC incidence and severity (186–188).

Amniotic fluid-derived stem cells (AF-MSCs) have also been investigated as a potential source for NEC treatment. A study by Zani et al., established that intraperitoneal injections of AF-MSCs in a murine model are significantly associated with a reduction in the incidence and severity of NEC and improved gut barrier function (5). Subsequent confirmatory studies verified that AF-MSC injections decrease histologic injury in experimental NEC models (189). Thus, there is indication that AF-MSCs have considerable beneficial effects as an inflammatory modulator and should be examined further as a therapeutic for NEC.

Experimental results of supplementation with probiotics and potentially fecal microbiota transplant (FMT) has also shown promising outcomes to treat NEC, however, appropriate donor selection, screening of FMT material, and a dosing strategy still need to be standardized (190–192).

Conclusion

NEC is a common gastrointestinal disease in premature infants associated with high morbidity and mortality. In recent years, substantial progress has been made to delineate the molecular mechanisms underlying the pathogenesis of NEC. The holistic approaches with scientific advancement to understand the risk factors predisposing an infant to NEC, including maternal, genetic, nutritional, and immunological changes in infants, clearly hold the potential to improve and lead to development of preventative measures and treatments to combat NEC. Although translating fundamental experimental discoveries to the bedside in the NICU is substantially challenging, continuous scientific efforts and collaborations between those working "at the bench" making discoveries in laboratories with those clinicians "at the bedside" caring for infants with NEC can lead to ground-breaking discoveries and transform the management of this devastating disease.

Author contributions

DKS, CM, KAO, MD, SM and MG reviewed the relevant literature, drafted, revised, and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Early antibiotics and risk for necrotizing enterocolitis in premature infants: A narrative review

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While prompt initiation of antibiotics at birth due to concerns for early onset sepsis is common, it often leads to many preterm infants being exposed to treatment despite negative blood cultures. Such exposure to early antibiotics can impact the developing gut microbiome putting infants at increased risk of several diseases. Necrotizing enterocolitis (NEC), a devastating inflammatory bowel disease that affects preterm infants, is among the most widely studied neonatal disease that has been linked to early antibiotics. While some studies have demonstrated an increased risk of NEC, other studies have demonstrated seemingly contrary findings of decreased NEC with early antibiotics. Studies using animal models have also yielded differing findings of benefit vs. harm of early antibiotic exposure on subsequent NEC susceptibility. We thus sought to conduct this narrative review to help clarify the relationship between early antibiotics exposure and future risk of NEC in preterm infants. Our objectives are to: (1) summarize findings from human and animal studies that investigated the relationship between early antibiotics and NEC, (2) highlight important limitations of these studies, (3) explore potential mechanisms that can explain why early antibiotics may increase or decrease NEC risk, and (4) identify future directions for research.

KEYWORDS
antibiotic stewardship, intestinal microbiome, prematurity, necrotizing entercolitis, antibiotics, postnatal intestinal adaptation, gut dysbiosis

Introduction

Necrotizing enterocolitis (NEC) is a devastating disease that develops in 5%–10% of preterm infants born less than 1500 grams (1). Exaggerated bacteria-induced gut inflammation and necrosis that in severe cases can cause a systemic inflammatory response are considered the central pathogenic mechanism of NEC (2). While the exact mechanisms underlying this exaggerated inflammation remains incompletely understood, prematurity, gut dysbiosis, genetic predisposition, formula-feeding, red blood cell transfusion, and intrauterine growth restriction are considered risk factors (3–5). Because NEC can develop suddenly, addressing risk factors that are potentially modifiable is a key strategy to prevent NEC and help improve outcomes (6). Antibiotics use in the first two weeks of life has been identified as one such risk factor that can potentially modulate risk for NEC (7). Several retrospective studies have demonstrated that early antibiotic use is associated with an increased risk for developing NEC (8–15). Each additional day of antibiotic exposure during the first 7–14 days of life despite sterile blood cultures is estimated to increase the risk for NEC by 7%–20% (8, 9). However, some studies have shown opposite results – that of a protective effect of early antibiotics and NEC. In fact, randomized controlled trials (RCTs) from the late 1970s to late 1990s indicate

that prophylactic treatment with oral antibiotics can reduce NEC by half (16–20); and other retrospective studies have demonstrated that early antibiotics is associated with a decrease in NEC incidence compared to infants not exposed to early antibiotics (21–23).

Because of the seemingly contradictory findings from different studies, we sought to conduct this narrative review to help clarify the relationship between early antibiotics and NEC in preterm infants. Our objectives are (1) to summarize human and animal studies investigating early antibiotics and NEC, (2) to highlight challenges and limitations of these studies, (3) to explore mechanisms that may explain how early antibiotics can modify the risk for NEC, and (4) to identify future directions for research.

Human studies of early antibiotics and NEC

Randomized studies: old studies indicating that prophylactic early antibiotics may reduce NEC

Five RCTs (16–20) done in the 1970 s-1990 s were conducted to determine whether prophylactic early antibiotics are effective at preventing NEC in preterm infants (**Table 1**). Oral antibiotics with poor systemic absorption – such as kanamycin, gentamicin, and vancomycin – were used by the studies to limit antibiotic effects to the gastrointestinal tract (24), and were generally administered for 7 to 24 days as enteral feeds were advanced. Overall, a beneficial reduction in NEC with prophylactic early antibiotics was found in four of the five RCTs; and a Cochrane meta-analysis summarizing the 5 trials demonstrated that early antibiotics was beneficial in decreasing NEC by half (RR 0.47, 95% CI 0.28–0.78) (25). Interestingly, beneficial reduction in NEC was observed with antibiotics that targeted gram-negative bacteria (i.e., kanamycin and gentamicin) or gram-positive bacteria (i.e., vancomycin).

Despite these positive results, several limitations have dampened adoption of prophylactic early antibiotics to reduce NEC in clinical practice. One limitation is antibiotic resistance. This limitation was demonstrated in the study by Boyle et al. (17) where infants prophylactically treated with kanamycin had higher incidence of kanamycin-resistant enteric gram-negative bacteria compared to controls. A second limitation is selective growth of other pathogenic bacteria (26). This limitation was demonstrated in the study by Siu et. al (20) where infants treated with vancomycin prophylaxis exhibited heavy predominant growth of enteric yeast and gram-negative organisms compared to controls. A third limitation is the questionable generalizability to current clinical practice. These RCTs were done in an era before effective strategies to reduce NEC such as early feeding (27), standardized feeding protocols (28, 29), widespread use of human milk (30, 31) and enhanced infection control practices (32, 33) were part of routine clinical practice. It is thus unknown whether early antibiotics as tested in these early trials would remain effective at reducing NEC in the current setting.

Retrospective studies: studies that suggest an association between prolonged early antibiotics and NEC

Several retrospective studies have identified a harmful association between early antibiotics and NEC (**Table 2**). Among the first to report of this harmful association was Cotten et al. (8). Using the Neonatal Research Network (NRN) database, Cotten et al. (8) evaluated 4,039 extremely low birth weight (ELBW) infants who received early antibiotics within 72 h after birth and had sterile blood cultures. The authors found that prolonged early antibiotics for ≥5 days was associated with an increased risk for NEC or death compared to antibiotic treatment for < 5 days (aOR 1.30, 95% CI 1.10–1.54). In another study, Esmaeilizand et al. (12) used data from the Canadian Neonatal Network (CNN) to conduct a matched case-control study of infants with and without NEC. Among the factors they found to be associated with an increased risk for NEC was prolonged early antibiotics (aOR 2.02, 95% CI 1.55–3.13). A population-based study from the Norwegian Neonatal Network also found similar results of higher NEC (aOR 2.27, 95% CI 1.02–5.06) among preterm infants <32 weeks' gestation who were exposed to antibiotics for 3–5 days compared to 0–4 days exposure (34). Other smaller retrospective studies demonstrated how each additional day of empiric antibiotic exposure in the first 7 to 14 days of life can increase the risk for NEC (9, 11, 15) or the composite outcome of NEC + late-onset sepsis + death (10, 13). Taken together, these studies seem to suggest that prolonged treatment with early antibiotics despite negative blood cultures can increase the risk for NEC and other poor outcomes (**Table 2**).

A major limitation of these retrospective studies is confounding by indication that comes from the possibility that prolonged early antibiotics is simply a marker of illness severity. In the majority of the studies, infants treated with prolonged early antibiotics were also more premature, had lower birth weight, and more likely to be

TABLE 1 Randomized controlled trials of prophylactic oral antibiotics to reduce NEC.

First Author and Year	Sample size	Intervention	Results
Egan 1976	75	Oral kanamycin vs placebo	Kanamycin decreased NEC (0/35) vs controls (4/40), $p = 0.038$.
Boyle 1978	99	Oral kanamycin vs placebo	NEC rates not different between kanamycin-treated (3/49) and placebo (9/50), $p = 0.2$.
Grylack 1978	42	Oral gentamicin vs placebo	Prophylactic oral gentamicin decreased NEC (0/20) vs placebo (4/22), $p < 0.05$.
Fast 1994	200	Oral gentamicin vs oral IgA-IgG	Oral gentamicin decreased NEC vs oral IgA-IgG (1/100 vs 13/10), $p = 0.0004$.
Siu 1998	140	Oral vancomycin vs placebo	Oral vancomycin decreased NEC (9/71) vs placebo (19/69), $p = 0.035$.

IgA, immunoglobulin A; IgG, immunoglobulin G; NEC, necrotizing enterocolitis.

TABLE 2 Retrospective studies showing the association between prolonged early antibiotics and NEC.

First Author and Year	Study design	Study Population	Results
Cotten 2009	Multi-center retrospective cohort study	4,039 ELBW infants treated with early antibiotics despite sterile cultures. Infants who received ≥5 days early antibiotics were compared to infants who received <5 days.	Increased odds for death (1.46, 95% CI 1.19-1.78) and increased odds for NEC or death (1.30, 95% CI 1.10-1.54) associated with ≥5 days exposure to early antibiotics.
Alexander 2011	Single-center retrospective case-control study	124 NEC cases (stage 2 or 3) were compared to 248 controls that were matched by gestational age, birth weight, and birth year.	Cumulative duration of antibiotic exposure associated with increased risk of NEC (aOR 1.10, 95% CI 1.02-1.19).
Kuppala 2011	Multi-center retrospective cohort study	365 VLBW infants ≤ 32 weeks' gestation exposed to early antibiotics despite sterile cultures. Infants were categorized into prolonged antibiotics (≥5 days), limited antibiotics (1–4 days) and no antibiotics (0 days).	Each day of early antibiotic treatment was associated with increased odds for composite outcome of NEC, LOS, and death (aOR 1.24, 95% CI 1.07-1.44).
Ghany 2012	Single-center retrospective cohort study	207 VLBW infants who received early antibiotics despite sterile cultures. Antibiotic treatment for ≥5 days were compared to <5 days.	Each day of early antibiotic treatment was associated with increased odds of NEC (aOR 1.32, 95% CI 1.05-1.65).
Cantey 2018	Single-center retrospective cohort study	374 VLBW infants with gestational age <33 weeks at birth. Infants with composite outcome of interest (NEC + LOS + death) were compared to infants without this composite outcome.	Each day of early antibiotic treatment in the first 14 days of life was associated with increased risk for the composite outcome of NEC + LOS + death (aOR 1.24, 95% CI 1.17-1.31).
Esmaeilizand 2018	Multi-center retrospective case-control study	224 NEC cases (stage 2 or 3) were compared with 447 controls that were matched by gestational age, birth weight, and gender.	Early antibiotic treatment for ≥5 days was associated with increased NEC (aOR 2.02, 95% CI 1.55-3.13) compared to antibiotic treatment for 0–4 days.
Raba 2019	Single-center retrospective case-control study	22 NEC cases (stage 2 or 3) were compared with 32 controls that were matched by gestational age, sex, maternal chorioamnionitis exposure, and mode of delivery.	Prolonged early antibiotics for >5 days associated with NEC (OR 3.6, 95% CI 1.13-11.47).
Chen 2022	Single-center retrospective cohort study	132 VLBW infants were investigated by multivariable logistic regression to determine the association of antibiotic treatment and NEC.	Each day of early antibiotic treatment in the first 14 days of life was associated with increased odds of NEC (aOR 1.28, 95% CI 1.03-1.59).
Zhu 2022	Single-center retrospective cohort study	51 NEC cases (stage 2 or 3) were compared with 516 with no NEC. Infants were all VLBW and <32 weeks' gestation at birth.	Early antibiotic therapy duration was associated with increased odds of NEC (aOR 1.27, 95% CI 1.13-1.42).
Vatne 2022	Population-based retrospective study	4,932 VLBW infants were studied using nationwide registry of Norway. Association between empirical antibiotics and NEC was assessed using multivariable logistic regression models, adjusting for known confounders.	Antibiotics ≥ 5 days were associated with higher odds of NEC (aOR 2.27, 95% CI 1.02-5.06).

ELBW, extremely low birth weight; NEC, necrotizing enterocolitis; LOS, late-onset sepsis; VLBW, very low birth weight.

born in the setting of chorioamnionitis compared to infants treated for <5 days (8–10, 13). It is well-known that the incidence and severity of NEC is inversely correlated to prematurity and birth weight (35, 36). Moreover, maternal chorioamnionitis is an important risk factor for early-onset sepsis that often informs the decision to use early antibiotics treatment and has also been shown to increase risk for NEC (37). It is thus unclear whether it is prolonged early antibiotics or these differences in underlying baseline characteristics that truly increases risk for NEC. Efforts to control for these differences, such as by propensity matching or logistic regression, are likely not able to fully adjust for the impact of these differences in NEC risk.

Retrospective studies: studies that suggest a potential protective effect of limited early antibiotics against NEC

Other retrospective studies have demonstrated contrary findings of a protective association between early antibiotics and NEC (**Table 3**). The first two studies to report of this protective association were small, case control studies with approximately 200 to 350 infants (38, 39). Krediet et al. (38) conducted a matched case-control study ($n = 208$ infants) to identify risk factors that may explain an increase in NEC incidence at their local institution. The authors found that treatment with antibiotics within 48 h after birth was associated with a reduction in NEC (OR 0.3, 95% CI 0.2–0.6). Berkhout et al. (39) also conducted a matched case-control study ($n = 336$ infants) and found a similar association of decreased NEC with early antibiotics. Three subsequent studies (21–23) were large, multi-center studies with approximately 1,200 to 14,000 infants. The largest of these studies was Ting et al. (22) ($n = 14,207$ infants). Using data from the CNN, Ting et al. (22) investigated the impact of early antibiotics on neonatal outcomes and found that limited early antibiotics (≤3 days) was associated with a reduction in NEC compared to untreated controls (aOR 0.74, 95% CI 0.55–0.99). The second largest of these studies was Li et al. (21) ($n = 2,831$ infants). Using prospective data collected from 13 neonatal intensive care units from five continents, Li et al. found that NEC incidence was lower among infants treated with early antibiotics compared to infants with no antibiotic exposure (aOR 0.25, 95% CI 0.12–0.47). Lastly, Dierikx et al. (23) studied

TABLE 3 Retrospective studies suggesting that limited early antibiotics decreases risk for NEC.

First Author and Year	Study design	Study Population	Results
Krediet 2003	Single-center matched case-control study	104 NEC cases (stage 2 or 3) were compared to 104 controls matched by gestational age, birth weight, and period of admission.	Antibiotic treatment <48 h after birth was associated with decreased risk for NEC (aOR 0.3, 95% CI 0.2-0.6).
Berkhout 2018	Multi-center matched case-control study	56 NEC cases (stage 2 or 3) were compared to 280 controls that were matched by gestational age, birth weight, and postnatal age of NEC. Infants with 1–3 days and >3 days of antibiotics were compared to infants with no antibiotics as reference.	Decreased NEC occurrence was associated with antibiotic exposure for 1–3 days (aOR 0.21, 95% CI 0.08-0.54) and >3 days (aOR 0.23, 95% CI 0.08-0.65).
Ting 2019	Multi-center retrospective cohort study	14,207 VLBW infants with sterile cultures were divided based on antibiotic exposure of 0 days, 1–3 days, and 4–7 days.	Infants exposed to limited antibiotics for 1–3 days have lower odds of NEC (aOR 0.74, 0.55–0.99) than infants who did not receive any antibiotics.
Li 2020	Multi-center retrospective cohort study	2,562 VLBW infants who received early antibiotics within 72 h after birth were compared to 269 VLBW infants who did not receive early antibiotics.	NEC incidence was lower in infants who received early antibiotics (aOR 0.57, 95% CI 0.35-0.94).
Dierikx 2022	Multi-center retrospective cohort study	1,259 infants <30 weeks' gestation with sterile cultures were divided into no antibiotics, short antibiotics exposure (≤3 days), and long antibiotics exposure (>3 days).	Short antibiotic exposure had decreased NEC incidence compared to long antibiotic exposure (aOR 0.58, 95% CI 0.35-0.96) and no antibiotic exposure (aOR 0.39, 95% CI 0.19-0.80).

NEC, necrotizing enterocolitis; VLBW, very low birth weight infants.

1,259 very low birth weight (VLBW) infants from 9 centers in the Netherlands and Belgium and found that early antibiotics was associated with decreased risk for NEC compared to no antibiotics (aOR 0.47, 95% CI 0.23–0.96).

Analysis based on duration of treatment provided additional insights regarding the relationship between early antibiotics and NEC. In the CNN study (22), Ting et al. divided the study cohort based on duration of antibiotic treatment (0 days vs. ≤3 days vs. >3 days). The authors found that limited early antibiotics (≤3 days) was associated with a reduction in NEC compared to untreated controls (0.74, 95% CI 0.55–0.99); but prolonged early antibiotics (>3 days) was not associated with either increased or decreased NEC risk when compared to either 0 days or ≤3 days. Dierikx et al. (23) also performed additional analysis based on duration of treatment and found similar results of protective effects of limited early antibiotics given for ≤3 days; whereas prolonged early antibiotics (>3 days) was neither harmful nor protective. While these two studies suggest that a limited course of early antibiotics (≤3 days) may help reduce the risk for NEC in preterm infants, the study by Vatne et al. (34) had different results. In their large population-based study, limited treatment with early antibiotics for 1–3 days did *not* have a protective effect compared to untreated controls (aOR 2.02, 95% CI 0.22–18.3).

A potential limitation of studies suggesting that limited early antibiotics can protect against NEC is the use of infants with no antibiotic exposure as the reference group. This limitation was suggested by Berkhout et al. (39) as another form of confounding by indication that arises from the possibility that infants with no antibiotic exposure represent an underrecognized population with high baseline risk for NEC. In the three large multi-center studies referenced above, infants with no antibiotic exposure were more likely to be small for gestational age (SGA) and born by caesarian section without premature rupture of membranes compared to infants treated with early antibiotics (21–23). These differences in baseline characteristics suggest that infants with no antibiotic exposure were born prematurely due to poor fetal Dopplers and intrauterine growth restriction which, while considered low-risk for early-onset sepsis (40, 41), are associated with higher risk for NEC (42, 43). Thus, there is a possibility that using infants with "no antibiotic exposure" as the reference may make it appear that early antibiotics is protective against NEC.

Animal studies investigating the relationship of early antibiotics and NEC

Given the varying results and important limitations of existing studies in humans, studies using animal models have been conducted to provide mechanistic insights on the effects of early antibiotics on the developing neonatal gut. In this section, we will review findings from two experimental animal models of early antibiotics and NEC, explore potential mechanisms that explain their results, and discuss the differences and limitations of each model.

Piglet model of early antibiotics and NEC

The first animal model used to investigate the effects of early antibiotics on the newborn gut was the preterm pig model of experimental NEC. In this model, pigs that were delivered prematurely *via* caesarian section at ~92% gestation and transitioned gradually from parenteral to enteral nutrition over the next 5 days develop experimental NEC spontaneously (44). But when early antibiotics were administered concurrently starting from birth until time of sacrifice, substantial protection from NEC among antibiotic-treated pigs was demonstrated compared to untreated controls (45, 46). Interestingly, the protective effects against NEC were limited to when antibiotics were given orally and not parenterally (47, 48) – a finding that mirrors early RCTs of prophylactic early antibiotics (25). Thus, studies using this piglet

model provide evidence supporting findings from human studies which suggest that early antibiotics is protective against NEC.

However, it is important to note that more recent investigation (49) with the piglet model have identified important adverse effects of early antibiotics, including emergence of antibiotic-resistant gut organisms and suppression of systemic immune function. Combining oral antibiotics with fecal microbiota transplantation did not prevent the adverse effects of oral antibiotics as hypothesized, suggesting pervasive effects of antibiotics on immune function. Thus, while early antibiotics were protective against NEC in piglets, important adverse effects were also found that warrant further investigation.

Mouse model of early antibiotics and NEC

Another animal model used to investigate early antibiotics and NEC was the newborn mouse model of NEC. In this model, newborn mice delivered naturally at term were immediately exposed to 10 days of systemic antibiotics (50). After a washout period of 4 days, the pups were then exposed to oral bacterial challenge with *Klebsiella spp*. to induce NEC. The authors found that NEC-like intestinal injury was significantly worse in antibiotic-treated pups compared to untreated controls. Thus, in contrast to the piglet model, studies using the newborn mouse model provide evidence supporting findings from other human studies which suggest that early, prolonged, systemic exposure to antibiotics increases risk for NEC.

Differences between piglet and mouse model of early antibiotics and NEC

Several experimental differences between the piglet and mouse models may explain the opposing findings from animal studies (**Supplementary Table**). One difference is with regards to the duration of early antibiotics. In the piglet model, piglets were treated for only 5 days of antibiotics, whereas in the mouse model, pups were treated for 10 days. Modulating effects of antibiotic duration on intestinal injury can be seen in experiments with adult mice, where 4 days resulted in transient ileal injury that quickly reverses by stopping treatment (51), whereas 14 days of antibiotics caused several more intestinal impairments including gut dysbiosis, reduced short-chain fatty acid concentrations, disrupted intestinal tight junction barrier, and increased activation of autophagy (52). Additional experimental studies that vary duration of early antibiotics within the same animal model may help better elucidate the impact of duration of treatment on NEC susceptibility. Another difference that can explain these opposing findings is the differences in gestational age used in each animal model. In the piglet model, piglets were born *via* caesarian section at 92% gestation, whereas the mouse model used mouse pups born naturally at term gestation. Thus, it is possible that the experiments in pigs modeled the effects of early antibiotics in preterm infants, while the mice experiments modeled the effects of early antibiotics in term infants. Other differences that could explain the opposing findings between the two animal models include differences in route of antibiotic administration, method of induction of experimental NEC, and the presence or absence of a wash-off period from antibiotics before NEC induction (**Supplementary Table**).

Proposed mechanisms by which by early antibiotics might increase or decrease the risk of NEC

Delayed bacterial colonization allows preterm gut defenses to mature and decreases NEC risk

Delayed bacterial colonization is hypothesized as the main mechanism by which early antibiotics protect against NEC (**Figure 1**). Studies in mice as well as human samples from immature intestine have shown that the preterm gut is inherently predisposed towards excessive inflammation (53–55). Delaying gut colonization can potentially allow more time for the preterm gut defenses to develop and mature before encountering bacteria, viruses, and fungi that can otherwise trigger pathologic intestinal inflammation and NEC. In a physiologic study of infants, intestinal permeability was higher in preterm compared to term infants only in the first 2 days of life. By 3 to 6 days of life, intestinal permeability between preterm and term infants was already similar, suggesting rapid postnatal adaptation of preterm intestinal mucosal barrier (56). A limited course of early antibiotics during the first few days after preterm birth may thus be sufficient to achieve this delayed colonization and allow maturation of preterm gut defenses without harming the developing gut microbiome (57). This hypothesis is further supported by the several improvements in intestinal structure, function, and immunity that have been identified in preterm pigs treated with early antibiotics for the first 5 days of life. These include increased villus height, higher digestive enzyme activity, increased goblet cell density, reduced gut permeability, downregulation of genes related to inflammation and innate immune response, and upregulation of genes related to metabolism (45–48). Thus, there is supporting evidence – from experimental piglet studies and from older RCTs of preterm infants – that early antibiotics can be protective against NEC by delaying bacterial colonization and allowing the immature intestine to better adapt to postnatal milieu.

Aberrant gut colonization disrupts proper postnatal intestinal adaptation and increases NEC risk

On the other hand, aberrant gut colonization is the main mechanism by which early antibiotics is hypothesized to increase risk for NEC (**Figure 1**) (58). Early antibiotics can predispose to aberrant gut colonization in a few ways. One is by suppression of beneficial bacteria that contribute to the physiologic development of the postnatal gut (59, 60). While beyond the scope of this review, several studies have demonstrated that synergistic relationships between colonizing microbes and the host gut mucosa are crucial for successful postnatal intestinal adaptation (61–63). For example, studies in mice reveal that the interaction of commensal bacteria with intestinal TLR signaling plays a critical

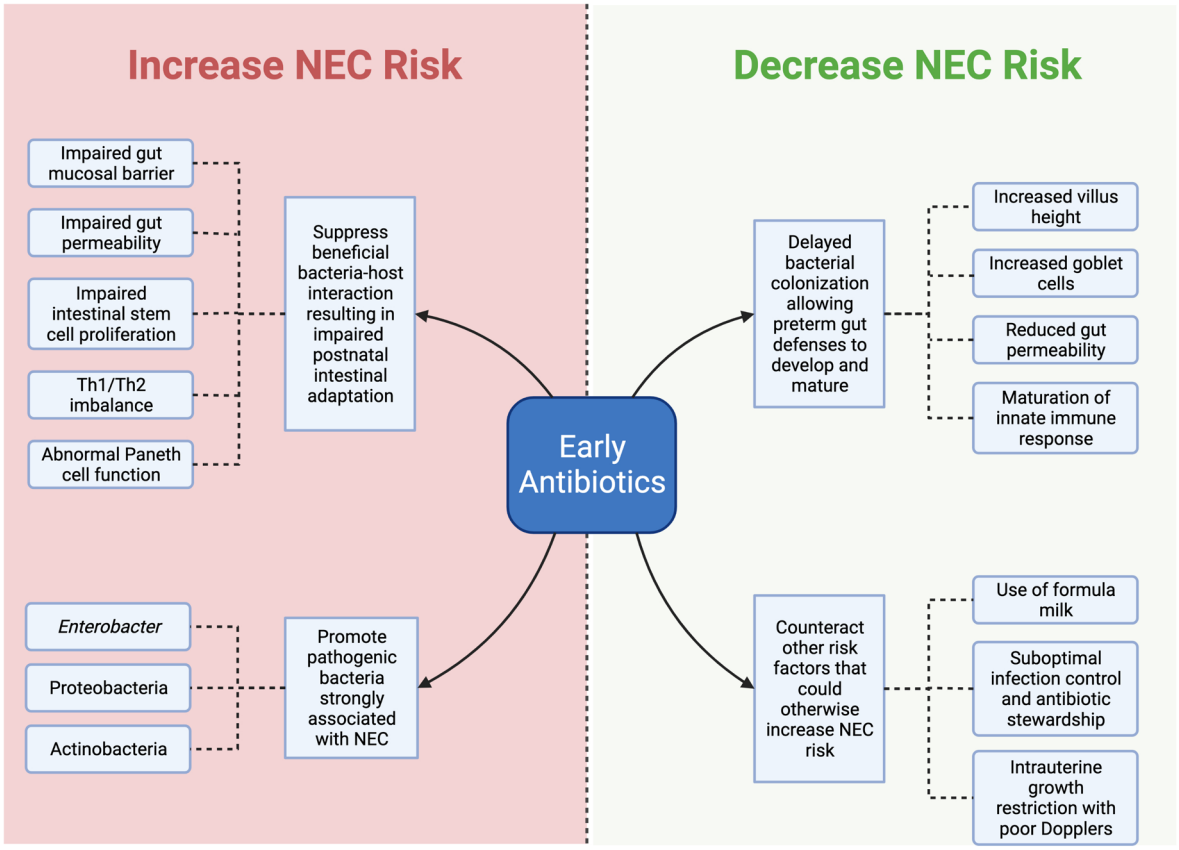

FIGURE 1
Diagram of potential mechanisms by which early antibiotics can increase or decrease NEC risk. Created with Biorender.com.

role for maintaining intestinal epithelial homeostasis and helps protect against gut injury (61). In another study, gut colonization of mice with the symbiotic bacteria *Bacteroides fragilis* was found to mediate establishment of proper Th1/Th2 balance through bacterial surface polysaccharide A (62). Disruptions to this normal process of gut colonization with commensals – such as with early antibiotic use – can thus lead to a dysfunctional gut mucosa predisposed to NEC (64–66). This hypothesis is supported by the mouse model by Chaaban et al. (50) where exposure of newborn pups to 10 days of antibiotics resulted in several impairments to gut mucosal barrier, intestinal permeability, intestinal stem cell proliferation, and Paneth cell function.

Another way by which early antibiotics cause aberrant gut colonization is by increasing the population of potentially pathogenic bacteria. Next-generation sequencing of stools from preterm infants demonstrated how antibiotic treatment is associated with increased relative abundance of *Enterobacter*, Proteobacteria, Actinobacteria in conjunction with decreased relative abundance of Firmicutes and Bacteroidetes (67–69). Moreover, this abnormal pattern of increased Proteobacteria and decreased Firmicutes and Bacteroidetes have been identified in gut microbiota studies to precede development of NEC in preterm infants (70–73). In animals, landmark studies have shown how antibiotic-treated animals but not untreated controls are susceptible to pathogenic bacterial challenges (74, 75), partly due to loss of colonization resistance afforded by commensals (76).

Thus, there is also supporting evidence – originating from mouse models as well as infant gut microbiome studies – that early antibiotics can be harmful to the developing neonatal gut by increasing pathogenic bacteria at the expense of beneficial commensals.

Additional speculations from human and animal studies

Is there an interaction between early antibiotics and other risk factors of NEC?

NEC is multi-factorial in origin, and early antibiotics exposure is only one of several risk factors that could modify NEC risk. One speculation is that perhaps harm or protection against NEC can depend on the interaction of early antibiotics with other risk factors of NEC (77, 78). For example, feeding with formula is a strong risk factor for NEC that is known to alter the developing gut microbiome; whereas feeding with human milk is protective and promotes colonization with beneficial commensals (79). It is thus possible that early antibiotics is protective when formula feeding is prevalent such as during RCTs of the 1970 s-1990 s; but is now harmful in the current era when human milk is the feeding standard for preterm infants. In addition to the type of milk,

variation in advancement of feeding in preterm infants could also play a confounding role in determining the impact of early antibiotics and NEC (80).

Another risk factor for NEC is intestinal colonization with harmful pathogens from the NICU environment. In the study by Li et al. (21), about half of the cohort came from Asia where antibiotic stewardship and infection control practices can be a challenge (81–83), and nosocomial infection with resistant strains is high (84–86). It is thus possible that early antibiotics is protective in NICU environments where the local antibiogram has a predominance of pathogenic and resistant bacterial isolates. However, the protective association of early antibiotics and NEC was also found in studies from developed counties such as Canada and Europe, where antibiotic stewardship and infection control practices are more robust. This consistency across developed and developing countries suggest that the beneficial effect of early antibiotics remain despite differences in these factors.

Another important factor that can interact with early antibiotics is supplementation with probiotics. While beyond the scope of this review, there is extensive literature supporting the protective effects of probiotics against NEC in general (87, 88). Looking specifically at the interaction of early antibiotics and probiotics, one study showed that antibiotic-treated mice supplemented with probiotics exhibited a reduction in pathogenic *Enterobacteriaceae* while promoting growth of commensal *Firmicutes* compared to antibiotic-treated mice with no probiotic supplementation (89). Similarly, in a prospective observational study, extremely preterm infants with high antibiotic exposure that also received probiotics had comparable microbial diversity and antibiotic resistome as more mature infants, suggesting that probiotic supplementation may have alleviated the harmful effects of antibiotics on the gut microbiota (90). Other factors that might interact with early antibiotics to modify future NEC risk include prior maternal exposure to antibiotics (91–93), genetic predisposition to NEC (4), and genetic predilection for antibiotic resistance (94).

Is limiting activity of early antibiotics key?

We also speculate that perhaps limiting antibiotic activity may be the key for reaping benefits of early antibiotics on NEC risk without harm. The early clinical trials that showed benefit of prophylactic antibiotics used oral agents with narrow spectrum and poor systemic absorption that limited antibiotic activity to the gastrointestinal tract (25). On the other hand, more recent studies that used broad-spectrum antibiotics given intravenously as part of clinical care seem to suggest that a limited exposure of less than 3 to 5 days can decrease subsequent risk for NEC (22, 23, 39). In animal models, prolonged treatment for 10 days with antibiotics resulted in several intestinal impairments and increased NEC severity compared to controls (50) but limited treatment for 5 days with poorly absorbed oral antibiotics caused improved maturation of preterm gut defenses and decreased NEC (45).

Studies that investigated the effects of antibiotics on gut microbiome also provide evidence that limited early antibiotics may not be as harmful as previously thought. In one study, Zwittink et al. (95) obtained fecal samples from preterm infants with no, short (≤3 days), or long (≥5 days) treatment with antibiotics. 16S rRNA sequencing revealed that while both short and long antibiotic treatment significantly lowered the abundance of the commensal *Bifidobacterium*, quick recovery of *Bifidobacterium* abundance was observed among infants exposed to short antibiotics while infants exposed to long antibiotics exhibited a persistent reduction of *Bifidobacterium*. In another study, Kim et al. (57) randomized preterm infants at low risk for sepsis to receive 2 days of placebo vs. ampicillin and gentamicin, analyzed their fecal microbiome, and administered early fecal supernatant to pregnant gnotobiotic mice. Surprisingly, in this study limited treatment with 2 days of antibiotics did not alter the fecal microbiome of treated infants compared to placebo; and pups of gnotobiotic pregnant mice exposed to the fecal supernatant of antibiotic-treated infants did not have any differences in gut microbiome, weight gain, and markers of intestinal health compared to controls.

Thus, there is evidence from both human and animal studies to suggest that limiting early antibiotics – whether by using narrow-spectrum, poorly absorbed oral antibiotics that limit activity in the intestinal tract, or by using broad-spectrum intravenous antibiotics but treating for shorter periods of time – may not be harmful and may have some benefit in decreasing NEC risk. One important caveat about poorly absorbed oral antibiotics in preterm infants is that in some studies, substantial systemic concentrations of these oral antibiotics can be found in the serum, especially when given in the first few days of life (96).

Do antibiotics have direct effects on host immunity and inflammation?

It is also possible that antibiotics have direct effects on immune cells and immune-mediated receptors that can modify risk for NEC (97, 98). For example, *in vitro* studies revealed that gentamicin, a first-line antibiotic drug of choice for neonatal sepsis, can directly inhibit the chemotactic response of human polymorphonuclear leukocytes (99). In another study, mice given Ampicillin or Vancomycin, two other antibiotics commonly used in neonates, exhibited significant downregulation of Th17-related genes in the ileum (100). In the piglet model of NEC, 5 days of antibiotic treatment resulted in significant downregulation of genes related to inflammation and innate immune response following compared to controls (45). Recent studies also suggest that antibiotic-induced elimination of bacterial pathogens can elicit the release of microbial components such as LPS that further worsens inflammation (101, 102). While it is difficult to discern whether these immune changes are independent of antibiotic-induced alterations in gut microbiome, there is accumulating evidence that antibiotics can have direct effects on host immunity and inflammation which may impact disease (103).

Summary and future directions

Although human and animal studies seem to suggest that treatment with early antibiotics can alter future risk for NEC (**Table 4**), inherent limitations of these studies must also be carefully considered for proper interpretation. RCTs done several

TABLE 4 Summary of human and animal studies regarding early antibiotics and NEC.

Study Design	Increased risk of NEC	Decreased risk of NEC	No difference in NEC
Randomized clinical trials		Egan 1976 Grylack 1978 Fast 1994 Siu 1998	Boyle 1978 Tagare 2010
Retrospective clinical studies	Cotten 2009 Alexander 2011 Kuppala 2011 Ghany 2012 Cantey 2018 Esmaeilizand 2018 Raba 2019 Chen 2022 Zhu 2022 Vatne 2022	Krediet 2003 Berkhout 2018 Ting 2019 Li 2020 Dierikx 2022	Greenberg 2019
Animal studies	Chaaban 2022	Sangild 2006 Jiang 2012 Jensen 2014 Nguyen 2016 Birck 2016	

decades ago with oral, non-absorbable, and narrow-spectrum antibiotics showed a reduction in NEC, but the relevance of such studies to modern NICU practice is uncertain. A more recent RCT of prophylactic intravenous antibiotics for 5 days vs. no antibiotics did not find any benefit with prophylactic antibiotics, but the study included low-risk infants (median gestational age 34 weeks) and was not powered to detect differences in NEC ($N = 140$) (104). Retrospective cohort studies suggest that prolonged duration of early antibiotics (>3 to 5 days) can increase risk for NEC, but these studies present only low quality of evidence as there is significant confounding by indication of antibiotic use and unequal exposure to other NEC-associated risk factors. Other retrospective studies suggest that limited duration of antibiotic use (<3 to 5 days) may decrease NEC risk, but these studies should also be interpreted with caution as using infants with no antibiotic exposure as reference may be a source of confounding bias. Interestingly, some animal studies seem to mimic human data with regards to duration of antibiotics and NEC risk, but additional experimentation to evaluate the impact of several other important variables – such as gestational age and mode of NEC induction – is needed. Of note, none of the human studies and few of the animal studies examined the effects of early antibiotics on the gut microbiome, further limiting mechanistic interpretation of results.

Additional studies in humans and animals are needed to attain a better understanding of the effects of early antibiotics on later NEC risk. Studies that evaluate effects of early antibiotics in intestinal immunity should also evaluate parallel changes in the gut microbiome. As stool samples may only reflect changes in either colonic mucosa or transient luminal contents, animal studies should endeavor to obtain intestinal mucosal samples from different parts of the intestinal tract to accurately investigate host and microbiota changes induced by antibiotics in the gut mucosa. In addition to antibiotic-induced changes on the gut microbiome, additional research into the direct effects of antibiotics on intestinal immunity is also needed. Ultimately, the inherent limitations of existing human studies warrant large prospective RCTs (105) as well as well-designed prospective observational studies (106) to study the impact of withholding early antibiotic use or limiting duration of exposure on NEC as well as other outcomes including late-onset sepsis and bronchopulmonary dysplasia. The NICU Antibiotics and Outcomes Trial (NANO) as well as other studies are beginning to address this question (106–108). Future clinical practice on early antibiotics use will likely be impacted by these ongoing studies. In the meantime, current efforts to implement sound antibiotic stewardship practices in the NICU should be followed (109, 110). This includes limiting prophylactic administration of early antibiotics only to infants with strong concerns for early-onset sepsis, such as those with prolonged rupture of membranes or maternal chorioamnionitis (40, 41). Antibiotics should also be promptly discontinued once blood cultures remain sterile for 24 to 48 h (111). Prolonged use of early antibiotics in the absence of positive blood cultures should be discouraged.

Author contributions

AC and VS conceptualized the study. AC wrote the first draft of the manuscript. VS and MM critically reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2023.1112812/full#supplementary-material.

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Gut-Brain cross talk: The pathogenesis of neurodevelopmental impairment in necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is a devastating condition of multi-factorial origin that affects the intestine of premature infants and results in high morbidity and mortality. Infants that survive contend with several long-term sequelae including neurodevelopmental impairment (NDI)—which encompasses cognitive and psychosocial deficits as well as motor, vision, and hearing impairment. Alterations in the gut-brain axis (GBA) homeostasis have been implicated in the pathogenesis of NEC and the development of NDI. The crosstalk along the GBA suggests that microbial dysbiosis and subsequent bowel injury can initiate systemic inflammation which is followed by pathogenic signaling cascades with multiple pathways that ultimately lead to the brain. These signals reach the brain and activate an inflammatory cascade in the brain resulting in white matter injury, impaired myelination, delayed head growth, and eventual downstream NDI. The purpose of this review is to summarize the NDI seen in NEC, discuss what is known about the GBA, explore the relationship between the GBA and perinatal brain injury in the setting of NEC, and finally, highlight the existing research into possible therapies to help prevent these deleterious outcomes.

KEYWORDS

gut-brain axis, necrotizing enterocolitis, perinatal brain injury, microbiome, neonatal brain, neurodevelopmental impairment

1. Introduction

Necrotizing enterocolitis (NEC) is a devastating condition that primarily affects premature neonates and is associated with high morbidity and mortality rates (1). The pathophysiology of this disease is multifactorial and is thought to be driven by an immature intestine and immune system, microbial dysbiosis, and a cascade of inflammatory responses (1, 2) that can result in intestinal injury and necrosis, which often progress to requiring surgery and intestinal resection (2). If these neonates survive, they are faced with several downstream complications including intestinal malabsorption, chronic lung disease, and neurodevelopmental impairment (NDI) (3, 4)—much of which is mediated by the complex interplay of the gut-brain axis (GBA). Although it is understood that infants with a history of NEC go on to have worse neurodevelopmental outcomes (3), the pathogenesis of perinatal brain injury in NEC, the causes of downstream development of NDI, and the role of the GBA are not well understood.

The gut-brain axis (GBA) is defined as the interaction of several systems including: the central nervous system (CNS); the autonomic nervous system (ANS); the microbiome; and the many neural, immune, and hormonal signaling pathways that exist between them (5–9). Alterations in the neonatal microbiome and the intestinal injury seen in NEC contribute to

pathogenic alterations in GBA signaling (5). This activation then triggers the downstream CNS inflammatory cascade seen in perinatal brain injury, which involves the activation of microglia —the main mediator of the innate immune system's response to brain injury (10). In addition, the stress of prematurity, maternal separation, and formula feeding can further activate the GBA in reverse and exacerbate this disease process (9). NEC usually occurs during a period of crucial and dynamic neurological development leaving the infant particularly susceptible to the pathogenesis of this disease (11), which leads to both short and long-term neurodevelopmental impairment (12). The purpose of this review is to summarize what is known about neurodevelopmental outcomes in NEC, the proposed interplay of the gut-brain axis in the pathophysiology of this disease, and to highlight research into possible therapies to help improve these detrimental outcomes.

2. Neurological and developmental delay seen in NEC

The presence of neurological changes and subsequent NDI in patients with a history of NEC is well established (13, 14). The systemic inflammatory response triggered by NEC may be mediated *via* bacterial products and cytokines released during intestinal injury. This, combined with the associated hypotension that is part of the systemic inflammatory response, results in signals traversing the GBA and causes well-documented white-matter injury (6, 15). Other neural changes noted include alterations of the brain parenchyma, decreased head circumference, and corresponding decreased volumes in total brain matter (16, 17). This stunted head growth and altered brain parenchyma in early infancy are detrimental to later cognitive outcomes and result in downstream NDI including: a higher incidence of cerebral palsy (CP), impaired motor function, visual and hearing impairment, and cognitive deficits (14, 16, 17).

2.1. Assessing neurodevelopmental impairment

There are a barrage of developmental screening tests for children used for early detection of developmental delays with the goal of identifying if a child has reached specific physical, cognitive, social-emotional, and behavioral milestones (18). It is important to keep in mind that these milestones are often modified by historical and cultural factors and the assessments themselves are limited by the training, availability of assessors, and the education level and socioeconomic status of parents. Despite these difficulties, there remain a series of established assessments that aim to evaluate these milestones, however, no established assessment and timing of assessments exist for looking at NDI in NEC. For this review, we will focus on describing a few assessments that are specifically targeted and validated for screening for developmental delays in high-risk populations (19, 20).

2.1.1. Ages and Stages Questionnaire -3rd edition (ASQ-3)

The ASQ-3 is a developmental screening tool that utilizes a parent-centric model. This questionnaire can be used in both general primary care and in higher-risk categories such as evaluating children that were born prematurely. The questionnaire is given at pre-determined ages (adjusted for corrected gestational age) and tracks the developmental progress of children between the ages of one month to just over 5 years. The benefit of this questionnaire is that it has an easy learning curve for administration, has several different language options, and is quick to administer (20, 21).

2.1.2. Bayley scales of infant and toddler development

The Bayley Scales of Infant and Toddler Developmental assessment is a widely used and the most psychometrically sophisticated assessment of development in infants and toddlers. This scale is advantageous because it is especially useful to screen high-risk populations such as those infants that are pre-term, have lower birth weight, or are from a lower socioeconomic status. It assesses cognition, language, motor, social-emotional, and adaptive behavior with an administration time ranging from 30 to 90 min. Most studies looking at NDI use this assessment, however, the drawbacks are that it is a difficult assessment to administer—requiring specialty training and a lengthy period of time with the patient and their families (19, 20).

2.1.3. Cognitive adaptive test/clinical linguistic auditory milestone scale (CAT/CLAMS) (20)

Like the Bayley Scales Assessment, the Cognitive Adaptive Test/Clinical Linguistic Auditory Milestone Scale (CAT/CLAMS) is another assessment that is practitioner-administered and specifically advantageous for high-risk children—especially from pre-term or low-birth-weight populations. It is a relatively newer assessment but compares favorably to the Bayley assessments and looks at language, problem-solving, and visual motor skills in children from birth to 3 years old. The CAT/CLAMS is also especially useful because it has high validity to target and identify early language delays (20, 22).

2.2. Clinical changes and neurodevelopmental impairment in NEC

A systematic review performed by Rees et al. in 2006, looked at 7,843 premature children (821 of which had NEC) and their neurodevelopmental milestones over an average of 20 months. These results demonstrated that infants with a history of NEC were more likely to have some form of neurodevelopmental impairment (NDI). Specifically, the breakdown showed that 20% of the patients with NEC developed CP, 3% developed visual impairments, 3% hearing deficits, 36% cognitive deficits, and 35% psychomotor impairments. Interestingly, when the data was further stratified, those with medical NEC were not found to have significant neurodevelopmental impairment when compared to the

cohort without NEC (prematurity alone), while those in the "surgical NEC group" had a more significant impairment, worse outcomes, and higher rates of CP and psychomotor impairment overall (12). Another review analyzed a database of 12,992 very low birth weight (VLBW) infants in Israel and looked at the association of several neonatal co-morbidities (including NEC) with the risk of head growth failure (HGF)—defined as head circumference z-score that was greater than two z-scores below the mean. Overall, the risk of severe HGF was associated with a nearly 3-fold greater odds with a diagnosis of NEC. These differences are even more disparate when surgery becomes necessary, and infants diagnosed with surgical NEC had an odds ratio of 7.62 associated with the development of severe HGF (23).

The pathogenesis of NEC progresses to requiring surgery for several reasons including, free intra-abdominal air and/or clinical deterioration despite optimal medical management—often translating to worse outcomes in infants with "surgical NEC" (24). The disparity between NDI in medical and surgical NEC is further illustrated by a multi-center retrospective review of 2,948 extremely low birth weight (ELBW) infants. At a corrected age of 18 to 22 months, infants with "surgical NEC" were found to have significantly reduced weight, length, and head circumference when compared to infants without NEC or with medical NEC. On Bayley Scales of Infant Development assessments, surgical NEC, but not medical NEC, was found to be an independent risk factor for lower scores on the mental developmental index (MDI), psychomotor developmental index (PDI), and resulted in an increased risk of neuro-developmental impairment (NDI) (25). This disparity in surgical vs. medical NEC is highlighted again by a study by Martin et al. looking at a cohort of 1,155 neonates for the development of surgical or medical NEC and accompanied prognostic factors. Those who had both surgical NEC and late bacteremia had worse NDI, with the group citing an increased risk of CP [OR = 8.4 (1.9, 39)] and microcephaly [OR = 9.3 (2.2, 40)]. Like the previous study, children with medical NEC with or without late bacteremia were not at increased risk of any developmental dysfunction (26).

It is also important to note that NDI seen in early childhood testing often persists in school-aged children. Rose et al. looked at neurodevelopmental outcomes of school-aged children with a history of surgical NEC or SIP (spontaneous intestinal perforation) and compared them with matched controls (27). Although this study combined outcomes from SIP and NEC, the data still showed that the combined cohort had more abnormal motor function scores (as assessed by Movement Assessment Battery for Children) and lower intelligence quotients (IQ)-(86 ± 14 compared with 97 ± 9 in the controls) (27), supporting the hypothesis that NDI persists past infancy.

2.3. Necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (SIP)

Spontaneous intestinal perforation (SIP) is a discrete entity from NEC and is characterized by an isolated perforation in the gastrointestinal tract. The presentation of an infant with SIP and NEC with perforation can be similar, however, the significant systemic inflammatory response of NEC isn't seen in SIP patients with these infants faring better after resection (28). Although NEC and SIP are often grouped together, NEC has been shown to have more significant NDI as evidenced by a retrospective study on preterm infants that compared neurodevelopmental outcomes within a cohort of NEC and SIP patients (29). A battery of neurodevelopmental assessments showed more significant abnormal findings in NEC compared to SIP in gross and fine motor skills as well as cognitive deficits (29), suggesting that the inflammatory process of NEC plays a greater role in brain injury and development of NDI.

The severity of NEC and the need for surgery demonstrating worse NDI lends itself to the question if surgery itself contributes to the NDI seen. The Necrotizing Enterocolitis Surgery Trial (NEST) looked at 310 extremely low birth weight infants (EBLW) and evaluated the difference between initial laparotomy vs. drainage on the rates of death or NDI (data collected from 18 to 22 months) in NEC and SIP. NEST ultimately determined that initial laparotomy was more likely to reduce rates of death or NDI in infants with a preoperative diagnosis of NEC when compared to placing a Penrose drain (30). This data echoes an earlier observational study in 2006 that showed that NEC (when compared to SIP) had a higher-odds of death and NDI at 18–22 months of adjusted age (31). These data indicate that surgical intervention itself, is not the primary driver of NDI, as those with worsening clinical NEC did better with more aggressive surgical intervention(laparotomy) vs. leaving a drain in place.

The above studies demonstrate that surgical NEC has worse NDI outcomes than SIP, and it is the progression of NEC pathogenesis to requiring surgery that leads to worse NDI (not surgical intervention alone). A retrospective analysis of preterm infants by Bell et al. clarifies this issue further and looks at outcomes of patients with NEC and SIP with or without short bowel syndrome (SBS). The risk of development of moderate to severe NDI was 77% in the cohort of infants with NEC/SIP and SBS when compared to 62% of those with NEC/SIP without SBS (aRR 1.22) and 44% with no NEC, SIP, or SBS (aRR 1.6). In addition, children developing short bowel syndrome had lower cognitive, language, and motor scores on Bayley assessments than the cohort with NEC/SIP that didn't develop SBS (32). Although this study didn't differentiate between surgical NEC and SIP, it did highlight that the surgical resection of intestine—resulting in short-bowel syndrome (SBS)—is another contributing factor to the development of long-term NDI. In summary, the existing literature on NDI indicates that surgical NEC has high rates of NDI when compared to SIP, medical NEC, and prematurity alone. In addition, the development of SBS results in even higher NDI.

2.4. MRI changes in parenchyma correspond to NEC severity

The presence of increased parenchymal abnormalities in NEC patients as seen on magnetic resonance imaging (MRI) has been validated in several studies. MRIs performed on a prospective cohort of 192 premature infants at birth and repeated at 2 years old showed that infants with sepsis and/or NEC had a higher prevalence and severity of white matter abnormality, and specifically that infants with NEC had higher rates of concurrent

gray matter abnormality. Unsurprisingly, infants with surgical NEC had more severe brain injury detected on MRI when compared with infants with medical NEC. When adjusted for other factors, this cohort was also found to have delayed cognitive and motor impairment (17) as demonstrated in the studies described earlier. In another study of 26 premature infants with NEC or SIP, infants with surgical NEC and SIP were found to have more significant brain injury seen on MRI, when compared with infants with medical NEC, even after adjustment for confounders (33). It is important to note that the patients with SIP were combined in the group with surgical NEC, so we are unable to extrapolate about the difference in significance between SIP and surgical NEC brain injury on MRI. More recently, another study looked at 69 infants with surgical NEC and found that 52% had some form of white matter brain injury as seen on MRI and were subsequently more likely to have a severe postoperative course. Those that survived with known white matter brain injury were found to have lower mean motor, cognitive, and language scores as well as higher rates of visual impairment at 2 years of age (34). These studies together show that NEC severity corresponds to parenchymal changes and especially white matter injury as seen on MRI. These observations along with others (17, 34) support the hypothesis that bowel injury initiates inflammation that potentially affects the developing brain (26).

3. The Gut-Brain Axis: an explanation for neurodevelopmental impairment

The gut-brain axis (GBA) is a bi-directional highway of communication involving neuro-immune-endocrine mediators that link the gut, the microbiome, and the nervous system—playing a critical role in the homeostatic processes of health and disease (9, 35). The alteration of the GBA has served as a framework for the explanation of many diseases for over three decades (36) and is now acknowledged as a crucial part of the development of the pathogenesis and downstream NDI in NEC (36, 37).

In the case of NEC, this begins as a combination of microbial dysbiosis and subsequent intestinal injury. This leads to signals traveling *via* the enteric nervous system (ENS) (6) residing within the intestinal wall, through the vagus nerve, and ultimately to the central nervous system (CNS) (6, 7). In addition to neural signaling, pathogenic bacteria release lipopolysaccharide (LPS) and a variety of other inflammatory mediators (such as fatty acids) into the systemic circulation. This initiates a cascade of inflammatory factors that causes systemic inflammation but also activates toll-like receptors on microglia. Activated microglia release pro-inflammatory cytokines, free-radicals, and help to activate other cells such as astrocytes as well as injure developing pre-oligodendrocytes. The combination of these insults results in white matter injury (7, 38). The interplay of this axis and its suspected role in NEC is further detailed in the following sections and is illustrated in **Figure 1**.

3.1. The role of the microbiome

The microbiome is a critical part of the GBA with microbiota influencing the CNS by interacting locally with intestinal cells and the ENS or directly *via* neuroendocrine and metabolic mediators (8, 9). The importance of the microbiome to the homeostasis of the GBA is best highlighted by the wealth of studies of germ-free animals which have shown a wide array of impairment or dysregulation in immune function, amino acid metabolism, hormone signaling, neurotransmission, and behavioral phenotypes when compared to their counterparts (9, 39–42).

Dysbiosis—or the change of the microbiome towards an unfavorable or pathogenic bacterial colonization—is a major contributing factor to the development of NEC (36, 43, 44). The infant microbiome is normally characterized by large amounts of *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* in the first few weeks to one year of life which is aided by normal vaginal delivery and feeding with human breast milk (45). Although there are no causal species to the development of NEC, an overgrowth of gram-negative organisms, specifically in the *Enterobacteriaceae* family (46, 47) and loss of intestinal diversity can contribute to dysbiosis and the multifactorial etiology of NEC (44, 47). Additionally, several experimental models have shown the pivotal role of bacteria in the pathogenesis of NEC as germ-free animals are protected from developing NEC (46, 48).

This unfavorable change in the microbiome triggers an acute inflammatory response that leads to further disruption of the already immature intestinal barrier and is further exacerbated when pathogenic bacteria release their endotoxins and pro-inflammatory mediators or translocate across the intestinal mucosa (49). The microbiome also directly influences the brain microenvironment by the generation of neurotransmitters, short chain fatty acids (SCFAs), and cytokines as well as *via* direct activation of immune cells and communication with neural networks that traverse up to the CNS (35, 50, 51). Bacterial toxins such as Lipopolysaccharide (LPS) can reduce ENS activity and inhibit the function of interstitial Cells of Cajal—often referred to as the pacemaker of the intestine and important to gut motility—resulting in the ileus that is often seen in NEC (36, 52). The microbiome also is an important regulator of the hypothalamic-pituitary axis (HPA) and is important for the postnatal development of an appropriate HPA stress response in mice. Activation of this axis can result in elevated levels of systemic cortisol which can further cause intestinal injury and stimulation of the pro-inflammatory cascade (8, 42). The microbiome also plays a role in the regulation of important epithelial barriers. Changes in the microbiome can cause direct influences on the intestinal epithelium and tight junction barrier activity (8). Additionally, studies in germ-free mice have shown that a healthy microbiome is essential to the development and function of the blood brain barrier (BBB), with germ-free mice showing increased BBB permeability that persisted into adulthood. In these studies, restoration of BB integrity was seen by postnatal recolonization of the intestine with a probiotic (9, 53).

3.1.1. Toll-Like receptor signaling

Toll-Like Receptors (TLRs) are pathogen-associated molecular pattern recognition receptors that participate in signaling in response to infection or disease (36). TLR-4 signaling, specifically, plays a pivotal role in the GBA and the pathogenesis of NEC. It has been shown that TLR-4 activation is unregulated in preterm infants and that TLR-4 knockout animals do not develop NEC (48,

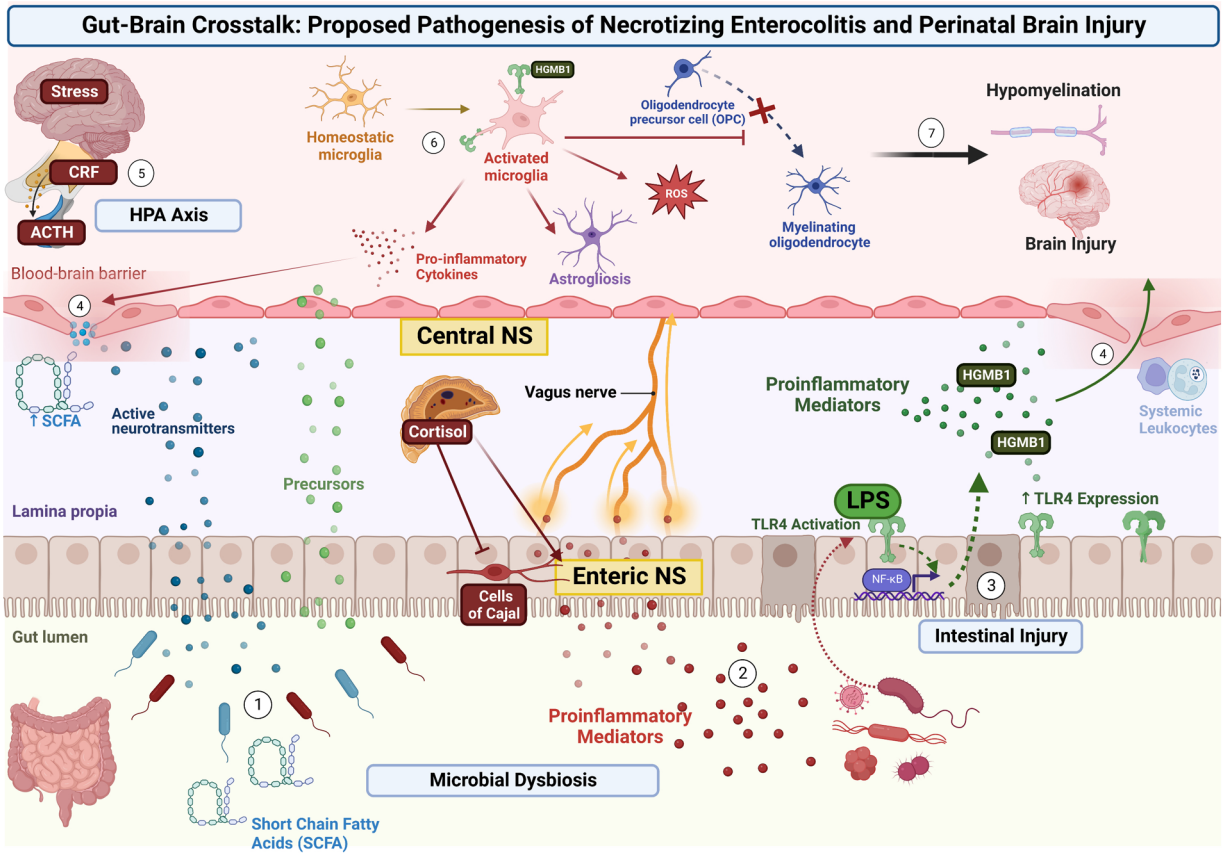

FIGURE 1
Proposed pathogenesis of NDI in NEC *via* the Gut-Brain Axis. Microbial dysbiosis and subsequent intestinal injury leads to the activation of several signaling pathways. (1) Pathogenic bacteria release signaling molecules including neurotransmitters, gasotransmitters, and short-chain fatty acids that cross the intestinal membrane and enter the systemic circulation. (2) Pathogenic bacteria release proinflammatory cytokines and other inflammatory mediators to stimulate the ENS within the intestinal wall, with signals traveling through the Vagus nerve, and ultimately to the CNS. (3) Intestinal injury and release of mediators (such as LPS) results in TLR-4 signaling and increased expression. This results in transcriptional changes *via* NF-κB and further release of inflammatory mediators into the systemic circulation. (4) Systemic and neural inflammatory mediators weaken the BBB and allow further passage of pro-inflammatory mediators and systemic leukocytes. (5) Concurrently, stress is processed in the limbic system of the brain, resulting in release of CRF from the hypothalamus, release of ACTH from the pituitary, and the release of cortisol from the adrenal gland. This signaling through the HPA axis and increase in systemic cortisol results in further activation of the ENS and intestinal epithelial cells, while inhibiting interstitial cells of Cajal (resulting in decreased gut motility). (6) Signals and inflammatory mediators reach the CNS and cause local brain injury as well as activate microglia *via* ligands (such as HMGB-1) binding to TLR4. Activated microglia release pro-inflammatory cytokines and free radicals, stimulate astrocytes, and injure developing pre-oligodendrocytes. (7) The combination of these insults results in brain injury and hypomyelination.

54). Lipopolysaccharide (LPS), an endotoxin produced by pathogenic bacteria, results in an excessive TLR-4 activation in intestinal cells that causes translocation of transcription factors such as nuclear factor kappa-β (NF-kB) leading to the transcription of various pro-inflammatory cytokines and other mediators (48, 55). These mediators cause intestinal inflammation, disrupt mucosal integrity, and enter the systemic circulation. From here, these mediators initiate a systemic inflammatory response and can travel through a weakened BBB to further initiate damage and activate the brain's immune response (48, 51, 54–56).

3.1.2. Short Chain Fatty Acids(SCFAs)/ neurotransmitters/gasotransmitters

Gut bacteria also independently produce metabolites that participate in the GBA. Both commensal and pathogenic bacteria produce SCFAs, such as butyrate, propionate, and acetate, that play a role in maintaining the barrier function of intestinal epithelial cells. Butyrate specifically can serve as a fuel source for colonocytes and improve tight junction integrity (35, 57). Alteration in the balance of gut SCFA is implicated in the altered function of both the intestinal barrier and BBB as well as the maintenance of homeostasis in the CNS (9). These molecules are also known to stimulate the sympathetic nervous system and influence the memory and learning process (8). SCFAs can diffuse through epithelia to exert their effects, typically through the inhibition of histone deacetylase (9, 58). In mice, intraperitoneal injections of butyrate have been shown to enhance learning, memory, and sociable behaviors while simultaneously decreasing depressive-like behaviors (9, 59–61). Studies in animal models have also shown that SCFAs can induce vagus nerve activation (9) and enhance mucosal barrier protection (9, 62). Although many studies promote the benefits of SCFA, the alteration of the homeostasis of SCFA has been implicated in certain disease processes (9). Specifically, excessive production of SCFAs has been implicated in NEC. One possible explanation for this can be secondary to overproduction of SCFAs by bacteria

and poor gut motility which can in turn cause local accumulation of SCFAs (56, 63).

In addition, bacteria can both directly release neurotransmitters (such as 5-HT and GABA) (8, 9), molecules that mimic local neurotransmitters (8), and can stimulate intestinal cells to release neurotransmitters, which traverse the intestinal epithelium. These molecules or their precursors can then pass through a weakened BBB and further influence the brain (64) and CNS physiology, resulting in possible brain injury and altered development (11, 65). Gasotransmitters are another important type of signaling molecule in the GBA. In NEC, an emerging gasotransmitter of interest is H_2S. Commensal bacteria such as *Lactobacilli* can produce hydrogen sulfide which further modulates gut motility (66). In fact, H_2S has been shown to have protective effects on intestinal injury in murine models of NEC (8, 9). Although the effect of H_2S on NDI has not been illustrated, there have been studies on neuroprotective effects of H_2S in secondary brain injury after a TBI (67). In rats, intraperitoneal injection of NaHS, a H_2S salt, resulted in improvement in TBI-induced memory impairment (67, 68) and H_2S decreased TBI induced lesion volume in brains (67, 69).

3.2. Neural communications and the Hypothalamic-Pituitary Axis (HPA)

There are several neural pathways that allow the peripheral components of the GBA to communicate with the brain (36). The ANS afferent pathway starts with signaling from the lumen which traverse through the enteric nervous system (ENS) and vagal nerve to reach the CNS (8). The efferent pathway (from the CNS back to the intestinal wall) (8) often serves an anti-inflammatory function. In healthy individuals, this pathway helps to balance out or "check" the responses secondary to pro-inflammatory cytokines such as TNF-α (tumor necrosis factor alpha), signaling molecules such as HMGB1 (high mobility group box1), and inflammasomes (multi protein cytoplasmic complex that triggers cascades to enhance secretion of proinflammatory cytokines)—thereby preventing unregulated pathogenic signaling (36).

The ENS is the first access point to the afferent pathway and resides within the intestinal wall (36)—receiving signals from microbiota, immune cells in the epithelium, and altered and injured intestinal epithelium (36). Enteric signals can then communicate through the vagus nerve, dorsal root, and nodose ganglia to the CNS (6, 35, 36). The vagus nerve serves as a major pathway between microbial mediators, the ENS, and the brain, which is well supported by animal models that show the absence of neurochemical and behavioral effects with the alteration of the microbiome in vagotomized animals when compared to controls (8).

The hypothalamic-pituitary axis(HPA) is a hormonal mediator in the GBA and works alongside other signaling pathways (36). The microbiome also is a regulator of the HPA and has shown to be important to for the postnatal development of an appropriate HPA stress response in mice (8, 42). If the gut is "stressed" or there is dysbiosis, the HPA processes this information up in the limbic system. This results in corticotropin-releasing factor (CRF) from the hypothalamus, adreno-corticoid hormone(ACTH) secretion from the pituitary (8, 9) and ultimately stimulates the adrenal gland to release cortisol (9). The activation of this system then allows neural-hormonal influence of immune and intestinal epithelial cells, interstitial cells of Cajal, and ENS neurons (8, 36). Stress and signaling from the brain can drastically affect the intestine by alteration of intestinal permeability (8) and immune cell activation (8).

3.3. The brain: immune cell signaling and the brain's effector cells

The brain itself, is a complex signaling system of regions that have their own sensory and motor functions and includes the cerebral cortex, the cerebellum, the limbic system, the HPA, and the brain stem (36). Injury to different parts of the brain, can have several downstream consequences to immune functioning, neurobehavioral disorders, and intestinal disease processes (36). For the purposes of this review, we are specifically interested in neural damage as signals travel up to the brain from injury in the intestine as in the case of NEC.

Once pathogenic signals have travelled through the vagus nerve and/or inflammatory mediators have reached the CNS, the brain becomes especially susceptible to injury. This process includes activation of microglia (*via* TLR4 stimulation) and subsequently astrocytes and glial cells within the brain (70). Activated microglia and astrocytes migrate to sites of injury and begin the neuroinflammatory cascade by releasing pro-inflammatory cytokines such as TNF-α, interleukin-1β (IL-1β), and interleukin-6 (IL-6) (70–73). The BBB (already weakened by SCFA release) is further destabilized by the release of cytokines and the inflammatory activation of enzymes such as matrix metallopeptidases (MMPs), which allows systemic leukocytes to enter and further exacerbate injury. This interplay is believed to cause abnormalities in normal myelination and white matter injury (70, 73). At the cellular level, white matter injury is defined by alterations in the developing oligodendrocytes and specifically the pre-myelinating oligodendrocyte cell which results in hypomyelination (38, 73). Understanding these cellular and molecular processes is important for identifying future targets for prevention of poor neurodevelopmental outcomes after NEC.

4. Animal studies of NEC and neurological impairment

Although the clinical and macroscopic neurological effects of NEC on infants are clear, the microscopic changes caused by NEC in the GBA remain to be elucidated. Several animal models of NEC have been studied that show early progress in this realm (13). In mice, Sampah et al. and Nino et al. showed the onset of NEC in the intestine leads to excessive TLR4 signaling and activation of an endogenous ligand HGMB1 (high mobility group box 1) which enters the systemic circulation and activates TLR4 receptors on microglia in the brain. The microglial activation and damage in the brain was confirmed by either an increase in Iba-1(a microglial marker) staining, increased radical oxygen species accumulation, or reduced myelin basic protein (51, 54). Nino et al. further

demonstrated that mice exposed to NEC had severe deficits in spatial working memory and novel object recognition memory by the time they reached postnatal day 60 (54). In another murine model of NEC, Biouss et al., showed that pups with NEC had higher brain-to-body weight ratios, thinner cortices, and increased levels of apoptosis and endoplasmic reticulum stress compared to breast-fed controls. In addition, the brains of mice with NEC had an associated reduction in the number of neurons, oligodendrocytes, and neural progenitor cells in specific regions of the brain. Finally, levels of pro-inflammatory cytokines, the density of activated microglia, and the density of astrocytes were increased in the brain, and correlated with an increase in the levels of pro-inflammatory cytokines in the gut and intestinal histologic damage (74).

Other animal models of NEC looking into the GBA remain sparse. A pig model by Brunse et al. showed that preterm pigs undergoing experimental NEC had increased BBB permeability and CNS inflammation (increased IL-6 production), but showed no effect on cerebral myelination or microglia density by day 5 (75). A rat model of NEC showed that animals with NEC demonstrated slower times to reach certain developmental milestones, increased anxiety-like behavior, and decreased cognitive function when compared to breast fed pups. These clinical observations were associated with increased numbers of "activated microglia" and decreased myelin basic protein (76).

The studies taken together highlight a few key findings of the pathophysiology of NEC: 1) intestinal inflammation and injury translates to neural changes that are proposed to occur through TLR4 signaling in the intestine, 2) endogenous ligands released from intestinal TLR4 activation go on to activate TLR4 on microglia and 3) downstream neurologic changes occur including microglial activation, increased neuroinflammation, and decreased myelination which can lead to downstream neurodevelopmental deficits.

5. Therapies in perinatal brain injury

The literature centered on therapies to prevent NDI in infants with NEC is scarce, and to date, no strong, randomized clinical trial data exist. However, there are several studies looking into therapies to prevent or ameliorate perinatal brain injury (of which NEC is a known risk factor). The following sections will summarize what is known about therapies to target perinatal brain injury which can be potentially applied to the brain injury seen in NEC.

5.1. Stem cell therapy

Mesenchymal stem cells (MSCs) are among the most widely studied stem cells because they are multi-potent cells that are relatively easy to isolate and maintain in culture (77, 78). Furthermore, they have a lower tumorigenic potential and are immune privileged with minimal host immune activation upon administration (79, 80). They have been used in various pre-clinical studies (81–84) and have been shown to reduce inflammation (85, 86), exhibit antioxidant properties (87), enhance neovascularization (88), and improve functional recovery of injured tissues. They can migrate to damaged tissues or organs in response to inflammatory mediators where they act in the local environment *via* secretion of paracrine mediators and interaction with surrounding cells (79, 89). The application of stem cells for the treatment of NEC, is largely still limited to pre-clinical animal studies, and very little is known about the effect of stem cells on NDI (82, 90–94). However, there are some studies that have looked at the separate effects of MSCs on the neonatal diseases of the gut, such as NEC, as well as the effects of MSCs on certain types of perinatal brain injury, including: periventricular leukomalacia (PVL), hypoxic-ischemic encephalopathy (HIE), and neonatal stroke. The combination of these findings helps us to extrapolate the connection between the effect of MSCs on the GBA in the pathogenesis of NEC (79).

5.1.1. MSCs and necrotizing enterocolitis

Over the past decade, stem cells have been studied as a potential avenue of treatment, however the therapeutic benefit of MSCs in the intestinal pathogenesis of NEC has yet to be fully explored in the clinical setting (77, 82, 95, 96). In fact, only one clinical case report shows a benefit of stem cells used in a case of surgical NEC where umbilical-cord-derived-MSCs (UC-MSCs) were given intravenously. Following administration of UC-MSCs, mesenteric doppler imaging showed improved perfusion to prior compromised portions of intestine by post-operative day 4 (97).

There are several animal studies that showcase the benefits of MSCs to mitigate the intestinal pathogenesis of NEC. In rat models of NEC, intraperitoneal(IP) injections of MSCs have shown an improvement in clinical sickness and intestinal histology injury—characterized by restoration of villi-crypt morphology and epithelium along with restoration of populations of Paneth cells, SOX9 cells, and LGR5 stem cells that occupy this crypt niche (98, 99). An adult mouse-model of ischemia and reperfusion utilized several different MSC's including umbilical cord (UC-MSC), bone-marrow (BM-MSC), and adipocyte-derived (AD-MSCs) cells and similarly showed improved overall survival, intestinal perfusion, restoration of normal intestinal histology, and a decrease in pro-inflammatory chemokines (84, 100).

5.1.2. MSCs and perinatal brain injury

Researchers have identified various causes that result in perinatal brain injury including neuronal cell death, ischemia from placental or umbilical cord disruption, accumulation of free radical oxygen species, persistent inflammatory cascades, and defective myelination of neuronal cells largely from microglia-mediated damage of pre-oligodendrocytes (101, 102). No human data exists that looks specifically at the effects of MSCs in neurodevelopmental impairment in NEC, however there are a few animal studies and clinical and preclinical trials that show promise in the field of perinatal brain injury. Oppliger et al. showed that UC-MSCs improved myelination and decreased microgliosis and astrogliosis in a rat model of white matter brain injury (103). A systemic review of 18 murine studies on the effect of neural stem cells (NSCs) on perinatal brain injury showed significantly improved motor function and cognitive function (104) consistently throughout most of the studies. In a preterm sheep model of LPS-

induced white matter injury, treatment with UC-MSCs reduced cell apoptosis/inflammation, promoted oligodendrocyte survival,and attenuated astrogliosis (105). Although the overall data behind MSCs in perinatal brain injury, and specifically from inflammatory causes (not ischemia/hemorrhage), is sparse, it shows promising results, indicating that continuing to investigate the benefits of MSCs on improving NDI in NEC would be beneficial. In the following sections, we will delve into specific neonatal brain pathologies and the studies that utilize stem cells to treat them.

5.1.2.1. MSCs in neonatal stroke

The pathogenesis of neonatal stroke involves an ischemia-reperfusion injury with disruption of arterial or major venous flow. Studies in a newborn rat model of neonatal stroke by Kim et al. showed that MSCs reduced brain infarct volume and enhanced astrogliosis and ultimately improved functional test scores (106). Another rat model study of neonatal stroke by van Velthoven et al. showed that intranasally delivered MSCs reduced loss of brain matter and ultimately improved motor function (79, 107).

5.1.2.2. MSCs in Hypoxic-Ischemic Encephalopathy (HIE)

HIE is a perinatal brain injury where insufficient blood flow and oxygen is delivered to brain tissue resulting in damage and disability such as CP. Currently, therapy for HIE centers around hypothermia which prevents secondary brain injury but offers no restorative function (108). There are a few preclinical and animal studies that demonstrate the benefit of MSCs in treating HIE. In a rat model of HIE, the combination of UC-MSCs and hypothermia resulted in a reduction of the previously injured brain region and improved sensorimotor function (109). In addition, MSC therapy showed improvement in the neuro-microenvironment with decreased pro-inflammatory mediators, decreased microgliosis and astrocytosis, and decreased permeability of the BBB (79, 109). Another rat model of HIE demonstrated that intranasally delivered MSCs reduced markers of neuroinflammation and restored neuronal cell numbers (110). In a mice model of HIE, a single MSC infusion treatment directly into the cerebrum resulted in inducible gene expression that promoted growth, proliferation, and survival of neural progenitor and glial cells (107).

Early clinical trials in preterm infants suffering from HIE indicate that autologous UC-MSCs delivered intravenously showed improved Bayley III Assessment scores by 1 year of age (111, 105). UC-MSCs therapy has also been used in older children with CP in which improved cognitive effects and gross motor function have been shown (112, 79, 105). Taken together, these studies show promise in the role of MSCs in neurogenesis and repair (79).

5.1.2.3. MSCs in Periventricular Leukomalacia (PVL)

PVL has a multifactorial etiology including HIE, trauma, immature brain development, and inflammatory changes (79) and is specifically characterized by a loss of pre-oligodendrocytes, loss of normal myelination potential, and diffuse gliosis (79, 113). In a neonatal rat model of PVL, rats receiving intracerebral injections of MSCs demonstrated increased anti-myelin immunoreactivity and glial cell migration and proliferation in injured areas indicating a neuroprotective and neuro-regenerative effect (114). Two different studies of a rat model of PVL illustrated that IP injections of UC-MSCs could replicate this improvement in brain injury with increased mature oligodendrocyte counts, decreased reactive astrocytes, and activated microglia (115) as well as a demonstrated reduction in IL-1B and reversed demyelination (measured by myelin basic protein staining. Interestingly, UC-MSCs pretreated with interferon-gamma resulted in even more significant effects, indicating that MSCs can be primed to deliver their protective effects (79, 116) These studies together show that MSCs delivered in the peritoneum can participate in the GBA to deliver neuro-regenerative effects in the setting of this disease.

5.2. Extracellular vesicles (exosomes)

There is a large body of research suggesting that stem cells can exert part of their regenerative effects through the release of extracellular vesicles (EV) or exosomes. EVs carry a wide range of bioactive cargo which includes nucleic acids, lipids, proteins, and a variety of intracellular mediators including cytokines. These can then fuse with other cells and incur transcriptional and translational modifications (77, 95) and facilitate intracellular communication (70). In a neonatal rat model of NEC, McCulloh et al. isolated EVs from four types of MSCs and injected them into the peritoneal cavity and found that EVs reduced the incidence of NEC in a dose-dependent manner and reduced histologic intestinal injury (83). No studies exist that specifically tie the use of MSCs and EVs in NEC and development of NDI, however, there are a few studies that look at the effects of EVs on perinatal brain injury.

A review of the therapeutic EV studies in experimental animal models of perinatal brain injury looked at 13 studies that administered EVs from MSCs *via* intravenous or intranasal administration in rats, mice, and sheep. The studies overall demonstrated an improvement in myelination and neuronal deficits following brain injury, decreased secretion of pro-inflammatory factors, and reduced microglia-mediated neuroinflammation (10). In rodent models of perinatal brain injury, long-term behavioral studies also demonstrated that EV treatment not only improved early neurological deficit scores, but improved long-term changes in motor coordination, spatial learning, and several types of memory testing (70). Thomi et al. looked at an *in vitro* model which showed that EV administration inhibited the production of pro-inflammatory cytokines by glial cells (including activated microglia) *via* interference of TLR4-signaling on microglial cells which prevented degradation of NFkB inhibitor and further downstream effects (10). In studies of HIE in preterm sheep, Ophelders et al. reported that intravenous administration of bone-marrow MSC-derived EVs to the fetus improved brain function (117). Collectively, these studies showed the benefits of EVs in improving neurodevelopmental outcomes, however, more studies with consistent cell lines and administration routes must be done to confirm these findings (70).

5.3. Nutritional supplementation

Probiotics are a group of supplements that have possible neuroprotective potential. They are an amalgam of micro-organisms that can help re-colonize the gut with commensal

bacteria and improve gut barrier function. Many studies have shown a benefit in the risk of NEC, but little is known about the effects on NDI. Clinical studies by Alfalfa et al. and Akar et al. showed that probiotic supplementation in preterm and VLBW infants reduced the risk, incidence, severity, and all-cause mortality in NEC, however there was no clear effect of probiotics on neurodevelopmental outcomes (118, 119). In preclinical animal studies, probiotics have been shown ameliorate brain injury by releasing inhibitors of TNF-a and NF-κB (36, 100, 101), blocking the transport of damaging bio-molecules *via* the GBA (36), alteration of mRNA expression in certain regions of the brain, and reducing HPA axis-induced release of cortisol (8, 120). In murine models, probiotics have been shown to alter anxiety and depression-related behavior in mice (8, 120) and in water-avoidance stress models strengthen tight junctional barrier integrity in the intestinal epithelium, which in turn attenuated the response of the HPA and ANS resulting in decreased end cortisol level and prevention of changes in the hippocampus (8, 121). Wang et al. showed that the probiotic *Lactobacillus reuteri* in a rodent model protected against several deleterious developmental behaviors such as cognition and anxiety and additionally prevented the increase in activated microglia and decrease in myelin basic protein that was seen in NEC (76).

Several studies have shown that early probiotic administration can help attenuate the effects of antibiotics and early life stress (9, 122–126), and prevent subsequent deleterious effects *via* the GBA. Cowan et al. performed studies that looked at maternal separation and early life stress in a rodent model. Pups in this model showed fear relapse and fear memories that more closely mimicked adult behavior (123). Female pups were shown to exhibit earlier onset of puberty while male pups exhibited an even later onset. Pups exposed to probiotics showed resistance to fear relapse and fear memories and restored normal onset of puberty in both sexes (124, 125). This maternal separation model also showed that by postnatal day 20, rats had hypercorticosteronemia, increased intestinal permeability, and altered gut microbiota—effects which were prevented in rats that were treated with probiotics. By postnatal day 56, rats exposed to maternal stress no longer showed serum changes in cortisol and their microbiome had largely normalized to control rats. However, the rats showed hypersensitivity when exposed to restraint stress with a significant increase in cortisol level and fecal frequency compared to controls. This hypersensitivity was also not seen in animals treated with probiotics (123). When applied to the pathophysiology of NEC and the GBA, probiotics could be a useful adjunct to attenuate the effects of early activations of brain-related circuits with fear and stress. However further studies need to be employed to look specifically at the effects of NEC and downstream NDI.

Prebiotics are defined as any substrate that is utilized by the host microbiota to confer a health benefit (9, 127). A main category is dietary fiber, which includes oligosaccharides, that may provide benefits to the developing preterm brain. These indigestible food components naturally occur in breast milk (human milk oligosaccharides) and have been assigned antimicrobial, immunomodulatory, and anti-inflammatory functions (127). Prebiotics have a high relative safety profile and can help the homeostasis of the gut microbiome (128). Fresh human milk provides up to a 4% relative risk reduction in the incidence of NEC (9) and helps to colonize the gut with healthy commensal bacteria and delivers important enzymes, immunomodulatory agents, and prebiotic oligosaccharides (36). Human milk contains many protective factors including secretory IgA, lactoferrin, and various oligosaccharides including glycosaminoglycans (GAGs). These carbohydrates are highly abundant and usually are not absorbed, but instead, serve as prebiotics for commensal bacteria in the intestine. They have been shown to exhibit immuno-modulatory effects in various disease processes (36). A prominent GAG gaining clinical interest in the treatment of NEC is chondroitin sulfate (CS), which comprises over half of the normal GAG content in human milk (129, 130) and is nonexistent in most major infant formulas (131). The concentration of CS in human breast milk is higher in preterm mothers than in term mothers indicating some evolutionary importance for preterm infants (44, 131, 132). In addition, maternal health characteristics have been shown to modulate the levels and function of GAGs, indicating that some element of maternal transfer is important to the health of infants (47) that are important to immune function and the development of a healthy microbiome (36). This in turn can prevent the deleterious activation of the GBA that can result in brain injury and downstream NDI (36).

Dabydeen et al. studied the effects of a high-calorie (120% of normal) and protein diet during the first year of life. With the altered diet, infants had dramatic improvements in head growth, weight gain, and increased axonal diameters in their corticospinal tracts. Unfortunately, neurodevelopmental data were unable to be obtained as the trial was aborted due to obvious benefits in the cohort with the diet. However, the importance of nutritional supplementation as an adjunct in the treatment of NEC and the potential for reducing NDI is important to continue to investigate (133). Taken together these studies demonstrate that nutrition, prebiotics, and probiotics can be important adjuncts to the treatment of NEC and amelioration of downstream NDI.

6. Conclusion

The morbidity and mortality of NEC in infants and the downstream neurodevelopmental complications after survival is well elucidated. Studies show that of infants that survive neonatal NEC, up to 45% of children show neurodevelopmental impairment (12). The pathophysiology of NEC involves a complex signaling cascade of the Gut-Brain axis driven by dysbiosis and inflammatory signaling within the intestine. This triggers inflammatory mediators that enter the systemic circulation, participate in TLR-4 signaling, or directly communicate between neural networks involving the ENS and the vagus nerve. Together these result in downstream microglial activation, subsequent astrocytic hypertrophy/astrogliosis, and impaired functioning of pre-oligodendrocytes, which ultimately cause white matter injury and impaired myelination (5–7, 70). This cascade inhibits normal brain development and growth, which is seen as white matter abnormalities on MRI (17, 25, 34). It becomes imperative therefore to make strides in therapies to protect against brain injury and downstream NDI. Although there is no clear therapeutic

intervention to improve or prevent NDI, there is some promising early research in the field of stem cells, extracellular vesicles, probiotic/prebiotic therapies, and aggressive nutrition. With the prevalence and emotional burden that NDI following NEC carries on our society, it becomes important to continue research in this field—with a specific focus on understanding gut-brain signaling and possible mechanistic targets of therapeutic and preventative interventions.

Author contributions

KM and TAM developed the concept of the manuscript, KM drafted the manuscript, and FMM, JL, WCS, JPB, and TAM provided critical revisions to the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

TAM serves as a consultant for Noveome Biotherapeutics. As such, he receives consulting fees for his services. The material presented herein is not in conflict with that position.

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Publisher's note

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Current and future methods of probiotic therapy for necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is a complex intestinal disease that primarily affects premature neonates. Given its significant mortality and morbidity, there is an urgent need to develop improved prophylactic measures against the disease. One potential preventative strategy for NEC is the use of probiotics. Although there has been significant interest for decades in probiotics in neonatal care, no clear guidelines exist regarding which probiotic to use or for which patients, and no FDA-approved products exist on the market for NEC. In addition, there is lack of agreement regarding the benefits of probiotics in neonates, as well as some concerns about the safety and efficacy of available products. We discuss currently available probiotics as well as next-generation probiotics and novel delivery strategies which may offer an avenue to capitalize on the benefits of probiotics, while minimizing the risks. Thus, probiotics may still prove to be an effective prevention strategy for NEC, although further product development and research is needed to support use in the preterm population.

KEYWORDS
necrotizing enterocolitis, NEC, probiotic, intestine, gut, microbiome, dysbiosis

Introduction

NEC is a severe inflammatory disorder of the premature intestine with complex pathophysiology and limited treatment options (1). One of the earliest reports of the disease was from Babies Hospital in New York City in 1965 (2). Despite several advances in the care of newborns since this time (3, 4), the overall incidence and mortality due to NEC remain high (5, 6). In contrast to respiratory distress syndrome (RDS), another common disease of prematurity, which was radically improved through the introduction of artificial surfactants (7), no such early preventative measure has yet been developed for NEC. In fact, the overall medical care for NEC has remained largely the same since the term was first coined: withholding feeds, antibiotics, and surgery when indicated. Today, NEC is the most common surgical emergency in neonates and the most common cause of gastrointestinal death in this vulnerable patient population. Given the high mortality of NEC, how difficult it is to treat, the significant financial burden it poses on society, and the long-term morbidity in survivors, there is an urgent need to develop novel preventative measures with an aim to eradicate NEC (8).

As NEC typically occurs in the first several weeks of life and is thought partly to be due to an altered gut microbiome (9–11), one potential and promising preventative measure is the prophylactic use of probiotics in susceptible neonates. Probiotics are defined per the World Health Organization (WHO) as live microorganisms such as bacteria that are

given in significant enough quantities to provide a specific health benefit (12, 13). While they have been formally studied in the western world since the early 1900s, it was not until the 1950s that they were first trialed in human neonates (14). More than a half-century later, probiotics have failed to gain traction in the USA for the prevention of NEC (15). However, interest in probiotics has increased over time; in 1997, almost no NICUS in the United States were using probiotics (16), but by 2015, that number had increased to 14% (15). Due to concerns regarding safety and efficacy, lack of clear protocolized guidelines for usage, and unavailability of FDA-approved products, neonatologists, pediatric surgeons, and other stakeholders are at present torn on the role of probiotics in preventing NEC. However, an improved mechanistic understanding of probiotic effects on neonatal intestine and immunity, careful selection and dosing of the most efficacious bacterial strains, and advancements in the production and delivery of next-generation probiotics, may warrant future reconsideration of this understandably cautious position. In this review article, we will explore the scientific rationale for the use of probiotics in human neonates, the current state of data in support or against the usage in human neonates, ongoing concerns and barriers to usage, and the future potential of probiotics in the prevention and eradication of NEC.

Understanding the pathophysiology of NEC and the rationale for prophylactic use of probiotics

The pathophysiology of NEC is known to be complex. This is in part due to early bacterial colonization and an excessive inflammatory response in the context of a premature gut and immune system. Several risk factors have been identified that increase the likelihood of NEC development, including premature birth, very low birth weight, exposure to asphyxia or hypothermia, and enteral feeding (8). This multifactorial pathophysiology underscores how difficult it is to fully prevent NEC with any one single intervention. However, one core component of the disease that may be modifiable, even in the earliest weeks of life, is the altered microbiome characteristic of NEC (17). Understanding the cause and characterizing the extent of this dysbiosis may be key to both understanding NEC and potentially preventing its occurrence.

When neonates are born, they acquire a small library of bacteria from the mother during delivery, from their environment, and from oral feeds, which rapidly expands in both size and diversity. This immature intestinal microbiome is believed to not only influence the immediate health of the neonate but also its life-long health. Most importantly, however, at this initial stage the microbiome is believed to be modifiable, providing a unique opportunity for early intervention (17). The earliest "pioneer" bacteria that seed the intestinal tract during this initial phase include facultative aerobes such as *Escherichia*, *Enterococcus*, and *Streptococcus*, that shift the intestinal luminal environment to an anaerobic one. This shift subsequently allows obligate anaerobes such as *Clostridium*, *Bacteroides*, and *Bifidobacterium* to thrive (18, 19). However, this process can vary tremendously depending on the specific bacteria that neonates are first exposed to, which is influenced by the mode of delivery. Neonates that are delivered vaginally appear to acquire gut flora that resemble their own mother's vaginal microbiome, whereas those delivered by cesarean section develop intestinal microbial communities with similarities to the maternal skin flora (20, 21). In addition to these early colonizers, breast milk feeding expands exposure to *Bacteroides* and *Bifidobacterium*, as well as lactic acid producers: *Lactobacillus* (i.e., *L. acidophilus*), *Limosilactobacillus* (i.e., *L. reuteri*), and *Lacticaseibacillus* (i.e., *L. rhamnosus*). These early gut bacteria are crucial to neonatal health as they are thought to play a role in educating the neonatal immune system and ensuring the evolution of a diverse intestinal microbiome, particularly through the production of beneficial bacterial metabolites (19).

Unfortunately, several factors can disrupt or alter the expected healthy gut colonization, including maternal disease or dysbiosis, cesarean section delivery, absence of breast milk feeding, prematurity, or early antibiotic use (22). Preterm neonates, the population most at risk for NEC, have several additional factors that contribute to dysbiosis, including early exposure to microbes *in utero* (i.e., preterm premature rupture of membranes or intra-amniotic infection), exposure to hospital microbes through prolonged NICU admissions after birth, and expected delays in enteral feeding due to prematurity. Consequently, preterm neonates acquire an abnormal over-representation of pathogenic facultative anaerobes within their intestines, including *Enterobacter*, *Escherichia*, and *Klebsiella*, all belonging to the Gammaproteobacteria class. Additionally, they have decreased proportions of the strict anaerobes that are a hallmark of the healthy developing microbiome, such as *Bifidobacterium* or *Bacteroides* (23).

While preterm infants are already noted to have a decreased diversity of intestinal microbes, the insufficiency is further exaggerated in infants that acquire NEC (24). At the same time, the proportion of Gammaproteobacteria in the intestine is further increased, which is predictive of disease development (9–11). Given these findings, there is an opportunity to target therapeutics towards improving the microbial diversity in the gut and reducing the relative abundance of Gammaproteobacteria, in the hope of preventing NEC. One obvious strategy for this is using beneficial bacteria such as *Bacteroides* spp. or *L. reuteri*. Through the production of anti-microbial compounds or direct competition, probiotic bacteria may be able to displace pathogenic bacteria that contribute to the dysbiosis preceding NEC (see **Figure 1**). For example, *L. reuteri*, in response to various pathogenic-type bacterial strains such as *E. coli*, can generate the antimicrobial compound reuterin, which inhibits bacterial resistance to oxidative stress (25, 26).

In practice, however, it is less clear to what extent this dysbiosis can be transformed into a healthy microbiota and whether this will truly prevent NEC. For example, in one preclinical study that evaluated the ability of a strain of *B. fragilis* to counter *Cronobacter sakazakii*-induced NEC in rodents, pre-treatment with the probiotic slightly improved the loss of microbial diversity and reduced the relative abundance of Proteobacteria. This finding was despite no observable increase in the relative abundance of the probiotic species itself in the gut (27). In

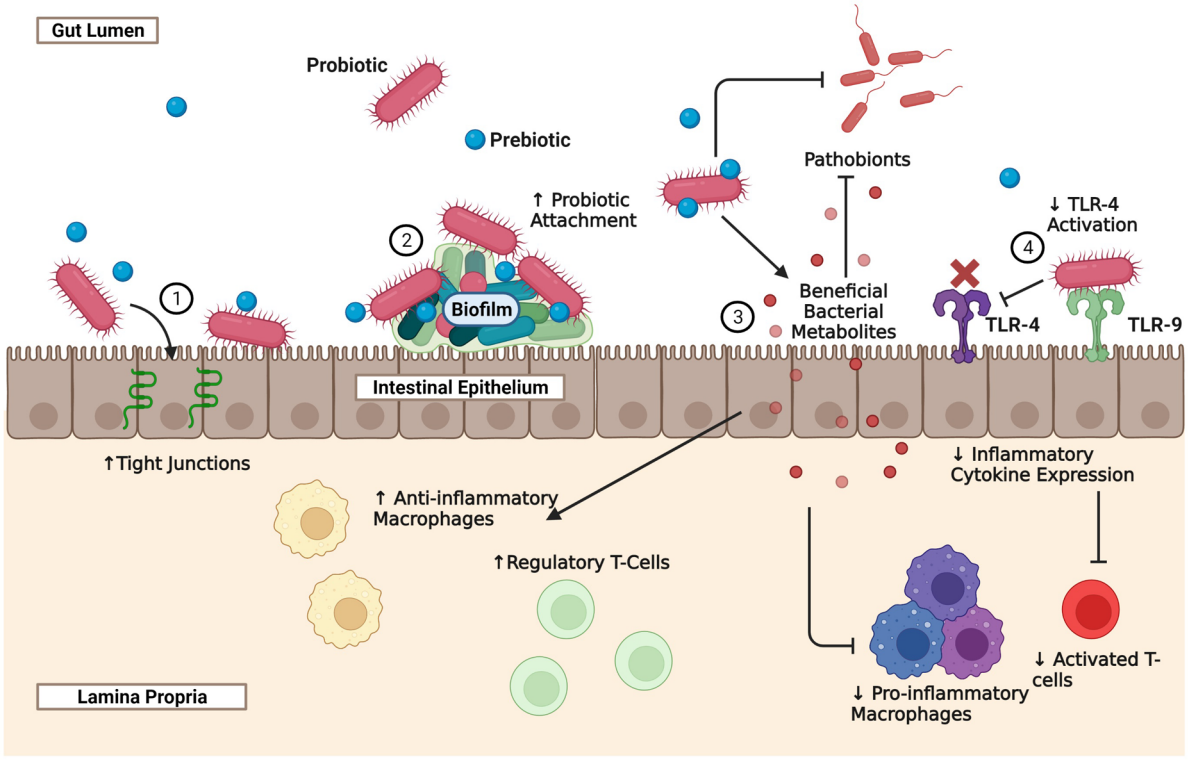

FIGURE 1
The effect of probiotics and prebiotics on the intestinal epithelium, immune system, and microbiome. Necrotizing Enterocolitis is a complex disease that is in part due to prematurity of the neonatal intestine, prematurity of the developing immune system, and dysbiosis. Probiotics, prebiotics, and synbiotics represent potential novel strategies for modulating all three of the intestine, the immune system, and the microbiome, in order to reduce the incidence and severity of NEC. The mechanisms through which probiotics provide benefits vary depending on the species and strain administered, the adjunct use of prebiotics, the use of novel probiotic delivery systems, and dosing regimens. The schematic illustrates some of the major known effects of probiotics on the developing gut that are relevant to NEC: (1) probiotics can improve gut-barrier function by preserving tight junction proteins such as claudin 4 and occludin. Probiotics also have anti-apoptotic and cytoprotective effects on the neonatal intestine; (2) probiotics that are highly adhesive to the gut intestine produce complex biofilms that improve the attachment and theoretically the efficacy of the probiotic; (3) through direct competition or the production of anti-microbial compounds, probiotics can reduce the presence of pathobionts that contribute to the dysbiosis seen in NEC. Probiotics can also metabolize environmental substrates such as tryptophan to produce beneficial bacterial metabolites that can reduce the presence of pro-inflammatory macrophages and activated T-cells, and increase the populations of anti-inflammatory macrophages and regulatory T-cells; (4) some probiotics are also able to indirectly inhibit the TLR-4 pathway, by interacting with TLR-9. TLR-4 is the receptor for LPS, a microbial cell wall product that is thought to play a role in the pathogenesis of NEC and is commonly used as a stressor in animal models of the disease. By inhibiting TLR-4 activity, there is a reduction in inflammatory cytokines and an increase in regulatory T-cells.

another study, administration of *B. infantis* in rodents prevented NEC in a hypoxia-hypothermia model of NEC, but had no impact on dysbiosis, and the probiotic was not detectable in the cecum (28). In contrast, higher dosing of *L. rhamnosus* was not only found to be protective against intestinal injury during experimental NEC, but also resulted in increased microbial diversity. Interestingly, the relative abundance of beneficial bacteria belonging to the phylum Bacteroidetes was also improved compared to lower dosing, underscoring the importance of optimal dosing in characterizing the impact of probiotics on dysbiosis (29). Taken together, these animal studies highlight the variable documented effects of probiotics on the microbiome during NEC, and the difficulty in comparing studies without controlling for differences in specific bacteria used or dosing regimens. Through a careful selection of the most advantageous strains and titration of dosing, the true effects of probiotics on dysbiosis can likely be better assessed in the future.

In addition to dysbiosis, exaggerated inflammation results in significant, patchy, intestinal injury during NEC. Through modulation of the developing immune system and the neonatal intestinal epithelium, prophylactic probiotics may also significantly minimize the intensity of this intestinal inflammation (see **Figure 1**). Several groups using different probiotic bacteria, including *L. reuteri*, *L. rhamnosus*, and *Bifidobacterium* spp., have shown that prophylactic use of these products can effectively reduce the incidence of NEC, the degree of intestinal injury, and the production of inflammatory cytokines in rodent models of the disease (25, 30–33). However, the mechanisms by which these benefits occur are far less clear, and the effects are likely to be bacterial species or even strain-specific. Several of these probiotic bacteria have been shown to influence gut barrier function, possibly through the regulation of intercellular tight junctions, preventing the translocation of pathogens and resulting sepsis (see **Figure 1**). For instance,

B. infantis given to mice prior to initiation of an experimental NEC protocol not only decreased the incidence of NEC, but also reduced the intestinal permeability to the test marker fluorescein isothiocyanate (FITC)-dextran and preserved tight junction proteins such as claudin 4 and occludin (32). Likewise, administration of *L. reuteri* has been shown to decrease intestinal permeability of FITC-dextran during rodent NEC (33). In addition to improving gut barrier function, probiotics can also have anti-apoptotic and cytoprotective effects on the neonatal intestine. For instance, *L. rhamnosus* has been shown to reduce caspase-3 cleavage during experimental NEC and this was associated with an upregulation of pathways involved in epithelial proliferation, migration, growth, and differentiation (34).

Probiotics have also been shown to play a role in modulating the neonatal innate and adaptive immune systems during NEC. For instance, activation of toll-like receptor (TLR) 9 by *L. rhamnosus* DNA has been found to be crucial to its protective abilities against experimental NEC. This is believed to be caused by TLR9 activation resulting in inhibition of TLR4 activation, a receptor that has been implicated in the pathophysiology of NEC and responds to the bacterial cell wall product lipopolysaccharide (LPS) (see **Figure 1**) (31). The probiotic *L. rhamnosus* can also reduce TLR4 activity during NEC by upregulation of TLR inhibitors such as single immunoglobulin interleukin-1-related receptor (SIGIRR) and A20, and the benefits appear to be dose-dependent (see **Figure 1**) (29). In addition to enhanced TLR4 activity, diminished regulatory type T cells (Treg), which play a role in modulating the severity of inflammation and promoting tolerance, have also been implicated in the pathophysiology of NEC. Administration of *L. reuteri* (DSM 17938) in a mouse model of the disease was found to reverse this reduction of $CD4^+$ $Foxp3^+$ Treg cells in the ileum and in mesenteric lymph nodes, which was not seen when *L. acidophilus* DDS was given (see **Figure 1**) (30). Furthermore, probiotics such as *L. reuteri*, have been shown to beneficially convert substrates such as dietary tryptophan from the environment into bioactive byproducts. Several of these tryptophan breakdown products can bind to a human receptor known as the aryl hydrocarbon receptor and promote an anti-inflammatory state, through reduced TLR-4 signaling in intestinal epithelial cells (35) and reduced inflammatory macrophage infiltration in the intestinal tissue (see **Figure 1**) (36). Regardless of the mechanisms, the potential for probiotics to beneficially modulate the intestinal epithelium and immune system are additional rationales for the continued development of probiotic therapies against NEC.

Comparison of current single versus multi-strain probiotics in the prevention of NEC in human neonates

The most studied probiotic bacteria in humans include *Bifidobacterium* spp., *L. reuteri*, or a combination of both (37). These bacteria are normally present in healthy, breastfed, term neonates (38). A study that examined 289 NICUs across the US from 1997 to 2016 found the most commonly administered probiotic products to be *Lactobacillus* (recently recategorized into several genera including *Lactobacillus*, *Limosilactobacillus*, and *Lacticaseibacillus*) formulations followed by Ultimate Flora (*Bifidiobacterium* and *Lactobacillus* spp.), ABC Dophilus (*Bifidobacterium*, *Lactobacillus*, and *Streptococcus* species), and Align (*Bifidobacterium* spp.) (16). Although there is no currently available FDA-approved probiotic, Viswanathan et al. (2016) reported that 14% of NICUs (70/500) in the United States were administering probiotics to very low birthweight (VLBW) infants. Surprisingly, only 4/16 of the probiotics being used in these NICUs were ever evaluated in a randomized controlled trial (RCT) (15). The following sections summarize different RCTs for single and multiple strain probiotic formulations in preterm infants weighing ≤1,500 g [i.e., very low birth weight (VLBW) infants] (see **Tables 1**, **2**).

Single-strain formulations

Lactic acid producers commonly found in breast milk, including *L. rhamnosus*, *L. reuteri*, and *L. acidophilus* are some of the most common bacteria in probiotic formulations administered in the neonatal population (see **Table 1** for comparison of single-strain probiotics in NEC). In 12 NICUs in Italy, 295 VLBW preterm infants were randomized to receive *L. rhamnosus GG* (Dicoflor®; Dicofarm, Rome, Italy), whereas 290 were given placebo. Treatment was given with the first feed, and after at least 7 days of treatment, there was no significant difference in the incidence of NEC. Nevertheless, all patients with NEC in the probiotic group did survive, whereas 25% died in the placebo group (39). Similarly, in a small single-institution RCT with 80 VLBW preterm infants, Dicoflor® reduced gastrointestinal colonization of Candida species. The clinical implications remain unclear as there was no significant difference in the incidence of invasive fungal infections, sepsis, surgical NEC, or death between treatment groups. The lack of significant findings may be attributable to the small study population (40).

The data for other commonly used lactic acid-producing probiotic strains against NEC is similarly mixed. A multi-center, double-blind RCT in Colombia also did not observe a significant decrease in the incidence of NEC between preterm babies who received *L. reuteri* DSM 17938 (Biogaia AB, Stockholm, Sweden) versus placebo. It is important to note that this study was not powered to detect a difference in NEC incidence (41). Likewise, Oncel et al. (2014) investigated the frequency of NEC in a single NICU as a primary outcome in VLBW preterm infants given *L. reuteri* DSM 17938 (Biogaia AB, Stockholm, Sweden) or placebo. After 7 days of treatment, there was no difference in NEC incidence or NEC-related mortality, even after patients were stratified to VLBW or extremely low birth weight (ELBW), defined as neonates weighing <1,000 g. However, there was a significant improvement in sepsis, feeding tolerance, and length of hospitalization in the probiotic arm (42). In contrast, a single-center NICU in Turkey administered *L. sporogenes* (DMG ITALIA SRL, Rome, Italy) to VLBW infants <33 weeks gestational age (probiotic $n = 110$ and control $n = 111$). The incidence of NEC and the incidence of either NEC or death decreased in the

TABLE 1 Randomized controlled trials studying incidence of NEC using single-strain probiotic formulations in premature neonates.

Probiotic strain	Probiotic dose	Date of publication	Country	Single center vs. multicenter	Number of patients enrolled (probiotic vs. placebo)	Enrollment criteria	Feeding type	Timing of probiotic administration	Incidence of NEC (probiotic vs. placebo)	Incidence of sepsis (probiotic vs. placebo)	References
L. rhamnosus GG (Dicoflor™)	6×10^9 CFU daily	2002	Italy	Multicenter	295 vs. 290	GA <33 weeks or birthweight <1,500 g	Both	First enteral feed	1.4% vs. 2.8%, ns	4.7% vs. 4.1%, ns	(39)
L. rhamnosus (Dicoflor™)	6×10^9 CFU daily	2006	Italy	Single	39 vs. 41	<1,500 g, >3 days old	Human milk	Third day of life	2.5% vs. 5%, ns	37.5% vs. 42.5%, ns	(40)
L. reuteri DSM 17938	1×10^8 CFU daily	2012	Colombia	Multicenter	372 vs. 378	≤2,000 g	Both	Between first 1–2 days of life	3.4% vs. 5.4%, ns (≤1,500 g) 1.5% vs. 2.6%, ns (>1,500 g)	Not reported	(41)
L. reuteri DSM 17938	1×10^8 CFU daily	2014	Turkey	Single	200 vs. 200	GA ≤32 weeks, birth weight ≤1,500 g	Both	First enteral feed	4% vs. 5%, ns	6.5% vs. 12.5%, $p = 0.041$	(42)
L. sporogenes	3.5×10^8 CFU daily	2011	Turkey	Single	110 vs. 111	GA <33 weeks or birth weight <1,500 g	Breast milk or mixed	First enteral feed	5.5% vs. 9%, ns	26.4% vs. 23.4%, ns	(43)
B. lactis BB12	12×10^9 CFU daily	2010	Germany	Single	91 vs. 89	GA <30 weeks	Both	Not reported	2% vs. 4%, ns	Not reported	(44)
B. breve BBG-001	10^8–10^9 CFU daily	2015	England	Multicenter	650 vs. 660	GA 23-30 weeks	Both	As soon as possible	9% vs. 10%, ns	11% vs. 12%, ns	(45)
B. breve OLB6378	2.5×10^9 CFU twice daily	2014	Japan	Multicenter	153 vs. 130	<1,500 g	Both	Within 48 h of birth	0% vs. 0%, ns	8.5% vs. 13.1%, ns	(46)
S. boulardii (Reflor™)	5×10^9 CFU daily	2013	Turkey	Single	135 vs. 136	GA ≤32 weeks, birth weight ≤1,500 g	Both	Within 48 h of birth	4.4% vs. 5.1%, ns	34.8% vs. 47.8%, $p = 0.030$ (clinical) 14.9% vs. 15.4%, ns (culture proven)	(47)
S. boulardii (Reflor™)	5×10^8 cell/kg twice daily	2013	Turkey	Single	104 vs. 104	GA ≤32 weeks, birth weight ≤1,500	Both	First enteral feed	6.7% vs. 6.7%, ns	24.3% vs. 18.3%, ns	(48)

ns, not significant.

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TABLE 2 Randomized controlled trials studying incidence of NEC using multi-strain probiotic formulations in premature neonates.

Probiotic strain	Probiotic dose	Date of publication	Country	Single center vs. multicenter	Number of patients enrolled (probiotic vs. placebo)	Enrollment criteria	Feeding type	Timing of probiotic administration	Incidence of NEC (probiotic vs. placebo)	Incidence of sepsis (probiotic vs. placebo)	References
B. infantis, *B. lactis*, and *S. thermophilus* (ABC Dophilus^{TM})	1.0×10^9 CFU daily	2013	Australia, New Zealand	Multicenter	548 vs. 551	GA <32 weeks, weight <1,500 g	Both	When infant was receiving at least 1 ml of milk every 4 h	2% vs. 4.4%, $p = 0.03$	23.5% vs. 26.5%, ns	(49)
B. infantis, *S. thermophilus*, and *B. bifidum* (ABC Dophilus^{TM})	1.05×10^9 CFU daily	2005	Israel	Single	72 vs. 73	Birth weight <1,500 g	Both	Recruited on first day of feeds	1% vs. 14%, $p = 0.013$	43% vs. 33%, ns	(38)
L. acidophilus and *B. bifidum* (Infloran^{TM})	10^9 CFU, twice daily	2008	Taiwan	Multicenter	217 vs. 217	GA <34 weeks, birth weight <1,500 g	Breast milk or mixed	Not reported	1.8% vs. 6.5%, $p = 0.02$	19.82% vs. 11.52%, ns	(50)
L. acidophilus and *B. bifidum* (Infloran^{TM})	1.0×10^9 CFU of each daily	2014	Thailand	Single	31 vs. 29	Birth weight <1,500 g	Both	First enteral feed	3.2% vs. 3.4%, ns	No sepsis observed in either	(51)
L. acidophilus B. bifidum and *B. infantis* (Labinic^{TM})	2×10^9 CFU daily	2022	South Africa	Single	100 vs. 100	GA <37 weeks, birth weight 750–1,500 g	Both	Not reported	0% vs. 5%, ns	Not studied	(52)
B. longum and *L. rhamnosus* GG	10^8 CFU, four times daily	2009	France	Multicenter	45 vs. 49	GA <32 weeks, birth weight <1,500 g	Both	First enteral feed	4.4% vs. 2.0%, ns	33.3% vs. 26.5%, ns	(53)
B. infantis (Align^{TM}) and *L. rhamnosus* GG (Culturelle^{TM})	5×10^8 CFU of each organism daily	2011	USA	Multicenter	50 vs. 51	Birth weight 501–1,000 g	Not specified	First enteral feed	6% vs. 8%, ns	26% vs. 31%, ns	(54)
L. acidophilus, *L. rhamnosus*, *L. casei*, *L. plantarum*, *B. infantis*, *S. thermophilus*	1.0×10^9 CFU/g, 4.4×10^8 CFU/g, 1.0×10^9 CFU/g, 1.76×10^8 CFU/g, 2.76×10^7 CFU/g, 6.6×10^5 CFU/g, respectively, daily	2011	Mexico	Single	75 vs. 75	<1,500 g	Both	First day of enteral feed	8% vs. 16%, ns	56% vs. 58.7%, ns	(55)
L. acidophilus, *E. faecium* and *B. infantum*	0.6×10^7 CFU, probiotic strains in ratio of 1.5:1:1.5	2015	Slovenia	Single	40 vs. 40	<1,500 g	Both	First enteral feed	0% vs. 12.5%, $p = 0.055$	40% vs. 72.5%, $p = 0.006$	(56)
L. acidophilus, *L. rhamnosus*, *B. longum* and *S. boulardii*	1.25×10^9 CFU daily	2017	India	Single	48 vs. 48	750–1,499 g	Colostrum or donor breast milk	Within 24 h of enteral feed initiation	4.1% vs. 12.5%, ns	Not reported	(57)
L. casei and *B. breve* (Yakult LB^{TM})	3.5×10^7 to 3.5×10^9 CFU daily	2011	Brazil	Single	119 vs. 112	750–1,499 g	Breast milk	Second day of life	0% vs. 3.6%, ns	33.6% vs. 37.5%, ns	(58)
L. acidophilus and *B. infantis* (Infloran^{TM})	Minimum 1.0×10^6 and 1.0×10^6 of each, respectively, twice daily	2005	Taiwan	Single	180 vs. 187	<1,500 g	Breast milk	Not reported	1.1% vs. 5.3%, $p = 0.04$	12.2% vs. 19.3%, $p = 0.03$	(59)
B. infantis, *B. bifidum*, *B. longum* and *L. acidophilus*	2.5×10^9 CFU of each organism, twice daily	2009	India	Single	91 vs. 95	GA <32 weeks, birth weight <1,500 g	Breast milk	Not reported	5.5% vs. 15.8%, $p = 0.042$	14.3% vs. 29.5%, $p = 0.02$	(71)

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probiotic group compared to infants who received the placebo; however, these trends were not statistically significant ($n = 211$) (43). The most recent RCT using *L. reuteri* DSM 17938 demonstrated that this probiotic can modulate the microbiome during the first month of life, improving microbial diversity and reducing the presence of potentially pathogenic bacteria. Although no significant effect on NEC was detected, only 54 neonates were evaluated per group and so this study was underpowered to detect any difference in the occurrence of NEC (61).

The other most studied category of probiotic is *Bifidobacterium* spp. For example, *B. lactis* BB12 was administered to VLBW infants who were <30 weeks gestational age at the Children's Hospital in Ulm, Germany between 2000 and 2003 (probiotic $n = 91$ and placebo $n = 89$). In this study, there was no significant difference in either the incidence of NEC (Bell's stage ≥2) or the incidence of nosocomial infections (primary outcome) between treatment and control groups (44). The largest trial, Probiotics in Preterm Infants (PiPs), investigated the use of *B. breve* BBG-001 (Yakult Honsha Co Ltd, Tokyo, Japan) in 650 babies compared to 660 infants who received placebo across multiple centers in the UK. The group found no protection by the probiotic against sepsis, NEC diagnosis, or death. A limitation of this study was the cross-colonization of the placebo cohort; 49% of infants who received a placebo were colonized with *B. breve* BBG-001 by 36 weeks postmenstrual age (45). A RCT in Japan between 19 NICUs provided *B. bifidum* OLB6378 (Meiji, Tokyo, Japan) ($n = 153$) or placebo ($n = 130$) to VLBW preterm infants within 48 h of life. This study did not identify any difference in NEC incidence, as no infant in either group developed the disease. However, there was significant improvement in feeding tolerance and late-onset sepsis in the probiotic group (46).

Aside from lactic acid producers and *Bifidobacterium*, other beneficial bacteria as probiotics have been studied in RCTs. *Saccharomyces boulardii* (*S. boulardii*) (Reflor®; Biocodex, Beauvois, France), a yeast-based probiotic, was administered to VLBW preterm infants at a single NICU within 48 h of birth. There was no significant difference in NEC (Bell's stage ≥2) or death amongst both groups. There was a significant improvement in feeding tolerance in the probiotic group (47). Another independent RCT also investigating *S. boulardii* (Reflor®; Biocodex, France) did not report a significant difference in the incidence of NEC between the probiotic and placebo group (48).

Overall, the results from current published RCTs on the use of single strain probiotics in preterm infants are not compelling regarding the ability of probiotics to reduce the incidence of NEC. Nonetheless, it is important to note that a number of these studies evaluated NEC only as a secondary outcome and enrolled a small study population. Future, more extensive studies using the most promising strains are warranted to detect any significant changes in the incidence of NEC.

Multiple-strain formulations

Although the results from RCTs using single-strain formulations have not been significant in decreasing NEC incidence, RCTs in preterm infants using multiple-strain formulations have been more promising (see **Table 2** for comparison of multi-strain probiotics in NEC). The ProPerms prospective trial evaluated a combination of *B. infantis*, *B. lactis*, and *Streptococcus thermophilus* (ABC Dophilus; Probiotic Powder for Infants, Solfar, Leonia, New Jersey) in 1,099 VLBW premature infants aged <32 gestational weeks in Australia and New Zealand. Although there was no significant effect on late-onset sepsis, the primary study outcome, the group did demonstrate a significant reduction in NEC in the probiotic group compared to the control (49). In another study, VLBW preterm neonates were randomized to receive ABC Dophilus (Solgar, division of Wyeth Consumer Healthcare, Bergen County, New Jersey), composed of *B. infantis*, *S. thermophilus*, and *B. bifidus*. The treatment group had a lower incidence of NEC (Bell's stage ≥2) and less severe NEC. There was an absolute risk reduction of NEC by 12% in the probiotic cohort (38).

Infloran™, a commonly discussed probiotic formulation composed of *L. acidophilus* and *Bifidobacterium* spp., was retrospectively studied in multiple centers in Germany and showed a significant reduction in the risk of NEC, overall mortality, mortality after NEC, and nosocomial bloodstream infection (62). A multi-center RCT in Taiwan with a total of 434 patients demonstrated similar results using *B. bifidum* and *L. acidophilus* (Infloran, National Collection of Dairy Organisms, Reading, United Kingdom and Laboratorio, Farmaceutico, Mede, Italy) in VLBW preterm infants (50). However, a single-center RCT with VLBW preterm infants using the same formulation did not demonstrate a difference in the incidence of NEC (Bell's stage ≥2). It is worth noting that only 31 infants were randomized to the *B. bifidum* and *L. acidophilus*, and 29 neonates to the placebo group (51). A more recent single-center RCT by Sowden et al. (2022) showed a decrease in the incidence of NEC in VLBW preterm newborns treated with a similar approach using Labinic™ (Bioflortech, Surrey, UK), composed of *L. acidophilus*, *B. bidifum*, and *B. infantis*. Although not statistically significant, zero patients in the probiotic arm had NEC, whereas two in the placebo group were diagnosed with the disease (52).

However, not all studies have found a clear benefit from giving multi-strain probiotic formulations to neonates. Another multi-strain formulation of *B. longum* BB536 and *L. rhamnosus GG* (BB536-LGG; Morinaga Milk Industry Co Ltd., Tokyo, Japan and Valio Ltd.) was studied in VLBW premature infants in two centers in France. There was no difference in the incidence of NEC between the study and the control group. This was partly attributed to a low overall incidence of NEC (53). Another multi-center RCT study showed that *L. rhamnosus GG* (Culturelle; Amerifit, Cromwell, Connecticut) and *B. infantis* (Align; Procter and Gamble, Cincinnati, Ohio) given to ELBW preterm infants did not affect the incidence of NEC or surgery for NEC. Only 101 patients were enrolled in this study, with 51 in the control group and 50 in the probiotic group (54). Several other RCTs have been performed around the world using various formulations of multi-strain probiotics but with low patient enrollments, and have also seen no significant effect on NEC (55–57).

Overall, it appears that the studies using multiple-strain probiotics are more promising than single-strain probiotics; however very few direct comparisons exist at present, making it difficult to recommend one over the other based on individual trial data. Interestingly, a study between single strain *L. acidophilus* and a multispecies probiotic formulation containing *L. acidophilus*, *L. rhamnosus*, *L. casei*, *L. plantroom*, *B. infantis*, and *S. thermophilus*, did not show a significantly different incidence of NEC (63, 64).

Meta-analysis of single and multiple-strain probiotics in NEC

One of the earliest, high-quality, meta-analyses performed using 7 randomized controlled trial data of preterm neonates that received prophylactic probiotics to prevent NEC, was from 2007 (64). These same data were later updated by the same group in 2010 with the inclusion of 4 additional trials (65). After developing a fixed-effects model using 2,176 preterm neonates with VLBW, they found that the use of probiotics was associated with a lower risk of NEC [RR = 0.35, 95% CI: 0.23–0.55], lower risk of all-cause mortality [RR = 0.42, 95% CI: 0.29–0.62], and an improved time to feed, with a mean difference of 5.03 days saved [−5.03, 95% CI: −5.62 to −4.44]. However, no significant difference was observed regarding impact on sepsis. They concluded that the number needed to treat to prevent 1 case of NEC or 1 death was 25 [95% CI: 17–34] and 20 [95% CI: 14–34], respectively (65).

These findings were validated in another large meta-analysis from 2015 by Lau et al. using 20 RCTs of preterm VLBW infants, in which 12 additional studies were included and 2 from the prior study were not included (66). The most recent Cochrane review from 2020 on this subject including 57 RCTs in total with an expanded study population including very preterm or VLBW infants ($n = 10{,}812$), added more weight to the emerging importance of probiotics (67). Their analysis revealed that probiotics were associated with a reduction in the risk of NEC [RR = 0.54, 95% CI: 0.45–0.65] and the number needed to treat to prevent one additional case of NEC was 33 [95% CI: 25–50] (67). Through meta-analysis of well-designed RCTs studying the utility of probiotics in preventing NEC in VLBW preterm infants, it is clear that probiotics remain an important strategy for prophylaxis against NEC and deserve continued study.

Interestingly, the work of Lau et al. also highlighted the importance of specific strains and multi-strain formulations in the prevention of NEC (66). Subgroup analyses from this meta-analysis revealed that in particular *Lactobacillus* or mixtures of *Lactobacillus* and *Bifidobacterium*, were most effective in minimizing the risk of NEC (RR = 0.573, 95% CI: 0.354–0.928), in contrast to *Bifidobacterium* alone or *Sacharomyces* alone, which were not significantly effective. Likewise, the multi-strain probiotic recipients had a significantly reduced risk of mortality compared to those that received single-strain formulations (RR = 0.669, 95% CI 0.505–0.886) (66). In fact, more recent meta-analyses have validated the importance of multi-strain formulations of probiotics over single-strain formulations in the prevention of NEC (68, 69). In particular, the 2017 meta-analysis by Chang et al. found *Lactobacillus* species to have a borderline effect against NEC and only multi-strain formulations to be effective in reducing mortality (69). Thus, future studies of probiotics in human neonates should focus on the most effective strains such as *Lactobacillus* species (reclassified into *Lactobacillus*, *Limosilactobacillus*, *Lacticaseibacillus*, among other new and relevant genera) or multi-strain formulations such as *Lactobacillus* and *Bifidobacterium*.

Confounders and the importance of breast milk in probiotic effects during NEC

There are several factors, regardless of whether single or multi-strain probiotics are used, that complicate analysis and comparison of the RCTs discussed here, including the use of different probiotic formulations and dosing, differences in gestational age of the study groups (degree of prematurity), whether VLBW or ELBW infants were included, and differences in the incidence of NEC. In addition, the use of human breast milk vs. formula to feed the neonate while they are on probiotics may alter the effect of probiotics on NEC (70). For example, probiotic supplementation of *B. breve* and *L. casei* (Yakult LB, São Paulo, Brazil) to human milk in VLBW preterm infants during the first month of life was associated with a reduction in the incidence of NEC (Bell's stage ≥2). In fact, there were only reported cases of NEC in the control cohort (4/112) (58). This was supported by a single-center RCT study, which demonstrated that VLBW infants who received breast milk supplemented with *L. acidophilus* and *B. infantis* (Infloran™; Swiss Serum and Vaccine Institute, Berne, Switzerland) had reduced NEC incidence and rates of NEC or death compared to infants who were fed breast milk alone (59). The importance of breast milk on the function of Infloran™ was again validated in 2015 in another RCT in Europe (60). In contrast, breast milk administration alongside a probiotic mixture of *B. infantis*, *B. bifidum*, *B. longum*, and *L. acidophilus* reduced NEC overall in preterm VLBW infants, but had no difference on Bell's stage ≥2 disease (71). It is possible that concurrent breast milk feeds alongside probiotic administration leads to the improvement of intestinal colonization allowing a greater protection against NEC (72). This is not surprising given the natural role that breast milk has been found to play in preventing NEC. Breast milk provides the developing neonate with valuable maternal IgA (73), immunomodulatory and anti-infective molecules such as lactoferrin (74), beneficial modulation of TLR-4 signaling (75), and specific healthy microbes such as *Lactobacillus* (see section on "*Understanding the pathophysiology of NEC and the rationale for prophylactic use of probiotics*"), packaged alongside the resources that these microbes need to succeed (see section on "*Advances in prebiotics*, *synbiotics*, and *postbiotics*"). Thus, future RCTs should also report the diet of the neonate as an additional variable that might contribute to the bioactivity and success of the probiotic. Overall, more work is clearly needed to identify the most beneficial strain or strains of probiotics to include in future research studies.

Concerns about sepsis and other major barriers to the use and development of probiotics

The American Academy of Pediatrics (AAP) recently published a statement in November 2021 addressing the use of probiotics in preterm infants. In this statement they decided that at this time they "[do] not support the routine, universal administration of probiotics to preterm infants, particularly those with a birth weight of <1,000 g." As justification for this conclusion, they cited that most recent modern trials have not demonstrated an apparent reduction in NEC within high-risk infant populations, that there is no pharmaceutical-grade probiotic product currently available in the United States, and that long-term safety remains unknown. However, they did acknowledge that there are conflicting data regarding the use of probiotics in preterm infants for the prevention of NEC. In addition, they encouraged centers choosing to administer probiotics to be selective about their use and to have a thorough discussion of the risks and benefits as a part of a formalized informed consent process (14).

The NEC Society, a non-profit organization dedicated to building a world without NEC *via* research, advocacy, and education, also recently added to this discussion. They acknowledged that further research was required to understand the role of probiotics in the prevention of NEC, to identify which patient populations would benefit most from probiotics, to determine which probiotic strain or strains were preferred, and to confirm the best dose and duration of treatment. However, they did recommend that probiotics be considered as a strategy to help reduce the risks of NEC and death in VLBW infants. Given the lack of clarity, they also recommended that families be better educated about the risks and benefits of probiotic use in NEC, and that clinicians be prepared to explain their NICU's rationale for offering or not offering probiotic administration (76).

This lack of consensus by multiple stakeholders has made it challenging to develop national policies regarding the use of probiotics in neonates. It highlights the essential need for more research on this topic. One of the most piercing concerns from opponents of probiotic use in neonates is the possibility of probiotic-associated sepsis, whether due to contamination or to the possibility of pathogenic behavior by the probiotic bacteria itself. Given that several prior cases of probiotic-associated sepsis or contamination have been documented in the literature, there is good reason to be cautious (77–82). For example, it was reported in 2004 that two pre-term infants in Washington with short bowel syndrome that were given *Lacticaseibacillus rhamnosus* GG to help prevent bacterial overgrowth, developed *L. rhamnosus* GG sepsis (77). The weight of this report was only increased by cases of *L. rhamnosus* GG sepsis after probiotic administration in neonates in Poland in 2014 (78), Italy in 2016 (80), and Taiwan in 2021 (81).

These cases of probiotic sepsis are not exclusively limited to any one species of probiotic bacteria, and have also been seen with currently available commercial formulations. A 2014 report from Switzerland detailed the case of two preterm infants that prophylactically received the probiotic Infloran^{TM}, which contains *Bifidobacterium* spp. and *Lactobacillus acidophilus*, to prevent NEC. Both infants unfortunately developed culture-proven *B. longum* bacteremia (82). In 2015, another three cases of *B. longum* bacteremia were reported in preterm infants who received prophylactic Infloran^{TM}. Although all three infants had blood cultures positive for *B. longum* either while on Infloran^{TM} or shortly after treatment, two of the three did not require additional antibiotic treatment. The third infant, however, developed NEC, despite treatment with Infloran^{TM}, and ultimately required both antibiotics and surgery (79). Although these cases are rare, the existence of these sentinel events is troublesome. Our lack of understanding as to why probiotic-related bacteremia occurs, which subpopulations of premature neonates are at the highest risk, and whether this is even preventable given the loss of intestinal barrier function in NEC, continues to be a significant barrier to the widespread use of probiotics in NICUs.

In addition to hesitancy due to a lack of defined guidelines for the role of probiotics in the treatment of NEC, and the rare but notable cases of probiotic-related sepsis, the absence of government oversight or regulation in this industry is another barrier to usage. At present, there are no FDA-approved probiotics on the market and the precise contents of non-FDA-approved probiotic formulations currently available cannot be guaranteed. Drago et al. conducted a study in 2009 to determine if products available in the USA market were correctly labeled and found that the contents of only 4 of 13 products matched their labels (83). A similar study by Toscano et al. in 2011 investigating products on the Italian and European market found that out of 24 products, 10 did not contain the expected amount of bacteria listed on the label and 4 did not contain any of the species included on the label (84). As recently as 2016, Lewis et al. aimed to validate the identity of *Bifidobacterium* species and subspecies in 16 different commercial products, of which only one probiotic perfectly matched its label (85). Beyond the discordance between product labels and their contents, there have been several probiotic recalls due to contamination (86–88). A widely known incident of probiotic-associated sepsis due to contamination was the death of a VLBW preterm infant in Connecticut, who unfortunately succumbed to gastrointestinal mucormycosis after receiving the probiotic ABC Dophilus Powder that was contaminated with *Rhizopus oryzae* (89).

These uncertainties and discrepancies demonstrate the importance of good manufacturing practice (GMP)-grade probiotic preparation for human administration as an important next step in developing probiotic drugs for NEC. However, given the exorbitant cost of producing a GMP-grade drug formulation, and the enormous effort required to test that drug and get it approved by the FDA, this is a significant hurdle. As our target population is newborns, the cost may be doubled as the FDA requires initial Phase 1 studies in adults prior to beginning Phase 1 studies in newborns (90). Funding this extensive effort is difficult without the support of pharmaceutical companies. Unfortunately, NEC is an orphan disease affecting less than 200,000 infants nationwide (91). As such, there is not a great incentive for pharmaceutical companies, hospitals, and government agencies to support new research and the development of novel therapeutics to treat NEC, compared to therapeutics for more prevalent diseases (90). Despite these clear difficulties in producing a probiotic drug for NEC,

several competing groups are working at present to test GMP-grade probiotics in the clinical setting, in order to gain full FDA approval. One such GMP-grade probiotic drug known as IBP-9414 (*L. reuteri*), developed by Infant Bacterial Therapeutics AB (IBT), is currently being studied in an ongoing, registered, phase 3 RCT known as the "Connection Trial" (NCT03978000). This study is presently in the recruiting phase and is slated to be complete by the end of 2023. In addition to uniquely being one of the few studies using GMP-grade products in an RCT, this study is also intentionally being powered to see an effect for NEC (92). If this GMP-grade product achieves full FDA-approval, this could change the landscape for the use of probiotics in NICUs, as it may be more universally accepted amongst neonatologists as a therapeutic option against NEC. Of note, IBP-9414 at present has received orphan drug status for the prevention of retinopathy of prematurity, but not for NEC (93). Preliminary data from this study was limited to establishing definitions for sustained feeding tolerance, a primary outcome for their trial, and researchers have not yet commented on the efficacy of their probiotic against NEC as they remain blinded. However, we do know that their overall incidence of NEC at this time, regardless of allocation to probiotic or control group, is 6% ($n = 13/216$) (94).

Next-generation probiotics in the prevention of NEC

Advances in prebiotics, synbiotics, and postbiotics

In addition to probiotics, prebiotics, synbiotics and postbiotics have emerged as potential prophylactic strategies against NEC (see **Figure 2**). A prebiotic is defined as a "substrate that is selectively utilized by host microorganisms conferring a health benefit" (95, 96). Breast milk contains prebiotics known as human milk oligosaccharides (HMOs), with HMO 2'-fucosyllactose (2'FL) being the most predominant (97). HMOs are selectively consumed by *Bifidobacterium* species, which colonize the gut in healthy breastfed infants (98). In an experimental rat model of NEC, HMOs or 2'FL alone were shown to reduce pathology compared to formula-fed only animals (99). Another important component of breast milk, particularly colostrum, is the iron-binding glycoprotein lactoferrin, which can promote the growth of *L. acidophilus* and *Bifidobacterium* species (100). A Cochrane review showed that lactoferrin decreased the incidence of NEC (Bell's stage ≥2) in pre-term infants when added to enteral feeds with or without probiotics (74). Thus, prebiotics remain a promising avenue in the treatment of NEC given their beneficial effects on commensal bacteria. If the right combination of prebiotics were discovered to help assure healthy maturation of the microbiome, it is possible that probiotics might not be needed at all; thus, eliminating the risk of probiotic-related sepsis and contamination.

Synbiotics, on the other hand, are a combination of prebiotic and probiotic products, in which the presence of the prebiotic benefits the growth of both the probiotic bacteria and commensal host flora (101). While this is a promising concept, further evaluation is necessary as the available data on their beneficial role and their innocuity are very limited. A group in Turkey performed a RCT where VLBW infants ≤32 gestational weeks received oral *Lactobacillus* species, *B. lactis*, oligosaccharides, and bovine lactoferrin with feeds. There was no difference between the treatment and control groups in terms of NEC severity, incidence, or death (101). On the contrary, a multi-center, international RCT revealed that bovine lactoferrin alone or in combination with *L. rhamnosus GG* was associated with a significantly reduced incidence of NEC compared to placebo (102). Another RCT found that enteral administration of multi-strain probiotics consisting of *L. rhamnosus*, *L. casei*, *L. plantorum*, and *B. animalis* (NBL probiotic®) alongside fructooligosaccharides and galactooligosaccharides to VLBW preterm neonates resulted in significantly decreased mortality and NEC incidence compared to placebo (103). Careful selection of prebiotic and probiotic combinations is important in the development of synbiotics to ensure long-lasting beneficial effects. For example, in an interim evaluation of an ongoing RCT, the enteral administration of *L. reuteri* in conjunction with *ω*-3 fatty acid treatment prenatally to the mother and then postnatally in the neonate resulted in synergistic epigenetic changes in allergy and immune-related pathways in T-helper cells (104). Thus, synbiotics are a clear new frontier for optimizing probiotic-based interventions for NEC.

Finally, a postbiotic is a bioactive metabolite with beneficial properties produced by a microorganism and used as a direct therapeutic in place of the microorganism (105, 106). For instance, Meng et al. (2020) identified anti-inflammatory indole-3-lactic acid (ILA) as a beneficial breakdown product of tryptophan produced by *B. infantis*. The addition of this postbiotic to enterocytes originating from a NEC patient *in vitro*, prior to addition of interleukin-1β (IL-1β) stress, resulted in reduced IL-8 secretion by the cells (107). Overall, there are fewer studies investigating the effect of postbiotics in NEC compared to prebiotics and synbiotics. However, this line of research will undoubtedly advance the field of probiotics overall, as it will allow for careful selection of strains based on their metabolic products. As we develop a more refined understanding of the optimal substrates and environment required by specific probiotics, we may be able to ensure the success of probiotics and even amplify their effects against NEC.

Developing novel delivery systems

Probiotics administered enterally face several inherent challenges before successfully colonizing the intestine, including exposure to gastric acids, turbulent intraluminal fluid forces, and competition with other microbes and the host immune response (108). One mechanism that some bacteria naturally employ to survive these harsh conditions, and to successfully attach to the intestinal wall, is the production of biofilms. Biofilms are an extracellular matrix composed of oligosaccharides, proteins, lipids, and DNA, produced by communities of bacteria to enhance their adherence to surfaces such as the intestinal wall (109). Interestingly, biofilms may also play a role in the ability of

some probiotics to attenuate intestinal inflammation. In adult mice, highly adhesive strains of *L. reuteri* have been shown to elicit a greater anti-inflammatory IL-10 response after LPS stress, compared to less adhesive strains (110). While several authors have tested strains of *L. reuteri* that happen to be biofilm-producing or highly adhesive, such as DSM 20016, this is not typically a variable that has been prioritized for probiotic selection in humans. However, enhancing biofilm production by *L. reuteri* DSM 20016 may improve the overall efficacy of the probiotic against human NEC by improving intestinal colonization.

One novel approach to capitalize on the inherent adhesiveness of *L. reuteri* DSM 20016 is by growing these bacteria on the surface of dextranomer microspheres such as Sephadex^{TM} (DM), as a vehicle for delivery of the probiotics (see **Figure 2**) (111). When *L. reuteri* DSM 20016 (*Lr*) is grown on DM (*Lr*-DM) there is enhanced biofilm production (112). In a rat model of the disease, we have shown that a single dose of *Lr*-DM (i.e., *Lr* administered in its biofilm state) administered after birth significantly protects the intestines against NEC (111). While most dosing strategies of probiotics in human NEC require daily usage, this delivery system could radically minimize the exposure of a premature neonate to probiotic bacteria, reducing the risk of probiotic-related sepsis. Also, DM can be loaded with beneficial substances such as maltose (*Lr*-DM-maltose) to further increase biofilm production, and we have shown that this further enhances the ability of the probiotic to protect the intestines against NEC in rats (33). We have now tested *Lr*-DM-maltose in a piglet model of NEC, and have confirmed these promising pre-clinical findings (unpublished observations). Unlike typical synbiotic strategies, where the probiotic and its substrate are fed separately, this delivery system allows for co-localization of the substrate to the microenvironment of the attached bacteria, avoiding any off-target effects of the prebiotic on potentially pathogenic organisms. Through the targeted selection of beneficial strains (i.e., *L. reuteri* DSM 20016) administered using novel delivery systems, it is possible that probiotics can be more safely and effectively delivered to human neonates in a prophylactic fashion.

Generating designer probiotics

Although not yet studied in the context of NEC, one next-generation approach to fine-tune probiotics to better address diseases while minimizing off-target effects is through genetic engineering. By editing specific disease-related genes, including those involved in inflammation, infection, or metabolism-related pathways, it may be possible to create enhanced probiotic strains, loosely known as designer probiotics, that can better address the diseases they are being developed for (see **Figure 2**). While there are significant ethical and safety issues with generating new bacterial strains and testing them in humans, the early efforts in this arena are encouraging and are likely to continue to evolve (113). Several examples of designer probiotics exist at present in the pre-clinical arena, targeting a diverse range of inflammatory and non-inflammatory diseases. For instance, the *L. plantarum* NC8 strain was modified to produce angiotensin-converting enzyme inhibitory peptides to successfully combat hypertension in rats (114) and *B. longum* was engineered to secrete fully functional glucagon-like peptide-1 to improve pancreatic function in type 2 diabetes mellitus (115).

With regards to addressing inflammatory and infectious disorders of the gut that might be relevant to NEC, *L. lactis* was modified to serve as a prophylactic vaccine against *C. difficile*, through the expression of non-toxic fragments of *C. difficile* cytotoxins. It was shown in an *in vivo* mouse model that this vaccination strategy improved survival and resulted in increased

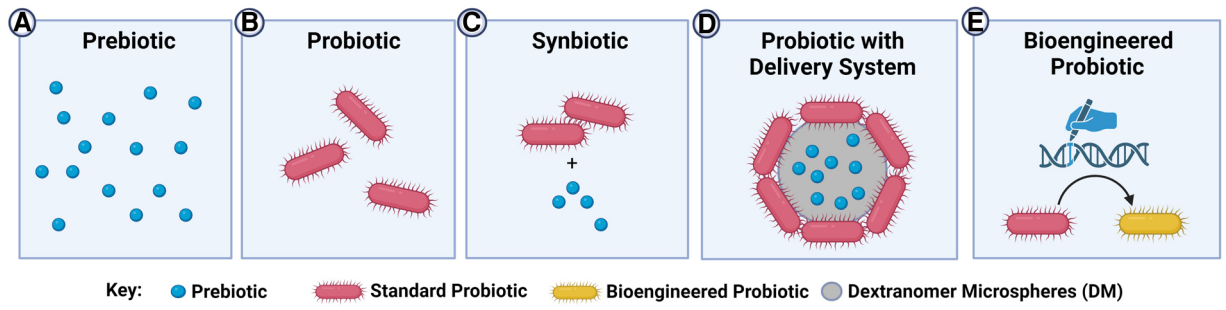

FIGURE 2
Current and next-generation probiotic-related therapies in the prevention of NEC. (A) Prebiotics are substrates that bacteria can utilize to confer a health benefit to the host. Since no bacteria are administered, this strategy eliminates concerns about probiotic sepsis. However, there are limited pathways being targeted compared to the complex interactions resulting from the use of whole bacteria; (B) probiotics are live bacterial species that confer a health benefit to the host. These products have a much broader range of targets/effects than simple prebiotics, but there is a theoretical risk of probiotic sepsis; (C) synbiotics are probiotics that are co-administered with beneficial prebiotics. The prebiotics can enhance the effect of the probiotic, however the prebiotic may not exclusively be used by the probiotic itself and could be utilized by other intestinal bacteria; (D) probiotics can be administered using novel delivery systems such as dextranomer microspheres (DM), which can be pre-loaded with prebiotics. These delivery systems can promote the formation of a biofilm, leading to increased attachment of the probiotic to the intestinal mucosa. Administration in the biofilm state improves survival of the probiotic against the harsh gastric and intestinal environment. The prebiotic and probiotic are co-localized to ensure maximal use of the prebiotic by the adherent probiotics, with no-off target effects of the prebiotic on other microbes; (E) bioengineered probiotics are theoretical or emerging probiotics in which specific pathways are enhanced or altered through bioengineering strategies. This can theoretically reduce safety concerns by eliminating pathogenicity and improve efficacy by selecting beneficial phenotypes. However, significant regulatory hurdles for the development and testing of bioengineered probiotics exist at present.

IgG and IgA titers (116). Another example of a bioengineered probiotic that might be relevant to NEC was the recent modifications of *E. coli* Nissle 1917, a harmless gram-negative bacterium, that was developed to combat *C. difficile* colonization (117). Given that conjugated bile acids have been found to play a role in *C. difficile* colonization, *E. coli* Nissle 1917 was bioengineered to deconjugate intestinal bile acids. Furthermore, it was modified to perform this deconjugation task only when dysbiosis was observed, through the detection of subtle changes in intraluminal sialic acid concentration, a reliable biomarker for dysbiosis (117). When testing this remarkable dysbiosis-sensing probiotic against *C. difficile in vitro*, it was found that the pathogen's germination and growth were significantly inhibited, and its toxicity was reduced. Most importantly, administration of this probiotic reduced histologic injury after *C. difficile* infection in mice (117). Another relevant approach that has been employed to reduce pathogen toxins in the intestine is the development of probiotics that express toxin receptor mimics to neutralize the toxin and minimize its binding to host toxin receptors (118).

As we develop a more rigorous understanding of NEC pathogenesis, it may be possible to create similar engineered probiotics that respond to early NEC-related changes, with targeted responses to neutralize pathogens or toxins and strengthen host defenses. Recently, it was shown that NEC may be associated with a reduction in IL-22 signaling and that recombinant IL-22 therapy during NEC could significantly reduce the severity of experimental NEC in mice (119). It will be interesting to study how probiotics engineered to deliver IL-22 or other disease-mitigating products might perform against NEC, a strategy that was very recently utilized with a modified IL-22 producing *L. reuteri* to protect against intestinal radiation in mice (120). While these "designer probiotics" are exciting alternatives as they might radically improve the efficacy of probiotics against NEC, it is important not to minimize the sheer volume of regulatory hurdles and preclinical work that would be required prior to such products being tested in neonates.

Conclusion

Despite decades of research on the use of probiotics in humans, the role of probiotics in preventing NEC remains controversial and unclear. Differences in dosing strategies, use of single versus multi-strain formulations, and co-administration of prebiotics or breast milk, have complicated comparisons and interpretations of previous work. However, the abundance of data available has helped to identify several specific strains of probiotic that merit further testing based on their anti-inflammatory, anti-microbial, metabolic, or highly adhesive properties. Current ongoing work in the field of probiotics has sought to amplify the effects of these strains and minimize concerns about safety, through the generation of next-generation synbiotics, delivery systems, and designer probiotics. Through careful strain selection and optimization of dosing strategies and effects, it is quite possible to use probiotics to effectively prevent NEC. FDA approval, GMP-grade production, and evidence-based guidelines are likely to significantly increase the routine use of probiotics in neonates in the future.

Author contributions

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Conflict of interest

GB is a scientific founder of Scioto Biosciences, Inc. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Probiotics to prevent necrotizing enterocolitis in very low birth weight infants: A network meta-analysis

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Objective: This study aims to review the evidence for the optimal regimen of probiotics for the prevention of necrotizing enterocolitis (NEC) in very low birth weight infants.
Design: Through searching PubMed, EMBASE, Cochrane Library, and Web of Science till September 30, 2022, only randomized controlled trials were included to evaluate the optimal regimen of probiotics for the prevention of NEC in very low birth weight infants. The methodological quality of the included studies was assessed by the Cochrane risk of bias assessment tool (RoB 2), and the collected data were analyzed accordingly using Stata software.
Results: Twenty-seven RCTs were included, and the total sample size used in the study was 529. The results of the network meta-analysis showed that Bovine lactoferrin + Lactobacillus rhamnosus GG (RR 0.03; 95% CI 0.00–0.35), Lactobacillus rhamnosus + Lactobacillus plantarum + Lactobacillus casei + Bifidobacterium lactis (RR 0.06; 95% CI 0.00–0.70), Bifidobacterium lactis + inulin (RR 0.16; 95% CI 0.03–0.91) were superior to the control group (Bifidobacterium lactis + Bifidobacterium longum) in reducing the incidence of NEC. The reduction in the incidence of NEC were as follows: Bovine lactoferrin + Lactobacillus rhamnosus GG (SUCRA 95.7%) > Lactobacillus rhamnosus + Lactobacillus plantarum + Lactobacillus casei + Bifidobacterium lactis (SUCRA 89.4%) > Bifidobacterium lactis + inulin (SUCRA 77.8%).
Conclusions: This network meta-analysis suggests that Lactobacillus rhamnosus GG combined with bovine lactoferrin maybe the most recommended regimen for the prevention of NEC in very low birth weight infants.

KEYWORDS
necrotizing enterocolitis, very low birth weight infants, probiotics, network meta-analysis, prevention

1. Introduction

Necrotizing enterocolitis (NEC) is a gastrointestinal disease that seriously threatens the life of newborns. Clinically the infant presents with feed intolerance, increased gastric aspirates, vomiting, blood in the stool which may progress to very severe illness including shock and perforation. It is a disease that has plagued neonatal care for a long time and is still relatively common in very low birth weight infants (1). NEC is associated with neurodevelopmental delays, growth retardation, intestinal strictures and adhesions, and short bowel syndrome with or without intestinal failure (2). The high incidence of NEC cannot be ignored, and according to large multi-center neonatal network databases in the

United States and Canada (3–5), NEC may occur in 7 out of 100 very low birth weight infants over the decades. Despite overall improvement in survival of preterm infants, a recent review suggests that the mortality and prevalence of NEC in very low birth weight infants has barely changed (6).

NEC, once established, is challenging to stop and has limited and expensive treatments. Treatment methods for NEC include antibiotics, gastric decompression, parenteral nutrition, etc. (7). It is not clear whether NEC is a single entity or a combination of similar entities and while progress has been made in understanding the pathogenesis of NEC there is still lack of clarity on many fronts which has perhaps contributed to a lack of significant progress in the treatment of NEC over the last many decades (8). On the other hand, although very low birth weight infants account for only a small proportion of newborns, the cost of treatment is indeed disproportionate. It has been reported that NEC causes more than 1 billion dollars in losses to medical institutions (9). It is worth noting that about 40% of NEC cases require surgical intervention (10), and the cost of treatment for infants requiring surgery has also significantly increased. All these factors lead to a considerable economic burden on the family and society.

Multiple research studies have explored various interventions for prevention of NEC including the provision of human milk and microbial therapeutics, with probiotic therapy garnering the most attention. Shiloh R. Lueschow et al. found that Bifidobacterium infantis EVC001 can prevent NEC in mice through anti-inflammatory and epithelial barrier-restoring properties (11). The study by Xiu-Li Zhu et al. has shown that probiotics supplementation can reduce the incidence and severity of NEC in preterm neonates (12), which seems to be related to the functions of probiotics in regulating immunity and inhibiting the imbalance of intestinal flora.

Most studies in the past decade have suggested that probiotics can significantly reduce the risk of NEC; However, it is unclear which probiotic or combination of probiotics is more effective (13) and what the optimal dose is. Moreover data on VLBW are scarce, and other related studies have not reported on specific strains of probiotics (14). Therefore, this study aimed to compare the effects of various probiotic regimens on NEC through a network meta-analysis, with direct or indirect comparisons, and to estimate the rank order of each combination. This hopefully will help target further research as well as facilitate improvements in practice.

2. Materials and methods

2.1. Search strategy

This network meta-analysis was conducted following the PRISMA statement, and the protocol for this study was registered in the International Platform of Registered Systematic Review and Meta-analysis Protocols (number INPLASY2022110001).

The researchers searched PubMed, EMBASE, Cochrane Library, and Web of Science till September 30, 2022. The search strategy was constructed around the PICOS tool: (*P*) Population: very low birth weight infants; (I) Intervention: probiotics; (C) Comparator: control group with only placebo or another probiotic usage; (O) Outcomes: necrotizing enterocolitis. (S) Study type: RCTs. The detailed search strategy is shown in (**Table 1**) (PubMed is used as an example).

2.2. Inclusion criteria

(1) Study designed as RCT; (2) Neonates with birth weight <1500 g; (3) Interventions included probiotics; (4) Reported outcomes included NEC stage ≥ II (Bell staging criteria); (5) The incidence of outcomes given by the study.

2.3. Exclusion criteria

(1) Studies from non-randomized controlled trials, including quasi-randomized controlled trials, non-human subjects, case reports, protocols, correspondence, or conference abstracts; (2) Studies with incomplete or unreported data.

2.4. Study selection

Literature was screened and excluded using the literature management software Endnote. Two researchers first screened papers by title to exclude duplication, non-randomized controlled trial studies, correspondence, review papers, and conference papers. Two researchers then read the abstracts to determine which studies to include and exclude. Finally, two researchers performed full-text readings to further identify the included literature. During this process, two researchers independently screened the literature and compared the remaining literature to determine whether they could be included in the study. Any conflicts were resolved by discussion with a third author.

2.5. Data extraction

A nine-item, standardized, and pre-selected data extraction form was used to record data from included studies under the following headings: (1) author, (2) year of publication, (3) country, (4) sample size, (5) mortality, (6) number of people progressing to NEC, (7) mean age, (8) details of the intervention, (9) overall risk of bias.

TABLE 1 Search strategy on PubMed.

#1	Enterocolitis Necrotizing [MeSH Terms]
#2	Necrotizing Enterocolitis [Title/Abstract]
#3	#1 OR #2
#4	Probiotics [MeSH Terms]
#5	Probiotic [Title/Abstract]
#6	#4 OR #5
#7	randomized controlled trials [Publication Type]
#8	#3 AND #6 AND #7

2.6. Risk of bias in individual studies

Two researchers independently assessed the risk of bias (RoB 2) according to the Cochrane Handbook version 6.1.0 tool for assessing RoB 2 in RCTs. Five items were considered: (1) randomization process, (2) deviations from intended interventions, (3) missing outcome date, (4) measurement of the outcome, and (5) selection of the reported result. The risk of bias for each domain can be classified into three levels: low risk, some concerns and high risk. If the risk of bias assessment for all domains is "low risk", then the overall risk of bias is "low risk"; If the risk of bias assessment in some domains are "some concerns" and there is no "high risk" domain, then the overall risk of bias is "some concerns"; As long as the risk of bias assessment in one domain is "high risk", the overall risk of bias is "high risk".

2.7. Data analysis

In studies using probiotics as an intervention, outcome variables were dichotomized and expressed as risk ratios (RR) and 95% confidence intervals (CI). Due to potential differences between studies, we decided to use a random-effects model rather than a fixed-effects model to analyze the data.

Data were compiled and analyzed using Markov chain Monte Carlo simulation chains of Stata software (version 15.1) based on a Bayesian framework according to the PRISMA NMA instruction manual. To quantify and demonstrate the agreement between indirect and direct comparisons, we used the nodal method calculated according to the instructions in Stata. The consistency test was passed if the *P*-value was > 0.05.

We presented and described network diagrams for different probiotic usage using Stata software. In the presented network diagrams, each node represents a different probiotic usage, and the lines connecting the nodes represent a direct comparison between interventions. The size of each node and the width of the connecting lines are proportional to the number of trials.

The evaluation of the intervention was summarized and presented in the form of a *P* score. The *P* score was considered as a frequency analog to surface under the cumulative ranking curve (SUCRA) values, a measure of the degree of certainty that one treatment is superior to another. The *P* score ranges from 0 to 1, with 1 representing the best treatment without uncertainty and 0 the worst treatment without uncertainty. The P score or SUCRA could be effectively expressed as a percentage of intervention effectiveness or acceptability, but this score should be interpreted with caution unless there is a genuine clinically meaningful difference between interventions. Small-scale studies could lead to publication bias in NMA, for which we created network funnel plots and checked them visually using symmetry criteria.

3. Results

3.1. Study and identification and selection

A total of 3,159 documents were retrieved from the electronic database, and an additional three documents were manually searched. After eliminating duplicates, the remaining 2,153 documents were read for titles and abstracts, and 1,994 documents were again excluded. The remaining 159 documents were read in full, and 132 documents were again excluded (for reasons including non-randomized controlled trials, incomplete data, conference papers, and failure to meet the interventions included in this review), leaving a final remaining 27 documents to be included in this study. (**Figure 1**).

3.2. Quality assessment of the included studies

Seventeen studies were defined as low risk, eight as some concerns, and two as high risk. Only three of these studies did not achieve simultaneous blinding of subjects and measures. Specific details are presented in (**Supplementary Table S1**).

3.3. Characteristics of the included studies

In total, we included studies from 27 randomized controlled trials, which included 529 patients diagnosed with NEC. Interventions included Bovine lactoferrin + Lactobacillus rhamnosus GG (2 studies) (15, 16), Bovine lactoferrin (2 studies) (15, 16), Lactobacillus rhamnosus + Lactobacillus planvtarum + Lactobacillus casei + Bifidobacterium lactis (2 studies) (17, 18), Bifidobacterium lactis + inulin (1 study) (19), inulin (1 study) (19), Bifidobacterium lactis + Bifidobacterium longum (1 study) (20), Bifidobacterium lactis (3 studies) (19–21), Bifidobacterium longum (1 study) (20), Bifidobacterium bifidum + Lactobacillus acidophilus (2 studies) (22, 23), Lactobacillus rhamnosus (3 studies) (24–26), Bifidobacterium infantis + Streptococcus thermophilus + Bifidobacterium lactis (2 studies) (27, 28), Lactobacillus sporogenes (1 study) (29), Lactobacillus reuteri DSM 17938 (4 studies) (30–33), Lactobacillus rhamnosus GG + Bifidobacterium infantis (2 studies) (34, 35), Saccharomyces boulardii (2 studies) (36, 37), Bifidobacterium breve + Bifidobacterium infantis + Bifidobacterium longum (1 study) (38) and Bifidobacterium breve (4 studies) (38–41). There were two studies from Asia, three studies from America, eighteen studies from Europe, and four studies from Oceania. The characteristics of the included studies are shown in (**Table 2**).

3.4. Network meta-analysis

The full NMA figure is shown in (**Figure 2**). All *P*-values for indirect and direct comparisons between all studies were tested for consistency and inconsistency, and most *P*-values were greater than 0.05, indicating that the effects of consistency between studies were acceptable. Details were shown in (**Supplementary Table S2**).

The results of the Network meta-analysis showed that Bovine lactoferrin + Lactobacillus rhamnosus (RR 0.03; 95% CI 0.00–0.35; **Table 3**), Lactobacillus rhamnosus + Lactobacillus

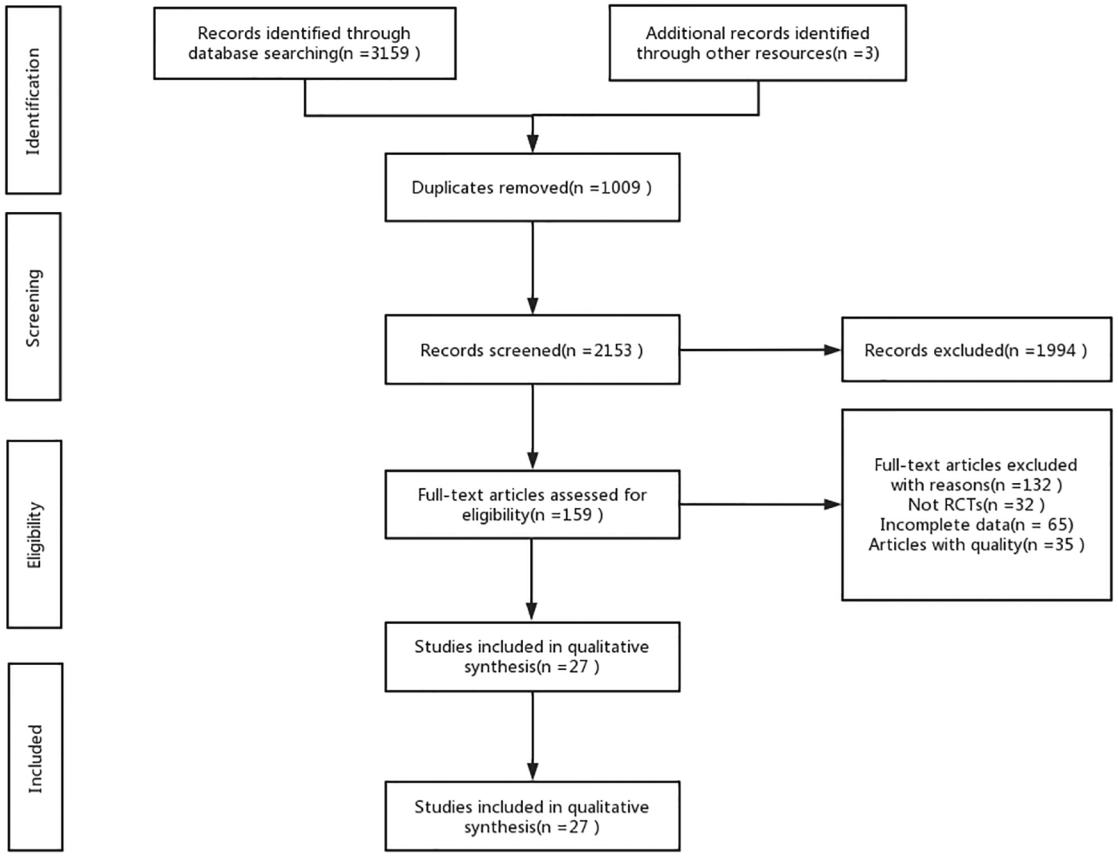

FIGURE 1
Flow diagram of literature selection.

plantarum + Lactobacillus casei + Bifidobacterium lactis (RR 0.06; 95% CI 0.00–0.70), Bifidobacterium lactis + inulin (RR 0.16; 95% CI 0.03–0.91), Bifidobacterium lactis (RR 0.20; 95% CI 0.05–0.82) were superior to the control group (Bifidobacterium lactis + Bifidobacterium longum) in reducing the incidence of NEC. Relative to the control group (placebo), Bifidobacterium bifidum + Lactobacillus acidophilus (RR 0.32; 95% CI 0.11–0.92) and Bifidobacterium infantis + Streptococcus thermophilus + Bifidobacterium lactis (RR 0.51; 95% CI 0.27–0.97) were superior to the control group (placebo) in reducing the incidence of NEC.

Bayesian Markov chain Monte Carlo modeling revealed that Bovine lactoferrin + Lactobacillus rhamnosus had the highest probability of having the lowest rate of NEC (SUCRA 95.7%; **Figure 3**), followed by Lactobacillus rhamnosus + Lactobacillus plantarum + Lactobacillus casei + Bifidobacterium lactis (SUCRA 89.4%), and Bifidobacterium lactis + inulin (SUCRA 77.8%).

3.5. Publication bias test

We constructed separate funnel plots for all outcome indicators to test for possible publication bias. Visual inspection of the funnel plots did not reveal any significant publication bias (42). Details were shown in (**Figure 4**).

4. Discussion

NEC is a gastrointestinal disorder that has plagued the field of neonatology for a long time. Considering the morbidity and mortality of NEC, as well as the high cost of treatment and socioeconomic loss, it is important to prioritize research on NEC prevention and treatment. This study included 27 trials with 18 interventions, including 9,501 very low birth weight infants. We aimed to investigate which probiotics effectively prevent NEC in very low birth weight infants. This network meta-analysis concluded that Lactobacillus rhamnosus plus bovine lactoferrin might be the most appropriate regimen for preventing NEC in very low birth weight infants compared to a placebo or other probiotic control group.

Lactobacillus rhamnosus GG belongs to the genus Lactobacillus, a naturally occurring gram-positive bacterium that was originally isolated from the healthy human intestine (43). Lactobacillus rhamnosus GG has strong adhesion to intestinal cells and can also exert its high immune activity in the acidic pH environment of the digestive tract (44), which are prerequisites for colonization in the human intestine. Lactoferrin is a transferrin-like protein with anti-infective and anti-inflammatory properties (45) and is found in high levels in human colostrum and low levels in breast milk, tears, saliva, and semen.

TABLE 2 Characteristics of the studies included in the meta-analysis.

Author	Year	Country	Sample		Mortality		Progression of NEC		Age.mean (SD)		Interventions		Interventions frequency	Overall Bias
			T	C	T	C	T	C	T	C	T	C		
Paolo Manzoni1	2014	Italy	①247	258	①5	18	①5	14	①29.7 (2.5)	29.4 (3.1)	①BLF	Placebo	Once a day	Low risk
			②238		②9		②0		②29.6 (2.8)		②BLF and LGG			
Gamze Demirel	2013	Turkey	135	136	5	6	6	7	29.4 (2.3)	29.2 (2.5)	S.b	Placebo	Once a day	Some concerns
Ozge Serce	2013	Turkey	104	104	5	4	7	7	28.8 (2.2)	28.7 (-2.1)	S.b	Placebo	Twice a day	Low risk
Kate Costeloe1	2015	UK	650	660	54	56	61	66	30.6 (6.5)	30.9 (6.6)	B.b	Placebo	Once a day	Low risk
Sanjay Patole	2014	Australia	79	80	0	0	0	1	29 (26-30; 23-32)	28 (26-29; 23-33)	B.b	Placebo	Once a day	Some concerns
Hung-Chih Lin	2014	China	217	217	4	20	4	14	/	/	B.bi and L.a	Placebo	Twice a day	Low risk
Mehmet Yekta Oncel	2014	Turkey	200	200	20	27	8	10	28.2 (2.4)	27.9 (2.5)	L.r	Placebo	Once a day	Low risk
Susan E. Jacobs	2013	Australia	548	551	27	28	4	11	27.9 (2.0)	27.8 (2.0)	B.i S.t and B.l	Placebo	Once a day	Low risk
Stephane Hays	2015	France	①50	52	1	1	①2	3	29.0 (28.1; 30.1)	29.4 (27.9; 30.6)	①B.l	Placebo	Once a day	Low risk
			②48				②1				②B.lo			
			③47				③5				③B.l and B.lo			
Dilek Dilli	2015	Turkey	①100	100	①3	12	①2	18	①28.8 (1.9)	28.2 (2.2)	①B.l	Placebo	Once a day	Low risk
			②100		②2		②12		②29.0 (1.7)		②inulin			
			③100		③3		③4		③28.9 (1.9)		③B.l and inulin			
Kate Costeloe2	2016	UK	611	619	54	56	57	62	/	/	B.b	Placebo	Once a day	High risk
İpek Güney-Varal	2017	Turkey	70	40	1	9	0	4	29.7 (1.9)	29.3 (1.7)	LGG, L.p, L.c and B.l	Placebo	Once a day	Low risk
M Al-Hosni	2012	USA	50	51	3	4	2	2	25.7 (1.4)	25.7 (1.4)	LGG and B.i	Placebo	Once a day	Some concerns
Ozge Serce Pehlevan	2019	Turkey	104	104	6	3	0	4	29 (1.9)	28 (2.2)	LGG, L.p, L.c and B.l	Placebo	Once a day	Low risk
W.A. Mihatsch	2009	Germany	93	90	2	1	2	4	26.6 (1.8)	26.7 (1.7)	B.l	Placebo	Once a day	Some concerns
Iwona Sadowska -Krawczenko	2012	Poland	30	25	1	0	1	4	29 (27-31)	30 (27-31)	LGG	Placebo	Twice a day	Low risk
Varaporn Saengtawesin	2014	Thailand	31	29	0	0	1	1	31.0 (1.82)	30.59 (1.76)	B.bi and L.a	Placebo	Twice a day	Some concerns
Carlo Dani	2002	Italy	295	290	0	2	4	8	30.8 (2.4)	30.7 (2.3)	LGG	Placebo	Once a day	Low risk
FN Sari	2011	Turkey	110	111	3	4	6	10	29.5 (2.4)	29.7 (2.4)	L.s	Placebo	Once a day	High risk
Paolo Manzoni2	2009	Italy	①153	168	①4	12	①3	10	①29.6 (2.5)	29.5 (3.2)	①BLF	Placebo	Once a day	Low risk
			②151		②6		②0		②29.8 (2.8)		②BLF and LGG			
Erica L. Plummer2	2021	Australia	229	230	/	/	11	18	28.6 (27.2-30)	28.1 (26.5-29.5)	B.i, S.t and B.l	Placebo	Once a day	Some concerns
T. Havranek	2013	USA	15	16	/	/	0	1	25.9 (1.3)	25.9 (1.5)	LGG and B.i	Placebo	Once a day	Low risk
Gayatri Athalye-Jape	2022	Australia	①86	29	①8	0	①3	0	①26.2 (24.4-27.2)	26.1 (25.2-26.9)	①B.b	Placebo	Once a day	Some concerns
			②87		②12		②3		②26.3 (24.7-27.1)		②B.b, B.i and B.lo			
P. Manzoni	2006	Italy	39	41	5	6	1	2	29.6 (5)	29.3 (4)	LGG	Placebo	Once a day	Low risk
JohanneE. Spreckels	2021	Sweden	48	57	0	0	2	5	25.4 (1.3)	25.8 (1.1)	L.r	Placebo	Once a day	Some concerns
Erik Wejryd	2019	Sweden	68	66	5	5	7	8	25.5 (1.2)	25.5 (1.3)	L.r	Placebo	Once a day	Low risk
Nancy Patricia	2015	Mexico	24	20	1	/	/	10	31.2 (2.39)	31.7 (2.47)	L.r	Placebo	Once a day	Low risk

Note: BLF and LGG, bovine lactoferrin and lactobacillus rhamnosus GG; LGG, L.p, L.c and B.l, Lactobacillus rhamnosus, lactobacillus plantarum, lactobacillus casei and bifidobacterium lactis; B.l and inulin, bifidobacterium lactis and inulin; B.l, bifidobacterium lactis; B.lo, bifidobacterium longum; B.bi and L.a, bifidobacterium bifidum and lactobacillus acidophilus; BLF, bovine lactoferrin; LGG, lactobacillus rhamnosus GG; B.i, S.t and B.l, bifidobacterium infantis, streptococcus thermophilus and bifidobacterium lactis; L.s, lactobacillus sporogenes; L.r, lactobacillus reuteri; LGG and B.i, lactobacillus rhamnosus GG and bifidobacterium infantis; S.b, saccharomyces boulardii; B.b, B.i and B.lo, bifidobacterium breve, bifidobacterium infantis and bifidobacterium longum; B.b, bifidobacterium breve; B.l and B.lo, bifidobacterium lactis and bifidobacterium longum.

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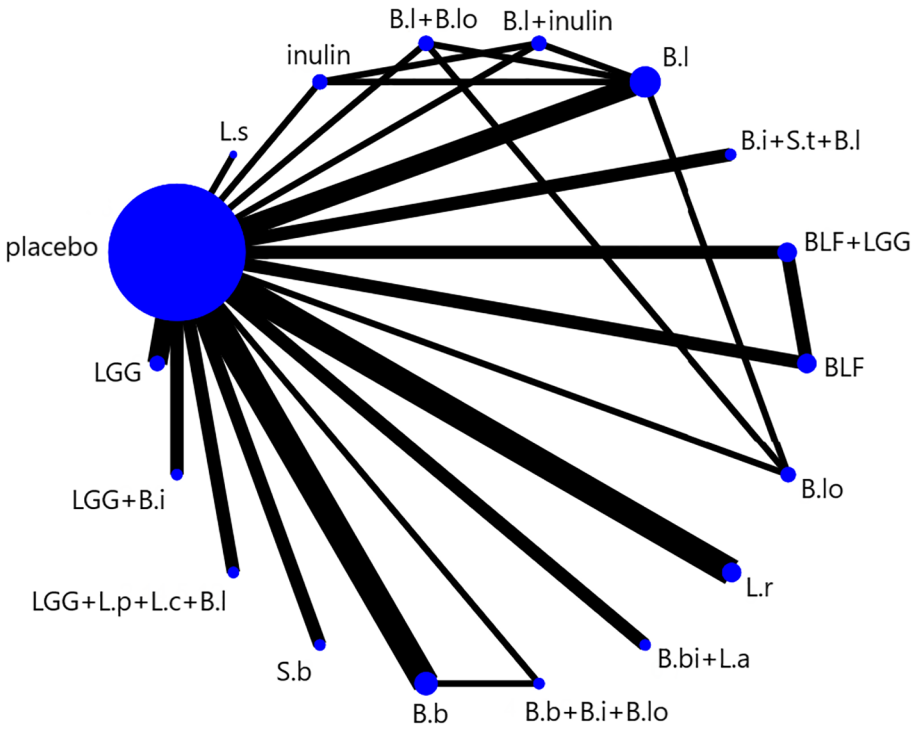

FIGURE 2
NMA figure.

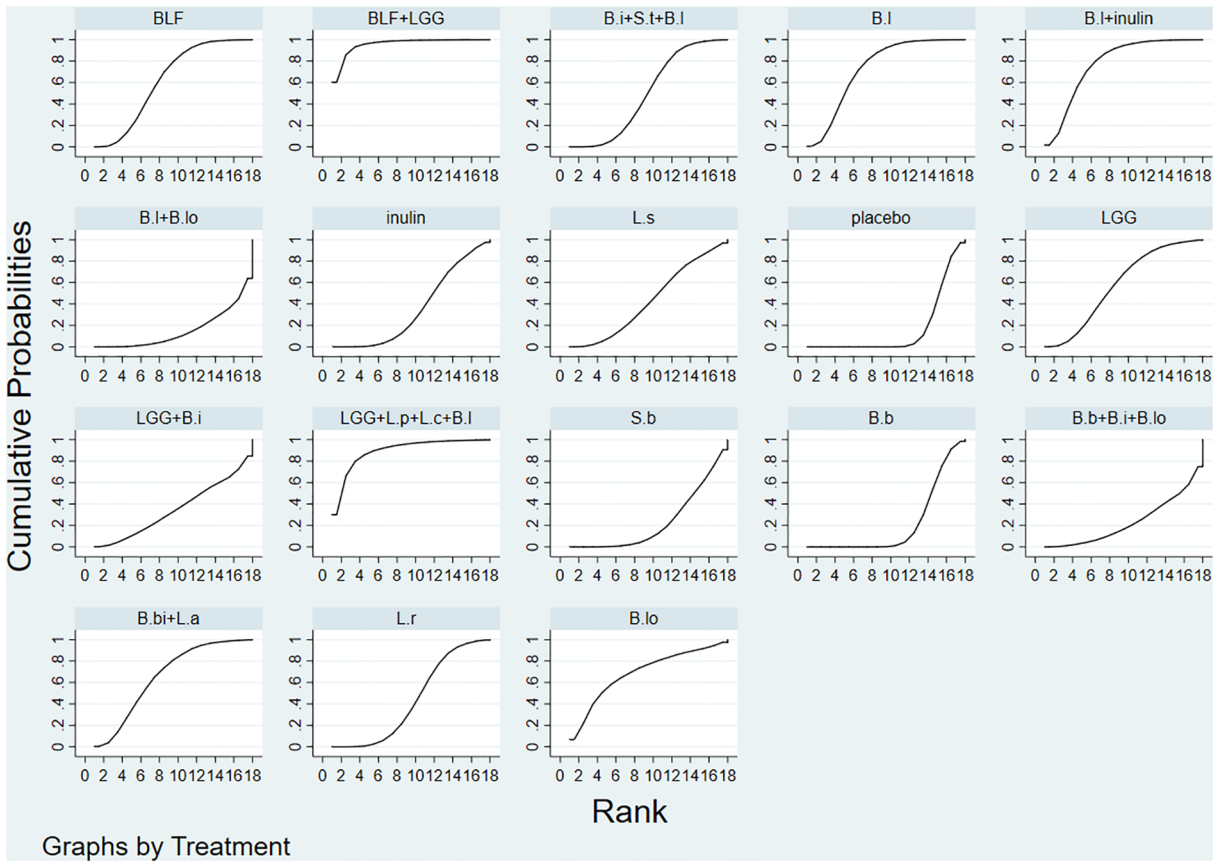

FIGURE 3
SUCRA plot.

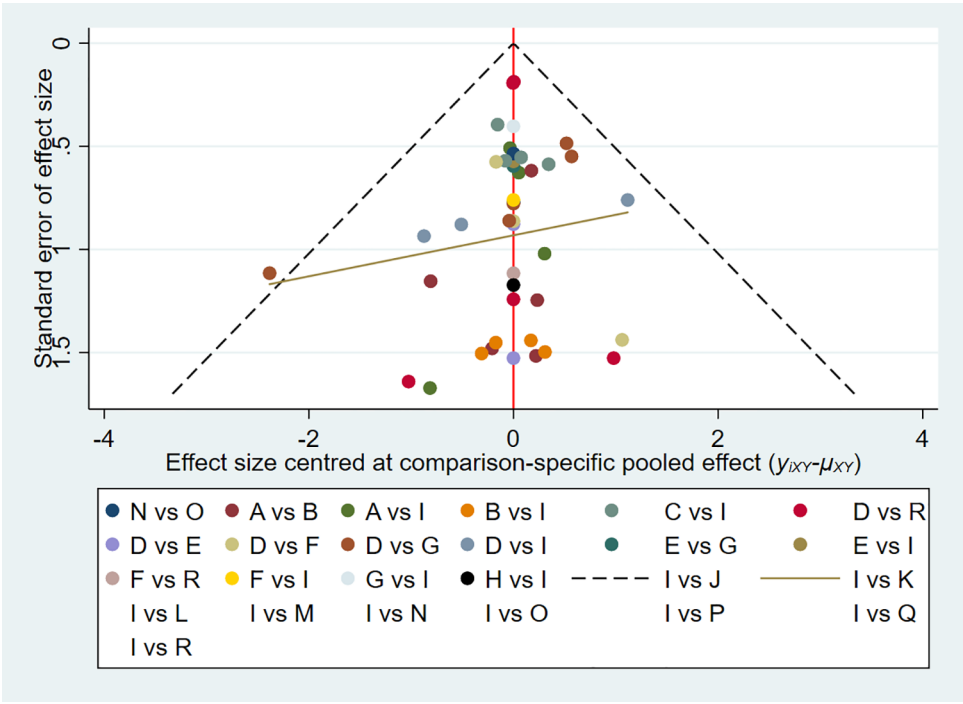

FIGURE 4
Funnel plot on publication bias.

Lactoferrin can be processed from bovine or human milk, with GM rice and GM corn currently under study as promising new sources (46). Since bovine lactoferrin is cheaper than human lactoferrin, it is more readily used.

The studies by Paolo Manzoni et al. (15, 16) have shown that bovine lactoferrin combined with Lactobacillus rhamnosus GG can significantly reduce the incidence of NEC in very low birth weight infants, which is consistent with the results of this network meta-analysis. They believe that this might be related to the ability of lactoferrin to provide some anti-infection, nutrition, and immune regulation activity in the intestine to synergize with the effect of Lactobacillus rhamnosus GG against NEC in premature infants. These findings are also supported by a retrospective cohort study by Michael P. Meyer et al., who also showed that the cost of prevention was significantly lower than the cost of treatment (47). Lactobacillus rhamnosus GG can adhere to the intestinal epithelium and generate biofilms and attenuate the pro-inflammatory effects of cytokines and protect the mucosal barrier (48, 49). In addition, Lactobacillus rhamnosus GG can play an anti-pathogen role by stimulating non-specific immunity, increasing the secretion of interleukin-6 and expressing over 90 antimicrobial or immunomodulatory proteins (43, 48–50). Bovine lactoferrin may provide a broad-spectrum anti-pathogen effect by directly lysing microbial cell membranes (51, 52). Moreover, lactoferrin can also protect intestinal epithelial cells by down-regulating the highly expressed pro-inflammatory cytokines in intestinal epithelial cells, inhibiting the activity of free radicals and reducing the levels of oxidative products (53, 54).

It is obvious that Lactobacillus rhamnosus GG and bovine lactoferrin have the similar effects and create good conditions for the growth of beneficial bacteria, and can also inhibit the colonization of pathogens. The combined use can enhance the overall effect (55). The study by Po-Wen Chen et al. has shown that when the growth of probiotics is not optimum, bovine lactoferrin provides a more substantial prebiotic effect and promotes the growth of probiotics, including Lactobacillus rhamnosus GG (56).

A Position Paper by the European Society for Pediatric Gastroenterology Hepatology and Nutrition Committee on Nutrition and the European Society for Pediatric Gastroenterology Hepatology and Nutrition Working Group for Probiotics and Prebiotics indicates that the question of which probiotic strain or combination to use is mainly based on known literature (mainly case series and author's expertise) (57), these recommendations are based on very low certainty of evidence. Compared with other studies, this study compared the effects of various probiotic regimens on NEC through network meta-analysis, and obtained the optimal probiotic regimen by ranking each intervention. In addition, this study also analyzed specific strains of probiotics. In the studies included in this network meta-analysis, the use of probiotics is described as being well tolerated and safe in very low birth weight infants. Feeding intolerance and clinical sepsis were significantly reduced in the probiotic group compared to the control group. Interestingly, these studies also suggest that different outcomes may be influenced by feeding type (human milk vs. formula). This appears to further support the benefits of lactoferrin in

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TABLE 3 League table for outcome.

BLF + LGG	LGG + L.p + L.c + B.l	B.l + inulin	B.l	B.lo	B.bi + L.a	BLF	LGG	B.i + S.t + B.l	L.r	L.s	inulin	LGG + B.i	S.b	B.b + B.i + B.lo	B.b	placebo	B.l + B.lo
BLF + LGG	1.87 (0.10,33.60)	5.11 (0.52,50.66)	6.33 (0.69,58.44)	5.66 (0.29,111.78)	7.68 (0.80,73.74)	8.81 (1.11,69.94)	9.68 (1.04,90.11)	12.11 (1.48,99.40)	13.95 (1.71,114.00)	13.87 (1.44,133.23)	16.73 (1.95,143.67)	17.94 (1.29,249.60)	22.09 (2.57,189.72)	24.76 (2.05,299.33)	22.12 (2.93,166.97)	23.81 (3.21,176.71)	31.66 (2.83,354.63)
0.53 (0.03,9.61)	LGG + L.p + L.c + B.l	2.73 (0.26,28.96)	3.39 (0.34,33.48)	3.03 (0.15,62.94)	4.11 (0.40,42.19)	4.71 (0.51,43.36)	5.18 (0.52,51.60)	6.48 (0.73,57.15)	7.46 (0.85,65.55)	7.42 (0.72,76.22)	8.95 (0.97,82.48)	9.60 (0.65,141.49)	11.82 (1.28,108.91)	13.25 (1.03,170.23)	11.83 (1.45,96.27)	12.74 (1.59,101.95)	16.93 (1.42,202.05)
0.20 (0.02,1.94)	0.37 (0.03,3.87)	B.l + inulin	1.24 (0.31,5.00)	1.11 (0.09,12.92)	1.50 (0.33,6.94)	1.72 (0.44,6.70)	1.89 (0.43,8.35)	2.37 (0.65,8.58)	2.73 (0.76,9.81)	2.71 (0.59,12.54)	3.27 (1.02,10.52)	3.51 (0.46,26.96)	4.32 (1.11,16.83)	4.84 (0.76,30.92)	4.33 (1.38,13.60)	4.66 (1.53,14.20)	6.19 (1.10,34.75)
0.16 (0.02,1.46)	0.30 (0.03,2.92)	0.81 (0.20,3.26)	B.l	0.89 (0.09,8.47)	1.21 (0.29,5.02)	1.39 (0.41,4.78)	1.53 (0.39,6.02)	1.91 (0.60,6.07)	2.20 (0.70,6.94)	2.19 (0.53,9.08)	2.64 (0.84,8.31)	2.83 (0.40,20.08)	3.49 (1.01,12.01)	3.91 (0.67,22.84)	3.49 (1.29,9.45)	3.76 (1.44,9.82)	5.00 (1.22,20.54)
0.18 (0.01,3.49)	0.33 (0.02,6.87)	0.90 (0.08,10.55)	1.12 (0.12,10.61)	B.lo	1.36 (0.12,15.66)	1.56 (0.15,16.19)	1.71 (0.15,19.19)	2.14 (0.21,21.38)	2.47 (0.25,24.53)	2.45 (0.21,28.30)	2.96 (0.29,30.21)	3.17 (0.19,51.74)	3.90 (0.38,40.65)	4.38 (0.31,62.58)	3.91 (0.42,36.18)	4.21 (0.46,38.35)	5.60 (0.63,49.84)
0.13 (0.01,1.25)	0.24 (0.02,2.50)	0.67 (0.14,3.07)	0.82 (0.20,3.41)	0.74 (0.06,8.49)	B.bi + L.a	1.15 (0.31,4.22)	1.26 (0.30,5.28)	1.58 (0.46,5.38)	1.82 (0.54,6.16)	1.81 (0.41,7.95)	2.18 (0.59,8.03)	2.33 (0.32,17.30)	2.88 (0.78,10.60)	3.22 (0.53,19.76)	2.88 (0.98,8.47)	3.10 (1.09,8.83)	4.12 (0.75,22.73)
0.11 (0.01,0.90)	0.21 (0.02,1.95)	0.58 (0.15,2.26)	0.72 (0.21,2.47)	0.64 (0.06,6.68)	0.87 (0.24,3.21)	BLF	1.10 (0.32,3.83)	1.38 (0.50,3.76)	1.58 (0.58,4.30)	1.58 (0.43,5.80)	1.90 (0.63,5.70)	2.04 (0.31,13.28)	2.51 (0.84,7.52)	2.81 (0.53,14.96)	2.51 (1.11,5.69)	2.70 (1.25,5.87)	3.59 (0.76,17.03)
0.10 (0.01,0.96)	0.19 (0.02,1.92)	0.53 (0.12,2.33)	0.65 (0.17,2.57)	0.58 (0.05,6.55)	0.79 (0.19,3.33)	0.91 (0.26,3.17)	LGG	1.25 (0.39,4.03)	1.44 (0.45,4.61)	1.43 (0.34,6.01)	1.73 (0.49,6.03)	1.85 (0.26,13.25)	2.28 (0.65,7.96)	2.56 (0.43,15.09)	2.28 (0.83,6.29)	2.46 (0.92,6.54)	3.27 (0.62,17.30)
0.08 (0.01,0.68)	0.15 (0.02,1.36)	0.42 (0.12,1.53)	0.52 (0.16,1.66)	0.47 (0.05,4.66)	0.63 (0.19,2.17)	0.73 (0.27,1.99)	0.80 (0.25,2.58)	B.i + S.t + B.l	1.15 (0.47,2.83)	1.15 (0.33,3.92)	1.38 (0.50,3.79)	1.48 (0.24,9.17)	1.82 (0.66,5.00)	2.04 (0.41,10.27)	1.83 (0.91,3.65)	1.97 (1.03,3.73)	2.61 (0.59,11.64)
0.07 (0.01,0.59)	0.13 (0.02,1.18)	0.37 (0.10,1.32)	0.45 (0.14,1.43)	0.41 (0.04,4.04)	0.55 (0.16,1.87)	0.63 (0.23,1.71)	0.69 (0.22,2.22)	0.87 (0.35,2.13)	L.r	0.99 (0.29,3.38)	1.20 (0.44,3.26)	1.29 (0.21,7.93)	1.58 (0.58,4.31)	1.78 (0.36,8.87)	1.59 (0.80,3.13)	1.71 (0.91,3.20)	2.27 (0.51,10.05)
0.07 (0.01,0.69)	0.13 (0.01,1.38)	0.37 (0.08,1.70)	0.46 (0.11,1.89)	0.41 (0.04,4.71)	0.55 (0.13,2.44)	0.63 (0.17,2.34)	0.70 (0.17,2.93)	0.87 (0.26,2.99)	1.01 (0.30,3.41)	L.s	1.21 (0.33,4.45)	1.29 (0.17,9.59)	1.59 (0.43,5.88)	1.79 (0.29,10.96)	1.59 (0.54,4.70)	1.72 (0.60,4.90)	2.28 (0.41,12.60)
0.06 (0.01,0.51)	0.11 (0.01,1.03)	**0.31 (0.10,0.98)**	0.38 (0.12,1.19)	0.34 (0.03,3.46)	0.46 (0.12,1.69)	0.53 (0.18,1.58)	0.58 (0.17,2.02)	0.72 (0.26,1.99)	0.83 (0.31,2.27)	0.83 (0.22,3.06)	inulin	1.07 (0.16,7.00)	1.32 (0.44,3.97)	1.48 (0.28,7.89)	1.32 (0.58,3.01)	1.42 (0.65,3.10)	1.89 (0.41,8.73)
0.06 (0.00,0.78)	0.10 (0.01,1.54)	0.28 (0.04,2.19)	0.35 (0.05,2.50)	0.32 (0.02,5.15)	0.43 (0.06,3.17)	0.49 (0.08,3.20)	0.54 (0.08,3.86)	0.68 (0.11,4.18)	0.78 (0.13,4.80)	0.77 (0.10,5.73)	0.93 (0.14,6.09)	LGG + B.i	1.23 (0.19,8.04)	1.38 (0.14,13.23)	1.23 (0.22,6.93)	1.33 (0.24,7.32)	1.76 (0.20,15.55)
0.05 (0.01,0.39)	**0.08 (0.01,0.78)**	**0.23 (0.06,0.90)**	**0.29 (0.08,0.99)**	0.26 (0.02,2.67)	0.35 (0.09,1.28)	0.40 (0.13,1.20)	0.44 (0.13,1.53)	0.55 (0.20,1.50)	0.63 (0.23,1.72)	0.63 (0.17,2.32)	0.76 (0.25,2.28)	0.81 (0.12,5.30)	S.b	1.12 (0.21,5.97)	1.00 (0.44,2.28)	1.08 (0.49,2.35)	1.43 (0.30,6.80)
0.04 (0.00,0.49)	**0.08 (0.01,0.97)**	0.21 (0.03,1.32)	0.26 (0.04,1.49)	0.23 (0.02,3.27)	0.31 (0.05,1.90)	0.36 (0.07,1.89)	0.39 (0.07,2.31)	0.49 (0.10,2.46)	0.56 (0.11,2.81)	0.56 (0.09,3.44)	0.68 (0.13,3.60)	0.72 (0.08,6.94)	0.89 (0.17,4.75)	B.b + B.i + B.lo	0.89 (0.21,3.86)	0.96 (0.22,4.23)	1.28 (0.17,9.47)
0.05 (0.01,0.34)	**0.08 (0.01,0.69)**	**0.23 (0.07,0.73)**	**0.29 (0.11,0.77)**	0.26 (0.03,2.37)	0.35 (0.12,1.02)	**0.40 (0.18,0.90)**	0.44 (0.16,1.21)	0.55 (0.27,1.10)	0.63 (0.32,1.25)	0.63 (0.21,1.85)	0.76 (0.33,1.72)	0.81 (0.14,4.56)	1.00 (0.44,2.27)	1.12 (0.26,4.84)	B.b	1.08 (0.83,1.40)	1.43 (0.36,5.66)
0.04 (0.01,0.31)	**0.08 (0.01,0.63)**	**0.21 (0.07,0.65)**	**0.27 (0.10,0.69)**	0.24 (0.03,2.17)	**0.32 (0.11,0.92)**	**0.37 (0.17,0.80)**	0.41 (0.15,1.08)	**0.51 (0.27,0.97)**	0.59 (0.31,1.10)	0.58 (0.20,1.66)	0.70 (0.32,1.53)	0.75 (0.14,4.15)	0.93 (0.43,2.02)	1.04 (0.24,4.57)	0.93 (0.72,1.21)	placebo	1.33 (0.35,5.12)
0.03 (0.00,0.35)	**0.06 (0.00,0.70)**	**0.16 (0.03,0.91)**	**0.20 (0.05,0.82)**	0.18 (0.02,1.59)	0.24 (0.04,1.34)	0.28 (0.06,1.32)	0.31 (0.06,1.62)	0.38 (0.09,1.70)	0.44 (0.10,1.95)	0.44 (0.08,2.42)	0.53 (0.11,2.44)	0.57 (0.06,4.99)	0.70 (0.15,3.31)	0.78 (0.11,5.80)	0.70 (0.18,2.76)	0.75 (0.20,2.90)	B.l + B.lo

combination with probiotics. Combining probiotics and lactoferrin may be a good idea for future research studies.

5. Limitations of the study

This network meta-analysis also has some limitations. This study only discussed the selection of probiotics for the prevention of NEC in very low birth weight infants, while the questions of the dosage, the timing of the intervention, and when to start the intervention remains unresolved. Most interventions were evaluated in only one or two trials, and only a few options were tested in four randomized controlled trials. Therefore, most probiotic interventions were evaluated in small experimental populations. In conclusion, the results of this study should be interpreted with caution, as the number of included trials was insufficient, so there was limited evidence for direct comparisons of some interventions, and further related studies are needed.

6. Conclusions

Our analysis suggests that Lactobacillus rhamnosus GG combined with bovine lactoferrin is the most effective and recommended regimen for preventing NEC in very low birth weight infants. However further studies are required to confirm this and also answer questions about probiotic dosage, timing and duration of therapy.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

Author contributions

KZZ: interpreted the data, wrote the initial manuscript, and was involved in the data analysis; KW, LXD, MH and YXL: were responsible for the collection of all relevant papers; LYZ: was responsible for the supervision of the study. Both authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2023.1095368/full#supplementary-material.

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The role of neutrophil extracellular traps in necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) continues to be one of the most common causes of mortality and morbidity in preterm infants. Although not fully elucidated, studies suggest that prematurity, formula feeding, imbalanced vascular supply, and altered bacterial colonization play major roles in the pathogenesis of NEC. NEC is characterized by increased cytokine release and leukocyte infiltration. Recent data from preterm infants and animal models of NEC suggest that neutrophil extracellular traps (NETs) are released in intestinal tissue. The contribution of NETs in the pathogenesis and/or prevention/treatment of this disease continues to be controversial. Here, we review the available data on NETs release in NEC in human patients and in different NEC models, highlighting their potential contribution to pathology and resolution of inflammation. Here, we review the available data on NETs release in NEC in human patients and the different NEC models, highlighting their potential contribution to pathology or resolution of inflammation.

KEYWORDS

neutrophils, NETs (neutrophil extracellular traps), NEC (necrotizing enterocolitis), intestinal disease

Introduction

Necrotizing enterocolitis (NEC) is one of the most devastating diseases in the neonatal intensive care unit. This inflammatory bowel disease primarily affects preterm infants, with an incidence of 7%–12% in neonates born less than 1,500 g (1). Importantly, the incidence is steadily increasing, as improvements in neonatal care lead to enhanced survival of premature infants (2–4.) NEC is associated with mortality rates of up to 30% in very low birth weight infants (5), and up to 80% in the most severe cases (fulminant NEC) (6). Moreover, survivors of NEC are at increased risk of long-term morbidities such as growth failure, short bowel syndrome, and neurodevelopmental delay, all of which increase the physical and psychological burdens for patients and their families (7, 8).

Despite decades of investigations, the pathogenesis of NEC remains inconclusive, perhaps since NEC may not be a single disease, but comprised of several entities (e.g., classic NEC, ischemic intestinal necrosis, food protein intolerance enterocolitis syndrome) (9–11) NEC pathogenesis is multifactorial with prematurity, formula feeding, and dysregulation of perfusion, as well as dysbiosis, playing major roles. In preterm neonates, developmental immaturity of the mucosal barrier and increased expression of toll-like receptor (TLR) 4 in the intestinal epithelium render the gut highly reactive to stimuli (12, 13). The net effect is exaggerated inflammatory cytokine/chemokine release, leukocyte infiltration, epithelial necrosis, altered epithelial barrier, and bacterial translocation across the lumen (14). Of particular interest, TLR4 expression can be reduced by breast milk feeding (15). This excessive TLR4 expression in response to the dysbiotic microbiome can lead to the death of intestinal epithelial cells through apoptosis and necroptosis, as well as

impaired mucosal restitution, which in severe cases leads to intestinal perforation, multi-organ failure, and potentially death (16).

Notably, NEC is not solely a disease of the abdomen, rather it is a multisystemic disease that can also affect other organ systems (17–19). Systemic reviews have demonstrated that NEC is an independent risk factor for neurocognitive developmental delay and poor neurocognitive outcomes (20, 21). Moreover, studies suggest that common morbidities of the preterm infant such as bronchopulmonary dysplasia (BPD) and brain damage are affected by the development of NEC necrotizing enterocolitis, through interactions known as the "Gut-Lung-Axis" and "Gut-Brain-Axis", respectively (18, 19). Recently, it has been reported that the excessive immune response *via* TLR4 and neutrophil activation are associated with increased damage to not just the intestine but also the lung and brain tissue, suggesting a potential role of neutrophils in distant organ injury in NEC (17–19). Suggesting a potential role of neutrophils in distant organ injury in NEC.

Neutrophils in necrotizing enterocolitis

Neutrophils are the most abundant immune cells and first-line responders of the innate immune system (22, 23). As polymorphonuclear cells, neutrophils are very motile, which enables them to migrate from peripheral vasculature into the tissue of recruitment (23, 24). One of the key functions in NEC pathogenesis is the activation of intestinal epithelial cell toll-like receptor 4 (TLR4) which leads to accelerated apoptosis of enterocytes and reduced rate of healing through impaired intestinal restoration and proliferation. Upregulation in TLR4 expression results in the production of pro-inflammatory cytokines and chemokines leading to the recruitment of neutrophils to the location of inflammation (25). Neutrophil infiltration has been long recognized in NEC tissues. However, the beneficial and detrimental contributions of these cells specifically in this disease remain unclear (11, 26, 27). Neutrophils seem to be critical for mucosal homeostasis as NEC is aggravated by neutrophil depletion in a murine model of NEC (28). However, excessive recruitment and activation of neutrophils could also promote injury and exacerbate disease including mutual upregulation of TLR4 and neutrophil activation (29).

Upon contact with pathogens, neutrophils can react *via* (1) phagocytosis, (2) production of oxidative bursts like reactive oxygen species (ROS), and (3) degranulation and/or (4) formation of neutrophil extracellular traps (NETs) (30). NETs are large extracellular networks consisting of DNA fibers and spherical proteins. The protein contents of NETs include histones, neutrophil elastase (NE), myeloperoxidase (MPO), defensin, calprotectin, cathepsin G, protease 3, and actin, lactoferrin, gelatinase, lysozyme C, and cathelicidins (24, 31–33). Neutrophils release NETs *via* multiple mechanisms: (1) NETosis, a programmed cell death pathway distinct from apoptosis, pyroptosis, necroptosis, or ferroptosis, (2) non-lytic discharge of parts or their entire nucleus, and (3) mitochondrial DNA release, providing an additional DNA source for NET formation (34). The main processes involved in NETosis are neutrophil activation, cytoplasmic granule dissolution, neutrophil protease activation, chromatin decondensation, and swelling, followed by plasma membrane rupture. NETs are released after histones are citrullinated by peptidyl arginine deiminase 4 (PAD4) (35). The function of those structures is to capture pathogenic microorganisms and enhance phagocytosis by macrophages thus preventing the spreading of infection (24, 36–38). NETs are normally cleared by plasma DNAse 1 followed by removal by macrophages. Inappropriate or delayed clearance of NETs or NET components specifically the associated histones and proteases contribute to pathological conditions like sepsis (39), thrombosis (40), transfusion-associated acute lung injury (41), cancer development and metastasis (42), autoimmune diseases (23, 43), and impaired wound healing (44) - mostly through induction of INF, proinflammatory cytokines, and the NLRP3 inflammasome (45). A major mechanism for the cytotoxic properties of histones in NETs is through direct binding to the plasma membrane causing calcium influx and loss of membrane barrier function (46–48). Histones also activate TLRs 2, 4, 9 leading to cytokine production, leukocyte recruitment, and tissue injury (49–51). Furthermore, extracellular histones in NETs stimulate platelet adhesion and coagulation (52), which in severe cases can lead to multi-organ failure due to micro thrombosis, decrease microvascular perfusion, and subsequent tissue damage (53). This has been demonstrated by the association of NETs with various thrombo-inflammatory diseases such as stroke, autoimmune diseases, sepsis, lung injury (i.e., COVID-19), diabetes, and ischemia-reperfusion injury of the intestine and testicles (54–60). To further illustrate their double-edged nature, NETs released in the gut have been shown to reduce the translocation of bacteria and support the healing of the intestinal mucosa. On the other hand, excessive NETs formation can damage the barrier function of the intestinal mucosa and thus play a key role in the development of a variety of intestinal diseases (23, 42) (**Figure 1**).

In neonates, neutrophils exhibit an intrinsic delay in NET formation but are capable of releasing functionally competent NETs (61–64). In a series of elegant experiments, Yost et al. showed that neutrophils isolated from term and preterm infants fail to form NETs in response to ROS, LPS, and bacteria after an hour of incubation. This defect in NET formation was associated with a reduction in extracellular bacterial killing *in vitro* compared to neutrophils isolated from adults (62). Such differences could explain the increased susceptibility of neonates and preterms in particular to sepsis and infection. To explore the mechanism for this blunted neonatal NET deployment, Yost et al. identified peptides in cord blood from preterm and term infants that inhibit NETosis (63) *in vitro* and *in vivo* and appear to be an endogenous regulator of NET generation. Importantly, the authors assessed the ability of neutrophils from preterm neonates longitudinally over the first 28 days after birth for NET formation in response to LPS. NET formation was not demonstrated until day 3 after birth and reached maximum capacity between days 3 and 14. Proteomic analysis identified neonatal NET-inhibitory factor (nNIF) detected in plasma from

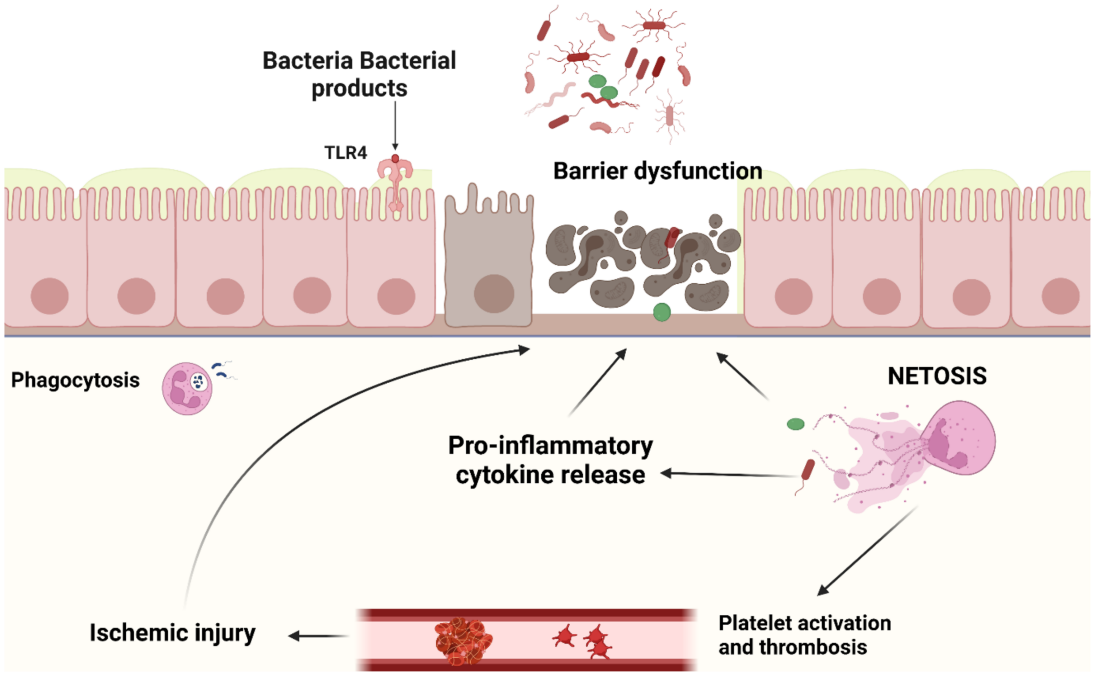

FIGURE 1
Neutrophil activation and NETs release in intestinal tissue of NEC patients occur as a result of various stimuli such as pathological bacteria, PAMPs and ROS. Though NETs and their components are important to prevent bacterial translocation through the intestine, dysregulated NETs release leads to adjacent epithelial and endothelial injury, cytokine release, platelet activation and thrombus formation. This would result in a vicious cycle of tissue necrosis, further immune activation, ischemia reperfusion injury, uncontrolled inflammation, multi-organ failure, and death.

cord blood of term and preterm infants in the first days after birth and is absent in plasma of adults. Importantly, nNIF use in animal models of inflammation and polymicrobial sepsis improved survival and multiorgan injury, supporting existing evidence that NETs are effectors of collateral vascular and tissue injury in certain pathologies.

Necrotizing enterocolitis and neutrophil extracellular traps

Previous studies showed that NETs release occurs in tissues, serum, and stool of infants and animal models of NEC (26, 65–67). In a prospective pilot study, McQueen et al. showed that infants diagnosed with NEC had increased fecal calprotectin levels compared to infants with NEC "ruled out". Further analysis using immunohistochemistry, showed an association between calprotectin staining, neutrophil activation markers, and NETs staining in the intestinal tissues of infants with surgical NEC. These data suggest that fecal calprotectin is released, at least in part, as a result of neutrophil infiltration, activation, and potentially NET formation in the intestinal tissue of infants with NEC (67). Other studies later confirmed NETs release in NEC patients and animal models of NEC. Nguyen et showed that preterm infants with NEC and sepsis had higher levels of cell-free DNA (cfDNA), a surrogate marker of circulating NETs, compared to controls (66). Similarly, Chaaban et al. showed increased levels of nucleosomes (histones-DNA), also a surrogate of NETs release, in the serum of infants with NEC stage II and above compared to gestational age-matched controls (65). Analysis of intestinal tissue confirmed neutrophil activation and NET release by immunohistochemical staining of intestinal tissue from preterm infants and a mouse model of NEC. Vincent et al. demonstrated that the pathogenesis of NEC is likely a NET-dependent process (26). They showed that markers of neutrophil activation and NET formation in both serum and histology directly correlate with NEC manifestation, severity, and mortality in a murine model of NEC that utilizes intermittent hypoxia/LSP, and formula feeding. Furthermore, the prevention of NET formation by PAD4 inhibition, using Cl-amidine, significantly reduced NEC histological injury, inflammation, and mortality in the model. The same group further showed that degradation of extracellular DNA in NETs by systemic application of DNase1 leads to a significant reduction in NEC severity, and mortality, suggesting an important role in the pathogenesis of NEC (68). The crucial role of NETs in NEC pathogenesis is further emphasized by the results of Klinke et al. wherein neutrophil concentrations of mice were elevated to match those of human neonates as a method to optimize intestinal injury in the NEC model. Of particular interest is that the NEC severity, tissue damage, and inflammation were significantly reduced, and similar to mice in the control group, in ELANE gene knockout pups, who are incapable of forming NETs (ELANE gene encodes for neutrophil elastase, so knockout results in lack of a key enzyme in NET formation) (69).

These data are in line with the recent studies that suggest, that the degradation of NETs by DNase1 significantly reduces gut-related inflammation, apoptosis of intestinal epithelial cells, and

intestinal damage (23). Martinod et al. also showed that suppression of NETs formation by PAD4 inhibition does not impair the ability of neutrophil granulocytes to defend against pathogens and, in particular, does not lead to higher bacteremia or mortality rates in a model of polymicrobial sepsis (70). Moreover, Silva et al. showed that another method of inhibition of NET formation by disulfiram improves organ function and lethality in sepsis (71). Finally, the neonatal NET-inhibitory factor (nNIF) appears to inhibit NET formation in fetuses and neonates in the first days after birth (63). Whether the maturation of NET formation which coincides with the timing for the development of NEC, plays a role in the pathogenesis of NEC, is yet to be determined. It is possible that preterm neonates develop NEC after a period of time, when the protective effects of the nNIF wear off.

In contrast, the use of PAD4 inhibition in another model of NEC characterized by bacteremia, known as the dithizone/klebsiella NEC model was associated with worsened outcomes. NETs inhibition in this model using cl-amidine was associated with increased inflammatory response, increased bacterial translocation, and mortality in the NEC mice (67). Similarly, Saha et al. showed that PAD4-dependent NET generation is indispensable for intestinal clearance of *Citrobacter rodentium* enteric infection, highlighting the beneficial effects of NETs release in an infectious context (72). These contradicting results strongly suggest that the effects of NET formation may be disease- and model-specific, and in NEC, they depend largely upon the level of intestinal bacterial translocation. They appear to play an integral role in innate defense, especially early on in the clearance of bacterial and bacterial products.

Conclusion

Our current understanding suggests that NETs may be a double-edged sword. They are relevant in the immune defense against pathogenic agents. However, excessive NET ormation induces hyperinflammation, tissue damage, and thrombo-inflammation, contributing to the pathogenesis of a wide variety of diseases such as sepsis, NEC, ARDS, lung injury in COVID-19, ischemia-reperfusion injury, and various oncological diseases. The effect of NETs in pathological conditions is perhaps disease and model specific. In NEC, it is likely dependent on the level of intestinal bacterial translocation. NETs seem crucial in the early phase of the disease to battle bacteremia and reduce bacterial translocation in NEC. However, after the initiation of antibiotic therapy, it may be reasonable to try reducing NET formation through the use of agents like DNases to avert the hyperinflammatory damage caused by NETs. Future studies are needed to further investigate the role of NETs in NEC and other human diseases and explore how best to optimize the beneficial effects and minimize the detrimental effects of NETs for therapy in various human diseases including NEC.

Author contributions

MK and MB performed the literature review, and drafted the first manuscript. HC reviewed the review, and drafted the first manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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State-of-the-art review and update of *in vivo* models of necrotizing enterocolitis

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NEC remains one of the most common causes of mortality and morbidity in preterm infants. Animal models of necrotizing enterocolitis (NEC) have been crucial in improving our understanding of this devastating disease and identifying biochemical pathways with therapeutic potential. The pathogenesis of NEC remains incompletely understood, with no specific entity that unifies all infants that develop NEC. Therefore, investigators rely on animal models to manipulate variables and provide a means to test interventions, making them valuable tools to enhance our understanding and prevent and treat NEC. The advancements in molecular analytic tools, genetic manipulation, and imaging modalities and the emergence of scientific collaborations have given rise to unique perspectives and disease correlates, creating novel pathways of investigation. A critical review and understanding of the current phenotypic considerations of the highly relevant animal models of NEC are crucial to developing novel therapeutic and preventative strategies for NEC.

KEYWORDS

NEC = necrotizing enterocolitis, animal model, preclinical (*in vivo*) studies, intestinal injury, necrotizing/intestinal diseases/intestine

Introduction

Necrotizing enterocolitis (NEC) remains a leading cause of morbidity and mortality in premature infants, with mortality rates as high as 10%–50% (1, 2). Clinically, NEC can rapidly progress from relatively mild feeding intolerance and abdominal distension to bowel ischemia and necrosis, fulminant septic shock, severe acidosis, multi-organ dysfunction, and death. Despite significant advances in neonatal clinical care in the last few decades, the prevalence of NEC has not significantly decreased globally (2, 3). Furthermore, the mechanisms driving the development of NEC remain poorly defined. This is in part because NEC is believed to result from a heterogeneous group of disorders or initiating pathways leading to a common final pathology (4). In addition, no current biomarkers predict the onset of NEC. Thus, it is difficult to study the mechanisms of NEC in human populations, making animal models that mimic NEC essential to determine the underlying pathophysiology and develop specific preventative and therapeutic targets (5).

Abbreviations

DSS, dextran sodium sulfate; DTR, diphtheria-toxin receptor; FF, Formula feeding; HF, Hypoxia-formula feeding model; HHF, Hypoxia-hypothermia-formula feeding; LPS, lipopolysaccharide; NEC, Necrotizing enterocolitis; NECteria, Bacterial culture stock derived from infant with *Nec totalis* (1); PCD, Paneth cell disruption; PIA, Phlebotomy-induced anemia; PN, parenteral nutrition; SMA, superior mesenteric artery; TLR4, toll-like receptor 4; TNBS, trinitrobenzene sulfonate; TNF, tumor necrosis factor; TPN, total parenteral nutrition; VLBW, very-low-birthweight.

Original models of NEC focused on adult animals undergoing experimental conditions such as ischemia-reperfusion injury, injections of pathogens into closed bowel loops, or combinations of hypoxia and hypovolemia (6). However, it quickly became apparent that the pathogenesis of NEC is a multifactorial process with four primary factors believed to be vital components driving disease manifestation. These include (1) immaturity of the intestine, (2) impaired mucosal barrier functions, (3) abnormal microbial colonization, and (4) dysregulated innate immunity (7). From this realization, the classic rodent model developed by Barlow et al. in 1974 became the mainstay of NEC research which involved exposing newborn rats to formula feeding, an oral inoculum of *Klebsiella pneumoniae,* and hypoxia (8). Since then, modifications have been made to the model, including adapting it to use in mice (9). In addition, new models have been developed that focus on the unique properties of the preterm infant, including the stage of intestinal development and immature immune systems (10). These have significantly contributed to our improved understanding of the mechanisms driving the increased susceptibility to intestinal injury in preterm infants and term infants with specific conditions associated with NEC.

Numerous animal models have been explored, including mice, rats, quails, rabbits, pigs, and baboons, each contributing to our understanding of NEC pathophysiology. However, given that NEC is a complex process with variable presentations and severity, no single animal model can truly and perfectly mimic NEC. Instead, each model captures a specific aspect of NEC, most aimed at recreating the predisposing clinical conditions that drive NEC susceptibility. In addition, animal models provide a means to manipulate variables that provide mechanistic insight and an ability to test therapeutic and preventative interventions in translatable preclinical models. This state-of-the-art review focuses on the highly relevant *in vivo* animal models of NEC, specifically the phenotypic considerations of each model and the research questions each model is best suited for. A comprehensive review of established animal models of NEC published since the 1960s was performed using search terms including but not limited to "necrotizing enterocolitis" "animal models", "necrotizing enterocolitis murine/rat/piglet model," "in vivo necrotizing enterocolitis," "experimental necrotizing enterocolitis." Once models were identified by keywords and previously published reviews, additional searches by corresponding authors and references were performed to identify the first publication using the original model and subsequent adaptations using a combination of Pubmed, Medline, and Google Scholar.

Ethical, governance and regulatory considerations

An in-depth discussion on these issues is beyond the scope of this review and are well summarized elsewhere(11–13). However, it is important to highlight that statutory and regulatory frameworks have improved practice globally. More importantly there is paradigm shift towards a "culture of care" which we need to continue to nurture and disseminate.

Risk factors for NEC

Prematurity remains the most critical risk factor for NEC. Roughly 90% of infants with NEC are born preterm, and the incidence is inversely related to gestational age (3). While the intestinal tract is one of the first organs to develop in humans, its development is not complete until term gestation. As a result, premature infants have immature intestinal barriers (impaired mucosal production, increased permeability), immunity (fewer Paneth cells, biochemically different mucous production, diminished regulatory T cells), and incomplete gut innervation with poor motility. Importantly, this combination of developmental immaturity of the preterm intestinal barrier function and the increased expression of toll-like receptor (TLR) 4 (14, 15) makes preterm infants particularly susceptible to the translocation of bacteria which can induce mucosal injury and lead to exaggeration of an already dysregulated inflammatory and immune response (**Figure 1**). These factors combine to induce further intestinal injury, ischemia, and necrosis seen in NEC (16).

In addition to prematurity, enteral feeding is a critical risk factor for developing NEC. Survival of the preterm infant depends on the delivery of adequate nutrition, often requiring supplementation with bovine and human-milk-based fortifiers for adequate growth. However, the combination of an immature intestine, a limited absorptive and digestive capacity, a dysbiotic microbiome, and delayed gut motility creates an intestinal environment marked by bacterial overgrowth and fermentation in the preterm infant (17, 18). These factors further contribute to the already dysbiotic and impaired mucosal barrier that renders preterm infants susceptible to mucosal injury (19). Studies have shown decreased incidence of NEC when infants are fed human milk (20). Furthermore, emerging evidence suggests that the absence of breastmilk and the critical components driving immunomodulation, barrier maturation, and growth promotion increase susceptibility to NEC rather than formula feeding itself (17, 21, 22). However, breastmilk does not completely prevent the development of NEC, and not all formula-fed premature infants develop NEC. We continue to lack complete mechanistic insight into how enteral feeding type can drive the increased susceptibility to intestinal injury, thus the critical need for multiple approaches and modeling to determine causality for intervention.

Other risk factors for developing NEC in the premature population include prolonged exposure to broad-spectrum antibiotics (23), severe anemia followed by transfusions (24), gastric acid suppression (25), sepsis/remote infection, and chorioamnionitis (26). While much of the recent studies have focused on the intestinal epithelium and inflammatory cells, given the histopathological characteristic of ischemia and necrosis seen in NEC, the microvasculature of the intestine is likely also involved. Establishing reduced nitric oxide synthase (eNOS) expression in patients with NEC has led to the recognition that decreased VEGF activity and expression in human neonates are independent risk factors for NEC (27). It is also important to note that NEC can also affect term neonates. However, NEC in this population typically occurs in conditions that compromise

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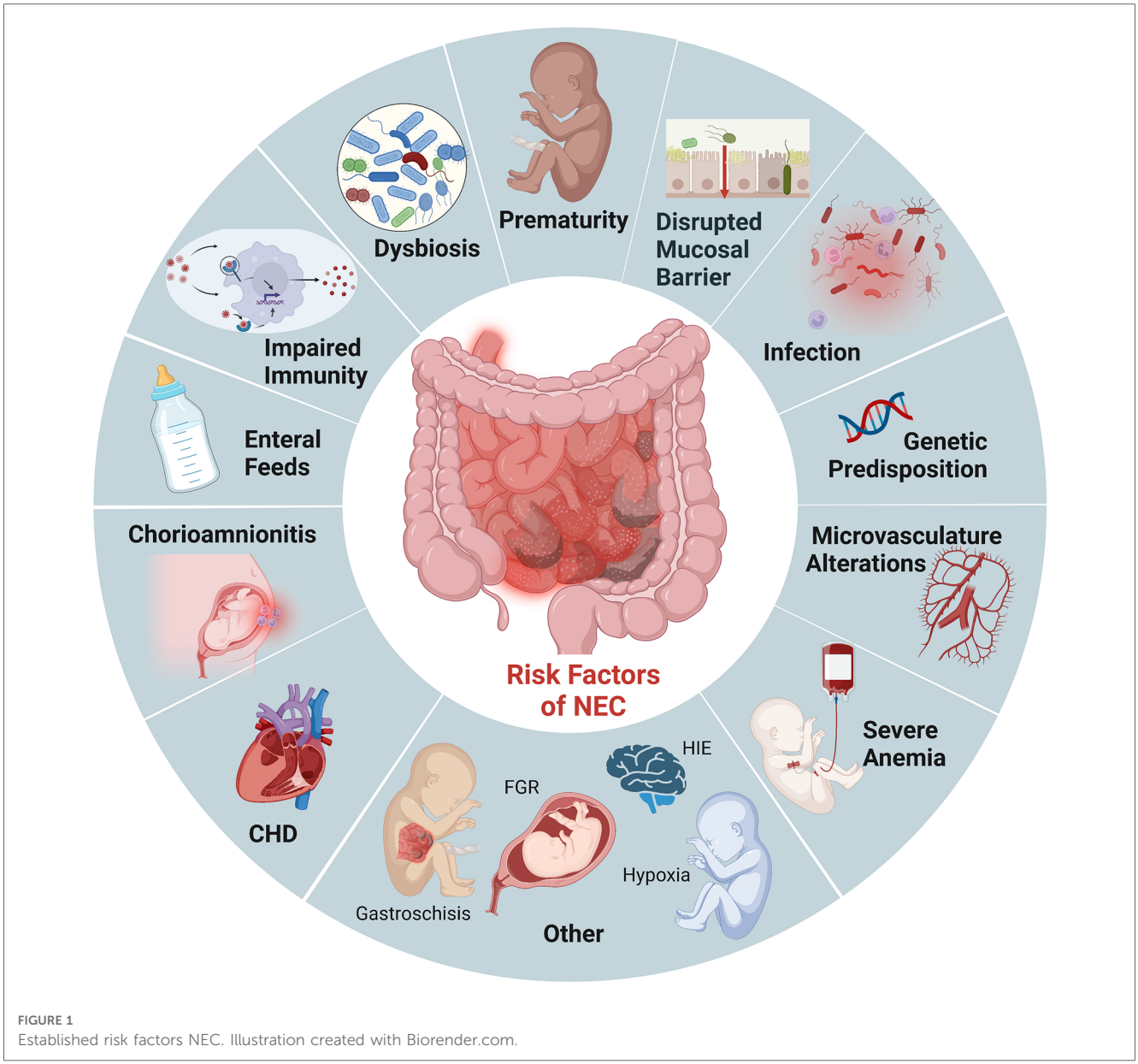

FIGURE 1
Established risk factors NEC. Illustration created with Biorender.com.

intestinal blood flow and oxygenation, such as ductal-dependent congenital heart defects (28, 29). Therefore, animal models that mimic ischemia/reperfusion injuries alone are likely more representative of this subset of neonates that develop NEC.

NEC has now been modeled in rats, mice, hamsters, piglets, rabbits, dogs, quails, and non-human primates, with piglets and rodents being the most commonly used. Perturbations of the intestinal environment in the neonate by directly or indirectly disrupting the protective mucosal epithelial barrier, innate immune functions, or the intestinal microvasculature/architecture are critical to inducing NEC-like phenotypes regardless of the animal model. It is essential to recognize that not all models of NEC have the same perturbations or disease phenotypes. Identifying predisposing factors and unique attributes for each model can help improve our understanding of NEC and is imperative for choosing the best model to answer the scientific question.

Histopathology of NEC in humans and animal models

The most common diagnostic pathologic finding of NEC is pneumatosis intestinalis. This pathognomonic finding can be seen on radiograph imaging (x-ray and ultrasound), on gross examination of the bowel, and on histopathology. Pneumatosis represents intramural gas within the bowel wall produced by bacterial fermentation within the gut lumen. Other hallmark features in human NEC include portal venous gas, mucosal edema, epithelial sloughing/villous atrophy, secondary bacterial infiltration, vascular thrombosis, and discontinuous coagulative necrotic segments intestine or "skip lesions" that vary in depth of the affected intestine (29, 30).

While pneumatosis and other signs are utilized clinically, histological grading of NEC severity is the gold standard in rodent models. The original grading system described by Barlow

et al. and subsequently validated by Caplan (31) and Dvorak (32) continues to serve as the basis for determining the incidence of and severity of NEC in rodent models today. In general, scoring is done on a Likert scale grading the extent of destruction of the intestinal mucosa: Grade 0—normal mucosa (intact epithelium); Grade 1—superficial epithelial sloughing or "lifting" (tip); Grade 2—mid-villous necrosis; Grade 3—complete villous necrosis; and Grade 4—complete loss of intestinal structure with transmural necrosis (31, 33). Generally, this follows one of two patterns depending on the model used: a top-down or bottom-up disease development (34). Additional features have been integrated, including separation of lamina propria, mucosal edema, coagulative necrosis, and depth of bacterial invasion. Scores of 2 or greater are considered to be representative NEC in humans.

The piglet model is unique in that the preterm piglet shares many overlapping features of gut anatomy, physiology, and microbiota with premature human infants (35). Thus, the grading system utilized in piglet models of NEC combines clinical features (e.g., abdominal distension, pneumatosis on imaging, cyanosis) with histological markers (coagulation necrosis, epithelial sloughing, and blunting mucosal edema, and leucocyte infiltration) to determine NEC-like intestinal injury (36). Furthermore, unlike most rodent models with NEC-like injuries occurring predominantly in the distal ileum, and taking 1–3 days to develop an injury, piglet models have an early onset of NEC (<24 h) that results in fulminant disease throughout the stomach to the large intestine, displaying a more widespread inflammatory response than typically seen in human neonates (37, 38).

Modeling necrotizing enterocolitis *in vivo*: basic concepts

Given the limitations, expense, and difficulty of utilizing clinically obtained surgical specimens from neonates and human tissue-derived *in vitro* models (39), *in vivo* animal models have been crucial in elucidating the mechanisms contributing to the pathogenesis and severity of NEC (5). However, the wide spectrum of clinical manifestations and disease severity of NEC makes modeling NEC in animals particularly difficult, with no "perfect" model. Instead, most models developed to date are based on specific predisposing factors and the subsequent phenotypic effect on the mucosal epithelial barrier, microbiota/dysbiosis, and/or the hyperactivation of the innate immune system of the animal studied.

The earliest models of NEC were performed in adult animals that induced ischemic/reperfusion injuries by occluding the superior mesenteric artery (SMA) or surgically creating closed loops of small bowel (5). However, it was not until the 1970s that predisposing factors associated with NEC development in human neonates, including prematurity, formula feedings, and bacterial colonization, were incorporated into animal models (8, 26). The most widely used animal models of NEC to date are based on this original principle, integrating experimental conditions that increase the susceptibility to intestinal injury based on clinical factors associated with human NEC known at that time. This increased susceptibility is combined with an exposure to a triggering event that leads to intestinal dysbiosis, disrupted mucosal barrier, and an exaggerated inflammatory response triggering subsequent ischemia and necrosis characteristic of NEC. This multiple-hit methodology includes factors such as exposure to formula feeds, medications that cause mucosal injury or enhance microbial disruption, hypoxia ± cold stress, anemia, ischemia/reperfusion, or disruption or loss of critical regulators of the innate immune system such as Paneth cells.

Specific animal models of NEC

Rat models of NEC

Barlow et al. (1974) described the first neonatal rat model of NEC, which demonstrated the importance of gut flora and lack of breastmilk (formula feeds) in the development of NEC-like injury (8), principles that are still pertinent today. This model was later expanded to include intermittent periods of hypoxia and hypothermia termed the HHF model, which serves as the foundation for many animal models of NEC subsequently developed (40). In addition, Caplan et al. (1994) later introduced bacterial pathogens in the formula given to neonatal rats, inducing manifestations of NEC-like intestinal injury, thus revealing a critical role of pathogenic bacterial colonization in developing NEC (31).

These original models have served as the basis for decades of subsequent models that have since modified, adapted, and improved these original concepts (41) (**Table 1**). However, there continues to be great variability in certain aspects of the rat models used today, including the use of both preterm and term neonatal rats, composition and frequency of formula feeds, duration, and degree of hypoxia and/or hypothermia. In general, rat pups are typically delivered *via* cesarean section or induction of labor by oxytocin administration, allowing for the avoidance of protective maternal milk feeds. The pups are then exposed to varying degrees and duration of hypoxia and/or hypothermia, followed by the introduction of a triggering agent such as lipopolysaccharide (LPS) and/or pathogenic bacteria (i.e., *Cronobacter sakazakii, Klebsiella*) administered enterally, intravenously, or intraperitoneally (52, 81, 82). These models generally take up to 3–5 days of exposure to various combinations of the above conditions before disease manifestation and development of NEC-like intestinal injury.

The advantages of using rat models to study NEC include (i) the similarities in intestinal immaturity between premature human neonates and of neonatal rats, (ii) their preterm viability post-cesarean section, (iii) their resilience and relative tolerance of stressors used to induce NEC-like injury (which may also be disadvantageous due to the variable manifestation of disease), (iv) their reasonably larger size (compared to mice) making gavage feedings and other manipulations more feasible, and (v) their relative low cost with high reproduction rate (**Figure 2** and **Table 3**). However, rat models have significant limitations in the ability to manipulate specific genes and pathways to aid in elucidating mechanistic processes and potential targets in disease development (83). Thus, studies use rat models primarily to test

TABLE 1 Commonly used and relevant animal models of NEC.

Model (Abbreviation)	Animal (Age)	Protocol Time	Key Points	Ref.
Hypoxia Hypothermia Formula (HHF)	Rats (E20–21)	3–4 days	Commonly used model, basis of several current models across species. Adaptations typically include gavage feeds with hyperosmolar formula creating impaired barrier/dysbiosis and exposure to bacterial pathogen (either colonized or administered *via* orogastric tube) ± LPS following exposure to periods of hypothermia, hypoxia (± hyperoxia to stimulate clinical conditions in certain variations). Activates TLR-pathway.	(9, 32, 40, 42–45)
	Mouse (P0–10)	3–7 days	Widely used adaptation of rat HHF model with vast number of variations that often include exposure to bacterial pathogens (*Klebsiella, C. sakazakii, E. coli, "NECteria")* ± LPS for increased disease manifestation. Commonly applied NEC induction protocol in transgenic mice testing specific genes/mechanisms (TLR4, VEGF, eNOS) ± exposure to various reagents/antibodies/modulators (amniotic fluid, HIF1α, IGF) to test therapeutic potential.	(9, 27, 33, 46–50)
	Piglet (Term, E115)	3–4 days	High rate of complications including 36% with pulmonary hemorrhage, 24% rectal perforations partly associated to technique (intubation, tonometer applied transanally), with severe manifestation of NEC.	(51)
Hypoxia Formula (HF)	Rats (E20–21, P0–3)	4–7 days	Requires exogenous or catheter colonization of bacteria to induce consistent NEC-like injury. Many variations utilized with varying exposures to hypoxia (decreased FiO2, 100% Nitrogen, etc) and different formula types (RMS, Ebsilac, human formula)	(8, 31, 52–56)
	Mouse (P0-P14)	3–4 days	Requires bacterial challenge ± LPS to trigger bacterial/inflammatory signaling; Important model for TLR4 signaling mechanisms.	(57, 58)
Formula Feeds (FF)	Mouse: SSC/Elecare (P8)	3 days	Use of hyperosmotic preterm human formula to induce NEC-like injury without exposure to hypoxia/hypothermia, has not yet been validated/replicated	(59)
	Mouse: Maltodextrin (P5–6 and P9–10)	10 days	Important model to study specific components (maltodextrin) of formula that create susceptibility to NEC. Consistent pattern of impairment with addition of maltodextrin combined Klebsiella (K) and/or hypoxia (H), worse with M/H. High survival rate after NEC induction protocol with milder severity.	(60)
	Piglet: Formula variations (E105–108)	1–4 days	Induction of NEC with formula feeding alone without exogenous hypoxic or hypothermic conditions. Prematurity of piglet (with transitional hypoxia and similarly impaired microvasculature to human premature infants), and presence bacterial colonization (no impact on germ-free piglets) necessary to induce intestinal injury.	(38, 48, 49, 61, 62)
	Piglet: Parenteral Nutrition (PN) (E105–109)	5 days	Gut dysfunction worsens with PN followed by FF, PN results in delayed gut maturation worsened by dysbiosis-induced FF. Several management changes in initiation of feeds, rate of feeding advancements, type of enteral feed, and PN-related applications derived from this model. Low true-NEC manifestation, wider distribution of disease to entire GI tract (including stomach, colon).	(51, 63–66)
Paneth Cell Disruption (PCD)	Mouse (P14–16)	16 h	Dithizone- or Diphtheria toxin-induced PC depletion. Model to study role of PCs in NEC, intestinal development closer to human development, model further simplified with either exposure to hyperosmotic formula (RMS) or bacterial challenge (*Klebsiella, NECteria*); short protocol time, TLR4-independent pathway	(10, 67–70)
Microvasculature Maldevelopment (MM)	Mouse: -VEGF/-IGF (E16–20, P0-1)	3 days	Models used to study intestinal vascular development and function using HHF NEC induction protocol in neonatal pups, using inhibition, deletion or down regulation on VEGF-related pathways. Fetal exposure to inflammation (using LPS) *in utero* (E16–20) followed by NEC induction at P0-1 also explored to determine chorioamnionitis impact on vasculature development and susceptibility to NEC. Addition of TNF shown to worsen NEC severity *via* decreased VEGF/VEGFR2 activity but prevented by DMOG (*via* HIF1a).	(27, 71, 72)
	Mouse: eNOS (P5)	4 days	Loss of eNOS in transgenic mice leads to greater gut and lung injury with altered inflammatory cascade, NO protective	(73)
Phlebotomy-induced anemia (PIA)	Mouse (P2)	10–12 days	Model investigating whether severe anemia ± RBC transfusion contributes to development and severity of NEC, activating TLR4-signalling mechanisms to drive inflammation and injury; Can be used to evaluate risk factor of iatrogenic anemia and gut perfusion.	(74, 75)
Antibiotic Exposure/Dysbiosis (ABT)	Mouse (P1–14)	14 days	Model to study role of prolonged antibiotic exposure leading to increased susceptibility to NEC when challenged at P14.	(76)
	Piglet (E 106)	5 days	Model to study effects of enteral vs. parenteral antibiotics in immediate post-natal period suggesting that enteral but not parenteral exposure protected from NEC, short duration (<5 days) of IV antibiotics with mild injury noted, no NEC.	(77)
Trinitrobenzene sulfonic acid (TNBS)	Mouse (P10)	1 day	Model using non-specific immunologic stimulant (TNBS) to induce NEC-like injury, highlighted critical role of gut microbiome with absence of injury in germ-free mice.	(78)
Dextran Sulfate Sodium (DSS)	Mouse (P3)	6 days	Adaptation of DSS model of IBD in adult mice, applied to neonatal mice to induce intestinal injury in the absence of hypoxia, hypothermia or LPS driven by humoral and cellular immune responses.	(79)
Anti-CD3 mAb	Mouse (P0)	2 days	Novel model illustrating the effect of T-cell inhibition to explore role of adaptive immunity in severity of NEC-like injury combined with dysbiosis from formula feeds (injury attenuated with antibiotics)	(80)

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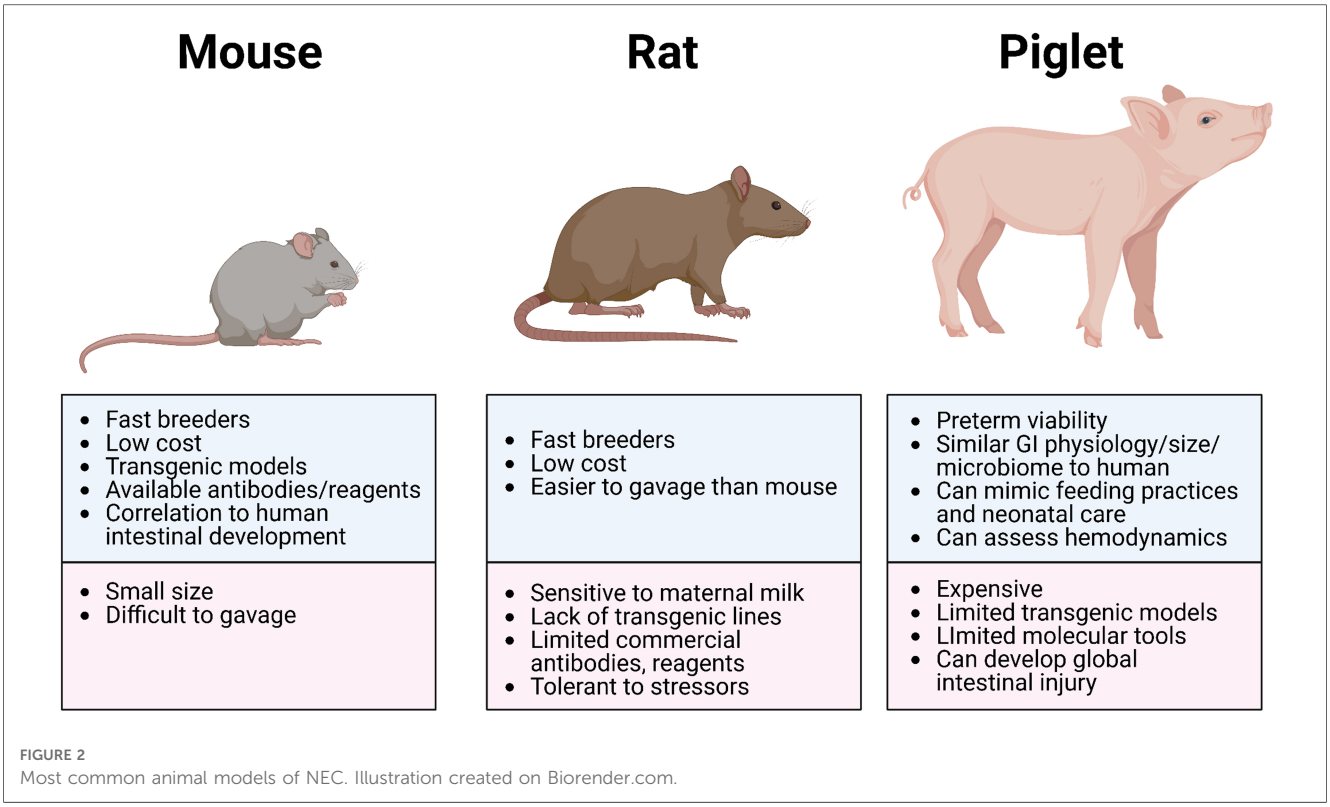

FIGURE 2
Most common animal models of NEC. Illustration created on Biorender.com.

feasibility and safety of interventions such as probiotics, while mice models became more ideal for mechanistic studies and elucidating the roles of growth factors, stem cells, human milk oligosaccharides, and tumor necrosis factor (TNF) blockers (21, 84).

Mouse models of NEC

Many early and existing mouse models of NEC were an adaptation of the rat HHF model (**Table 1**). These models subjected mouse pups to some combination of formula feeds, hypoxia, hypothermia, LPS, and/or bacterial dysbiosis/colonization to induce NEC-like injuries (9, 82). More recently, Mihi et al. (2021) described a version of these adapted HHF models that removes hypothermia but includes hypoxic stress, formula supplemented with LPS, and enteric bacteria derived from an infant who died from NEC totalis, the most severe form of NEC ("NECteria") (1). In addition, early mouse models of NEC initially attempted to deliver pups *via* cesarean section immediately before term to prevent exposure to maternal milk like in the rat models (9). However, subsequent studies confirmed that there is no need to immediately separate pups from their mothers since early dam feedings did not prevent the incidence of NEC (33). This is also demonstrated by the wide variability of postnatal ages of mice at the time of induction and subsequent disease manifestation of various mouse models of NEC.

Recognizing the emerging role of Paneth cells in the regulation of the innate immunity and protective mucosal barrier, the McElroy lab developed a two-hit model of NEC that requires both Paneth cell disruption and exposure to either enteral bacteria or formula feeds (68, 69). This model induces Paneth cell disruption by one of two validated methods: (i) chemically *via* the administration of dithizone, a heavy-metal chelator that reacts with zinc contained in Paneth cells leading to their disruption, and (ii) transgenically, using a human diphtheria-toxin receptor (DTR) that induce the selective necrosis of Paneth cells. This model does not require the combination of formula feeds, hypoxia/hypothermia, formula feeds, and bacterial challenge/dysbiosis to induce NEC, which most rodent models are based on. By limiting the number of experimental conditions and time required for disease manifestation (onset within 16 h vs. up to 5 days in other rodent models), this model may be more feasible. This model has uncovered new mechanisms and pathways that contribute towards the development of NEC that is independent of the well-studied TLR4 pathway and has now been validated and successfully replicated by other labs (76).

The advantages of using murine models of NEC include their relatively inexpensive cost, the ease of breeding, and the ability to genetically manipulate strains (**Figure 2** and **Table 3**). In addition, mice are born relatively early with relatively immature intestines, which continue to develop postnatally. Based on the presence and abundance of 20 epithelial genes shared by mice and humans, the mouse intestinal epithelium has been shown to develop similarly to the human intestine from mouse birth (equivalent to a human fetus around 16–20 weeks) until the mouse reaches four weeks of age (equivalent to a term human infant), making the mouse an excellent model to study premature gut development (85). Furthermore, many of the biochemical and genetic pathways implicated in the development of NEC in mouse models have also been observed in clinical

NEC, such as pathways involving TLR4, EGF, IgA, and HMGB1 (9, 86, 87). The primary disadvantage of using mice is their relatively small size, which makes them difficult to handle and gavage feed with formula, thus increasing the likelihood of complications and inconsistency. Still, mouse models of NEC have greatly advanced our understanding of the immature intestine and the factors contributing to injury susceptibility.

Piglet models of NEC

Touloukian et al. (88) were the first to describe a neonatal piglet model of NEC by inducing asphyxia followed by resuscitation, leading to hallmark features of intestinal necrosis. However, because this model utilized mature piglets (7–20 days old) and severe asphyxia approaches, Cohen et al. (51) modified this approach using moderate asphyxia (50% reduction in PaO2 ×30 min) in neonatal piglets (3–96 h old). Subsequent adaptations and modifications were made, shifting to the use of premature piglets without active asphyxia induction (89, 90).

With some minor variations, the piglet model of NEC generally involves the delivery of neonatal piglets at about 90% of full gestation (104–107 days of normal term at 114–118 days) (**Table 1**). Since the intestinal maturation of the piglet is not complete until a few weeks after birth, this period correlates with more premature intestinal physiology of human infants born at 75% of full gestation (28–30 weeks gestation) (91). Similar to the HHF rodent models, these piglets are exposed to a period of either natural or induced hypoxia/hypothermia followed by formula feeds to induce injury (91, 92). This model was later expanded to introduce the administration of total parenteral nutrition (TPN) prior to transitioning to enteral feeds. Exposure to TPN resulted in delayed intestinal growth and development that was characterized by mucosal atrophy, impaired mucosal barrier, and digestive functions that increased the development of NEC (65). Other piglet models of NEC include the combination of cow-based formula with high fat (3.5%) and ischemia/reperfusion (93) or *via* administration of iso-osmolar acidified casein solution into surgically created bowel loops in neonatal piglets (<3 days old and 2 weeks old) (94).

The greatest advantages of the piglet model are the size of the animal, similarities in metabolism and microbiome to humans, and a greater degree of similarities with human neonatal intestine, making this model highly translatable (**Figure 2** and **Table 3**). Piglets can also be sustained prematurely and receive total parenteral nutrition (TPN) *via* central venous access, mimicking similar clinical situations and management as the preterm infant, making the piglet model truly unique (91). However, besides being extremely costly to maintain, piglets have limited molecular analytical tools, such as antibodies, and it is difficult to create transgenic strains for genetic manipulation. Additionally, while HHF induces similar histological changes that resemble NEC, the inflammation triggered in this model can be widespread involving the stomach and jejunum and not limited to the ileum as seen in human and rodent models of NEC. Regardless, given the similarities of clinical manifestations of NEC in piglets and human neonates, piglet models of NEC have been critical in elucidating specific aspects of the pathogenesis of NEC, evaluation of feeding regimen compositions and rates, preclinical drug studies for potential preventative and therapeutic targets, and the development of radiological diagnostic approaches (37, 95).

Other animal models

Other less frequently used animal models have been developed to study specific aspects of NEC, rabbit models of NEC consisted of variations of the HHF model with endotoxin, hypoxia, and cold stress (96), as well as intraluminal insults on closed intestinal loops (97, 98), resulting in the generation of free radicals and exaggerated release of leukotrienes causing NEC-like injury. In addition, a preterm rabbit model was also developed that incorporated anal blockage to simulate preterm neonates' poor intestinal function and dysmotility, resulting in NEC-like pathologic changes in the small and large bowel (99).

Notably, two studies described the development of spontaneous NEC in 5%–16% of preterm non-human primates (14, 100). In one study, baboons were delivered prematurely *via* cesarean section at 125 days gestation, correlating to 27 weeks gestation in humans (100). The baboons underwent identical management to premature neonates in neonatal intensive care units (NICUs) with mechanical ventilation, antibiotics, enteral feeds, etc., simulating the conditions that make them susceptible to NEC. Over two years, they reported the development of spontaneous NEC at the age of 7 to 18 days in 5% of the preterm baboons. In addition to the similar incidence and postnatal age, baboon NEC had a striking clinical, radiological, and histopathological resemblance to human NEC. The possibility of creating an NEC model in non-human primates would offer multiple advantages due to the high degree of genetic similarities, the similar gastrointestinal anatomy and physiology, and comparable immune response to humans. However, difficulty in animal procurement and lack of availability to many investigators, increased ethical considerations, and extremely high husbandry costs are major limitations for establishing such a model. Gnotobiotic quails have also been used to elucidate the mechanisms connecting specific bacterial species and the fermentation process of undigested nutrients that contribute to the development of NEC by inoculating germ-free quails (101, 102).

Critical components and considerations when choosing a NEC model

Developmental stage correlation

As our understanding and management of infants with NEC evolved, so have the applicability of existing and new models (**Table 1**). Given that prematurity remains the most consistent risk factor for NEC, models have been developed to target the conditions of prematurity that may be driving the risk of NEC. Thus, understanding the stages of intestinal development in the model being used and how well correlated to the premature human infant will aid in determining whether the right model and age are being utilized.

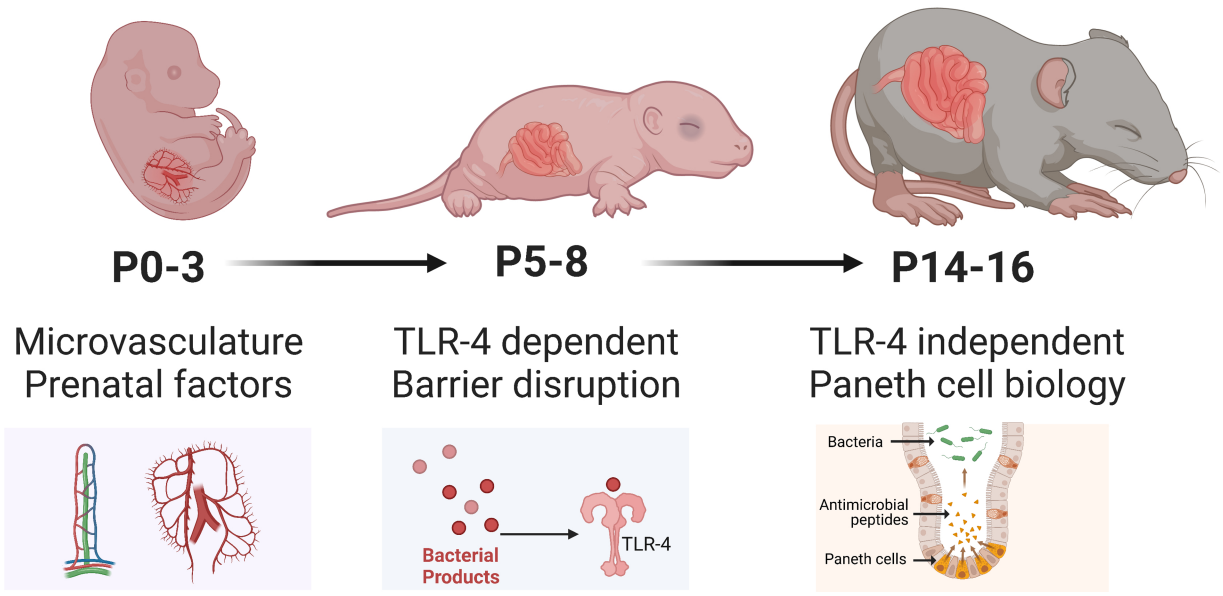

FIGURE 3
Developmental considerations of mice used to model NEC. Illustration created on Biorender.com.

The piglet model of NEC more closely matches the overall stage of development in the premature human infant (91). By delivering these animals at 90% of full gestation, there is better alignment with the premature state of human development on a multi-organ level, making the piglet model truly unique. The rat model is typically delivered just prior to term, closer to 94%–97% of full gestation, driven by inadequate lung development until that stage. Gut development, on the other hand, continues to mature postnatally, but unlike in the mouse model, many of the rat models of NEC rely on the prevention of maternal milk exposure to avoid its protective effects.

While maternal milk is extremely protective in rat models, mouse models of NEC are still able to activate mechanisms that drive intestinal injury despite being dam fed, possibly due to a comparatively less developed intestinal epithelium. Compared to rat models, there is greater variability in the modeling of NEC in mice (**Figure 3**), particularly in the age of induction, ranging from postnatal day 0 (P0) to P16. This is particularly relevant since neonatal mice intestinal maturation continues postnatally, with the emergence of critical cell types and factors occurring at later time points. Since NEC most likely is a common endpoint of various pathways and pathogenetic mechanisms, disease manifestation at various postnatal ages is critical to determining which process may be triggered. For example, induction of NEC at earlier postnatal ages (P0-P7) in mice appears to trigger TLR4-related pathways despite the absence of Paneth cells in the neonatal mouse until at least P7. At the same time, NEC can occur with Paneth cell disruption in the absence of TLR4 (68).

Mucosal barrier disruption

The HHF model used in the rat, mouse, and piglet models of NEC is the foundation upon which subsequent models have developed (**Table 1**). This model applies a multiple-hit approach that disrupts the protective mucosal barrier and alters the microbiota environment creating more dysbiosis. This then leads to bacterial translocation and the triggering of the inflammatory cascade that follows in NEC.

The mucosal epithelium is the key interface between the environmental microbiota, the neonatal host system, and its immune system (76, 103). This physical barrier includes tight junctions which modulate permeability, goblet cells that produce mucus (aids the trapping of pathogens and absorption of nutrients), and Paneth cells (produces antimicrobial peptides and a critical regulator of the innate immune system and stem cell niche) (67, 69, 104). The mucosal barrier in premature infants is immature, with increased permeability or "leakiness" that can lead to altered gut microbiota, nutrient deficiencies, and bacterial translocation to systemic organs. Also, premature babies have decreased mucin production, impacting the ability to trap pathogens and allowing increased penetration of the epithelium (105). Several animal models of NEC mimic conditions that ultimately lead to the disruption of the mucosal barrier, subsequently triggering the inflammatory cascade characteristic of NEC.

Dysbiosis and prolonged antibiotic exposure

The intestinal microbiota is critical to maintaining epithelial barrier functions (106). The integrity of the mucosal barrier symbiotically interacts with the intestinal microbiota, protecting from the overgrowth of opportunistic bacterial invasion and promoting continued gut epithelium maturation. Changes in the healthy microbial populations are critical for postnatal intestinal development, particularly in the underdeveloped intestinal barrier of preterm infants (107–109). However, in the preterm infant, the intestinal microbiota is impacted by several often-unavoidable

factors such as mode of delivery, antibiotic usage, type of enteral feeds, and need for blood transfusions (110), further increasing their susceptibility to developing NEC.

Numerous studies in mouse, rat, and piglet models of NEC have consistently demonstrated a link between bacterial colonization and the pathogenesis of NEC (111). In addition, several animal models have repeatedly shown a greater incidence of NEC-like lesions when animals are colonized or challenged with bacterial strains combined with an acute stressor to increase further susceptibility and disease manifestation. Other models that do not directly introduce a bacterial pathogen introduced variables that are now known to cause alterations in the microbiota populations, increasing the risk for bacterial translocation (31, 52, 68, 91).

Prolonged exposure to antibiotics, while often necessary in the premature population, has also been shown to increase the risk of developing NEC, likely due to the shifts in microbiota (112). Chaaban et al. (2022) describes a mouse model subjected to 10 days of the same empiric antibiotics used in neonates (ampicillin and gentamicin) of which more than half develop NEC following an oral bacterial challenge (76). This study nicely describes how prolonged use of systemic antibiotics lead to impairments in intestinal development, resulting in decreased cell proliferation, villi height, crypt depth, and numbers of goblet and Paneth cell expression. Interestingly, Birck et al. demonstrated that a shorter duration of enteral rather than parenteral antibiotics confers some protection from developing NEC in the preterm piglet model (77).

Enteral feeding types

While the exact etiology and pathogenesis of NEC remain poorly understood, enteral feeding type is recognized to play an important role (20, 113). It is surmised that enteral feeds combined with insufficient digestive capacities and an incompletely formed vascular system lead to bacterial overgrowth and increased metabolic demand on the immature intestine, further creating a susceptible environment to injury. Animal models typically utilize hyperosmolar formulas to aggravate the disruption of the mucosal barrier (**Table 2**). This concept has been used to mimic NEC in various animal models, particularly in rodent and piglet models. Importantly, hyperosmotic formula feeding is insufficient to create NEC-like injury, requiring a secondary insult such as hypoxia, cold stress, and/or bacterial pathogens to develop intestinal injury. Numerous studies have demonstrated that the lack of breastmilk and all the important components within it, rather than formula, increases susceptibility to NEC (69). Furthermore, animal studies have shown that the level of hyperosmolality to drive gut injury would need to be extremely high and beyond what is currently used in human neonates.

The models that utilize formula as an inciting factor to develop NEC-like injury utilize additional aspects of prematurity in combination or with an added inflammatory response. Formula-feeding-associated dysbiosis, in combination with factors that increase mucosal inflammation, has been shown in several models. As a recent example, Singh et al. (2020) describe a model that uses a maltodextrin-dominant formula, combined with either hypoxia and/or bacterial challenge with *Klebsiella* induce NEC in P5–6 and P9–10 murine pups without hypothermia (60).

Importance of innate immunity in modeling NEC

Premature neonates have intestinal immaturity that leads to a disrupted mucosal barrier, an underdeveloped immune defense system, altered vascular development and tone, and delayed enteric innervation (110). Intestinal inflammation and sepsis can develop when exposed to luminal bacteria that is impacted by enteric feeds, antibiotic exposure, and delivery method. The neonatal intestine must quickly respond to the presence of both "good" and "harmful" bacteria after birth, making the role of the innate immune system and mucosal barrier critical to avoiding injury. Animal models have been vital to characterizing the massive inflammation that occurs with NEC that appears to be triggered by either a TLR4-driven pathway or a TLR4-independent mechanism *via* Paneth cell disruption.

The most widely studied mechanism contributing to NEC pathogenesis is the role of Toll-like receptor 4 (TLR4), a receptor

TABLE 2 Feeding type formulation and reported osmolarity/osmolality.

Feeding Type	Osmolality (mOsm/kg) Osmolarity (mOsm/l)	Models used	Ref.
Rat/mouse (dam) milk	352 mOsm/l	Mouse, rat	(114)
Rat milk substitute (RMS)	660–721 mOsm/kg	Mouse, rat	(70)
Hyperosmotic: 15 g Similac + 75 ml Esbilac	849 mOsm/kg	Mouse, rat	(22)
Diluted hyperosmotic: Similac lower iron + Esbilac	324 mOsm/kg	Mouse, rat	(22)
33% Esbilac	Not measured/reported	Mouse, rat	(47)
Elemental formula (Elecare)	455 mOsm/kg	Mouse	(59)
Similac Special Care (SSC)	303 mOsm/kg	Mouse	(59)
Elemental formula (Neocate)	360 mOsm/kg	Mouse	(57)
Elemental formula (Pregestimil)	710 mOsm/kg	Dog	(115)
Term formula (Similac)	295 mOsm/kg	Mouse, dog	(115)
Preterm formula (Neosure)	298 mOsm/kg	Mouse	(59)
Pig milk (colostrum, preterm)	344 mOsm/l	Piglet	(38)
Pig milk (unfortified, donor)	312 mOsm/kg	Piglet	(116)
Commercial pig milk formula	481 ± 41 mOsm/kg	Piglet	(117)
Custom pig milk formula	182 mOsm/l	Piglet	(38)
Hyperosmotic milk formula + sorbitol	872 ± 32 mOsm/kg	Piglet	(117)

TABLE 3 Animal models of NEC- advantages and limitations.

	Mice	Rat	Pig
Advantages	High reproductive rate Genetically modifiable Commercially available tools (existing antibodies, primers) Postnatal intestinal development Ability to induce NEC at various ages	High reproductive rate Relatively larger size than mouse Easier to gavage feed than mice Neonatal rats more resilient than mice	Preterm viability Ability to evaluate perfusion/hemodynamics Can perform sequential lab work Ability to mimic identical feeding practices (formula, TPN) and clinical exposure Similar GI physiology/size to human neonates
Limitations	Difficult to gavage feed Require regular feeds for hydration and glucose regulation	Lack of transgenic lines High endotoxin/bacterial tolerance Requires c-section to avoid dam milk	Limited molecular diagnostic tools Can develop global intestinal injury
Cost	Low	Low	High
Ideal for:	Elucidating mechanisms, pathways and single gene effects driving pathogenesis	Testing safety/feasibility Temporal biomarker studies	Translational evaluation for therapeutic strategies
Models:	HF, HHF, ABT, PCD, PIA, I/R, MHK, FF	HF, HHF, I/R	HHF, ABT, I/R, FF, FF/PN

HHF, hypoxia-hypothermia-formula feeding; ABT, antibiotic exposure; PCD, Paneth cell disruption; PIA, phlebotomy-induced anemia; I/R, Ischemia/reperfusion; MHK, Maltodextrin ± hypoxia ± Klebsiella; FF, formula feeding; PN + FF, parenteral nutrition followed by formula.

for LPS, a component of the outer membrane of Gram-negative bacteria critical for developing NEC (14). A large body of work Hackam et al. and others has shown that the activation of TLR4 results in the inappropriate activation of the NF-kB pathway, resulting in mucosal damage *via* the production of proinflammatory cytokines, leading to damage of the intestinal mucosa. This then leads to bacterial translocation, further activating endothelial TLR4 leading to a reduced expression of the nitric oxide-generating enzyme eNOS in mice and further activating the inflammatory cascade in NEC (118, 119). In addition, TLR4 activation can also significantly inhibit the β-catenin signaling that is important for enterocyte proliferation in the ileum of newborn mice, which further leads to apoptosis and can lead to NEC (120).

Genetic alterations in the TLR4 pathway have also been found to increase susceptibility to NEC in humans. This includes variants of single immunoglobulin interleukin-1-related receptor (SIGIRR), which is associated with the inhibition and regulation of TLR signaling. Variants of SIGIRR have been associated with widespread inflammation and severity in NEC (15, 121). This was confirmed in SIGIRR −/− transgenic mice subjected to experimental NEC, leading to increased intestinal inflammation, apoptosis, and NEC severity (122).

However, TLR4 activation is not always associated with the development of NEC in premature infants, and NEC can develop in the absence of Gram-negative bacteria (123). An alternative mechanism was further established in the murine model of Paneth cell disruption that demonstrated that NEC-like intestinal injury could occur in TLR4 −/− mice subjected to Paneth cell disruption developed by the McElroy et al. (68). Human neonates with NEC have decreased expression of Paneth cells (124). Paneth cells are critical regulators of the innate immunity of the gut, producing essential antimicrobial peptides in the epithelium as part of the mucosal epithelial barrier and regulating the innate immune system (104). The innate immune system of the gut requires a careful balance between maintaining homeostasis on the one hand and rapid inflammatory response to pathogens and other threats on the other; thus, impaired Paneth cell function can create a proinflammatory state more susceptible to injury.

Modeling impaired microvasculature in NEC

One of the hallmark features of NEC is intestinal ischemia and necrosis. Earlier models attempted to recapitulate the ischemia that is believed to contribute towards the development of NEC. These models typically involved the occlusion of the superior mesenteric artery (SMA), effectively blocking blood flow to the small bowel and then allowing for reperfusion. However, these models replicated ischemia that occurs before NEC without also inducing inflammation, thus not an accurate model of NEC (28, 97, 125). Although not directly targeted, several models developed and currently utilized have some component that drives the ischemic changes seen in NEC. Whether it is hypoxia exposed *via* subjecting the animal to decreased oxygen concentration or nitrogen gas or "transitional" hypoxia that occurs when animals such as the piglet are delivered prematurely and require some mode of oxygen support.

Neonates, particularly premature infants, are uniquely vulnerable to hypovolemic or ischemic injury to the intestine compared to adults in part due to their relatively low resistance to blood flow (126). Postnatal hypoxia and other diseases that result in decreased blood flow, disruption of intestinal vascular development, and /or oxygen delivery resulting in impaired perfusion increase the risk of NEC in neonates and experimental animal models (42, 127, 128). Preterm infants with NEC also have been shown to have increased levels of TLR4 with reduced nitric oxide synthase (eNOS) expression, suggesting that intestinal endothelial dysfunction by endothelial TLR4 activation contributes to the development of NEC (118, 129). The role of inflammation is thus believed to trigger a secondary vasoconstriction that worsens the intestinal ischemia process leading to a vicious cycle of ischemia and inflammation characteristic of NEC (130).

Work done by De Plaen and colleagues have advanced our understanding of the mucosal microvasculature that is impaired in NEC, explicitly highlighting the importance of VEGF and VEGF-receptor 2 signaling pathways (27, 71). Specifically, this group has shown that inhibition of VEGFR2 with kinase inhibitors led to more severe intestinal necrosis with a higher mortality rate, decreased endothelial cell proliferation, and decreased microvascular network density. While the administration of macrophage-derived IGF-1, which promotes VEGF expression and endothelial cell proliferation, leads to protection against experimental NEC. These models applied a modified HHF NEC induction protocol on neonatal P0 transgenic mice. Data gathered from these experimental models are critical to our understanding of how the most commonly utilized models of NEC can result in ischemic changes coupled with a dysregulated inflammatory response (either *via* bacterial/LPS exposure or PC disruption), making this a truly unique aspect of studying the pathogenesis of NEC (71, 72).

Anemia and packed red blood cell (pRBC) transfusions in the development of NEC

Premature infants often develop severe anemia either early on secondary to iatrogenic blood loss from lab draws/procedures or later classically as anemia of prematurity, which is related to several factors, including insufficient erythropoietin production, immature bone marrow functions, high turnover of neonatal RBCs with shorter half-lives, infections, and nutritional deficiencies (74, 131). In addition, anemia alone has been shown to directly alter the intestinal barrier (increased mucosal hypoxia and barrier permeability) and innate immunity (increased proinflammatory macrophage activity) in a neonatal mouse model of phlebotomy-induced anemia (PIA) (75).

Mohankuma et al. (2019) combined the PIA model with RBC transfusions, creating a novel model to determine the combined and separate effects of each (74). In this study, severe anemia was found to cause inflammatory changes in the intestinal mucosa with macrophage infiltration, and the subsequent RBC transfusions further activated these cells *via* a TLR4-mediated mechanism to cause injury. Transfusion in anemic but not control mice was associated with intestinal injury within 28 h after transfusion, characterized by coagulative necrosis, inflammation, submucosal edema/separation, and interstitial hemorrhages (74). These studies highlight how severe anemia is an independent risk factor for NEC and that transfusion-associated NEC occurs only in the setting of severe anemia, likely due to a similar phenomenon as seen in ischemia/reperfusion models of NEC.

Other inflammation and immune-modulating approaches to NEC

Other models have been developed that attempt to induce the exaggerated inflammation seen in NEC. For example, Mohankuma et al. (2017) described a model that incorporates the enteral administration of trinitrobenzene sulfonate (TNBS), a non-specific immunologic stimulant that leads to an increase in chemotaxis for macrophage infiltration, resulting in a mucosal injury similar to that of NEC. In this model, TNBS was administered *via* gavage and enema to 10-day-old pups to induce enterocolitis. Interestingly, this model is ineffective when applied to germ-free mice, illustrating the critical role of the gut microbiota in developing TNBS-induced enterocolitis and NEC-like injury (78, 132).

Ginzel et al. (2017) administered formula containing dextran sodium sulfate (DSS), a mucosal irritant, to 3-day-old pups, which resulted in NEC-like disease of the small and large bowel in the absence of hypoxia or hypothermia (79). This model resulted in NEC-like lesions with both humoral and cellular immune responses throughout the intestine. This model is unique in that mucosal tissue damage was induced in the absence of any physical stressors in a relatively short period and produced a greater degree of intestinal injury than LPS alone.

Klinke et al. (2020) developed a mouse model that targeted the inflammatory cascade that occurs in NEC by altering neutrophil concentrations. In this model, neutrophilia by the administration of G-CSF leads to an increase in the disease manifestation of NEC when induced using hypoxia, formula, and LPS (133).

Subramanian et al. (2022) recently described a model of NEC that combines formula-feeding-associated dysbiosis with mucosal inflammation driven by anti-CD3 mAb treatment. This model uniquely illustrates the potential role of T-cell inhibition using anti-CD3 mAb. In addition, the severity of the NEC-like injury was attenuated with the administration of antibiotics and dam feeds (80).

Conclusion

The multifactorial processes driving disease manifestation in NEC makes the development of an exact animal model of NEC difficult, if not impossible, to achieve. Instead, each unique model provides a different perspective on how multiple factors independently lead to the alteration of complimentary and overlapping signaling pathways that ultimately lead to NEC-like injury (**Table 3**). While Barlow's original neonatal rat HHF model continues to be the foundation on which many of the current models are based, unique approaches and considerations have emerged that offer new insight into the predisposing factors, pathogenesis, and more global effects of NEC. In addition, the continued advancement of molecular tools, data and collaborative science allows the discovery of new aspects and correlates to the human conditions of NEC that we seek to answer. Best practice in science requires the use of animal models only when other alternatives are not applicable, but because of the multifactorial pathophysiology of NEC and the difficulty obtaining human samples, animal models are needed to move the field forward (134). In developing these models, one must make every effort to implement the "3Rs" to guide the humane treatment of animals used in research. These include ***r***educing the number of animals used in research, ***r***efining procedures and studies to minimize pain, and ***r***eplacing animal experiments with *in vitro* models whenever possible (135). The Animal Welfare Act and specific governing bodies such as the Institutional Animal Care and Use Committee (IACUC) in the U.S. have been established to specifically aid research institutions

and investigators in maintaining ethical practices and the most efficient use of animals in all research endeavors (136, 137).

By understanding the basis of each model that currently exists and the unique aspects it can provide, new and current investigators will be able to determine the best tools available to elucidate the particular aspect of NEC they seek to explore further. By directing our efforts and using the optimal model, we can further delineate the various pathways disrupted in NEC, determine how modifiable factors such as enteral feeding types and environmental exposures specifically impact these pathways, and uncover potential genetic susceptibilities, leading to the successful identification of novel therapeutic targets and prevention strategies that will be crucial to our vision of a world without NEC.

Author contributions

Conceptualization: GMB, SJM; Methodology: GMB, SJM. Investigation: GMB, AJC, SJM. Writing – original draft: GMB, AJC, SJM. Writing – critical review & editing: GMB, AJC, HC, SJM. Visualization: GMB, AJC, HC, SJM. Supervision: GMB, SJM. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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State of the art review on machine learning and artificial intelligence in the study of neonatal necrotizing enterocolitis

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Necrotizing Enterocolitis (NEC) is one of the leading causes of gastrointestinal emergency in preterm infants. Although NEC was formally described in the 1960's, there is still difficulty in diagnosis and ultimately treatment for NEC due in part to the multifactorial nature of the disease. Artificial intelligence (AI) and machine learning (ML) techniques have been applied by healthcare researchers over the past 30 years to better understand various diseases. Specifically, NEC researchers have used AI and ML to predict NEC diagnosis, NEC prognosis, discover biomarkers, and evaluate treatment strategies. In this review, we discuss AI and ML techniques, the current literature that has applied AI and ML to NEC, and some of the limitations in the field.

KEYWORDS

machine learning (ML), artificial intelligence (AI), necrotizing enterocolitis (NEC), biomarker discovery, disease modeling

1. Introduction

Necrotizing enterocolitis (NEC) is a devastating, inflammatory disorder, which impacts mainly preterm infants and remains one of the most common gastrointestinal emergencies in the preterm infant population (1–6). In the United States alone, it is estimated that up to 9% of infants weighing less than 1,500 g at birth will develop NEC (7). The mortality rate from NEC is significant and has been reported up to 30%–50% depending on disease severity (1–6). Treatment strategies have remained limited, non-targeted, and have not changed significantly in decades (8). Although NEC was formally described in 1965 by Mizrahi et al., the specific causes have yet to be fully elucidated (1–6). To help clinicians with NEC diagnosis, Bell et al. published the first clinical staging system for NEC in 1978 that was designed to help clinicians know when to surgically intervene (9). Eight years later, Walsh and Kliegman published a modified version of Bell's staging system (9, 10). The Bell and Modified Bell staging systems have consistently been the most widely used clinical definitions and are considered the "gold standard" in the field. However, most researchers and clinicians now focus on Bell ≥2 and believe that Bell stage 1 or Modified Bell stage 1A and 1B are considered largely non-specific (11). This has led to the development of six newer definitions for NEC, which all propose to be superior at NEC diagnosis than the Bell and Modified Bell staging definitions (12–18).

While many discoveries are being made within the NEC field, which may help prevent or treat NEC in the future, there remain fundamental limitations that clinicians and scientists in the field face. First, there is no universal definition of NEC. As discussed in the last paragraph, there now exist multiple definitions of NEC and clinicians and scientists can choose the one that suits their purposes best. This can lead to differences in what clinicians diagnose as NEC

at various institutions. An added challenge is that the etiology of NEC has yet to be fully understood. Many in the field believe that NEC is a multifaceted disease and is the common end point of several pathways and pathologies. This multifaceted nature of NEC has made biomarker discovery difficult. Despite the NEC field spending ample time, resources, and research focus attempting to discover better biomarkers to aid in better prevention and mitigation strategies, all biomarkers discovered thus far have been insufficient (19–21). Therefore, NEC as a disease has the potential to benefit greatly from artificial intelligence (AI) and machine learning (ML) (21–24). So far, AI has shown promise in identification and prediction of diseases, biomarker discovery, disease risk evaluation, and development of improved treatment plans for many diseases both for adults and neonates (25–31). While AI and ML studies applied to the healthcare setting have rapidly increased in recent years, most instances have been applied to common and more well-defined diseases such as sepsis or cancer and only a few published studies have applied AI and ML to NEC. This review will summarize basic concepts of AI and ML (Section 2), present and summarize the current published literature on AI and ML in NEC (Section 3), as well as describe some of the limitations and pitfalls of AI and ML (Section 4).

2. Artificial intelligence and machine learning in healthcare

Artificial intelligence (AI) has become an increasingly relevant topic in most aspects of life and has offered particular promise in the healthcare sector (32–35). Computers have the unique ability to quickly find patterns in massive datasets that would take the human eye and brain far longer to identify (33, 36). Because of this, as early as the 1980's it was thought that through machine learning (ML), AI had the potential to be used to identify disease patterns and ultimately improve healthcare. Although at the time the computational power and algorithms necessary for ML and AI to be used effectively were not available, within the past decade a massive amount of time and resources have been devoted into the advancement of computers, AI, and ML (33, 36, 37). These improvements have made applying AI and ML to electronic medical records (EMRs) and within the healthcare sector a real possibility (38). While many use AI and ML interchangeably, there is a distinction between the two. AI describes a machine/computer using math and logic to learn and problem solve similarly to how a human brain functions, which can be done with or without the use of ML (39–43). While ML, a subset of AI, is the use of mathematical modeling and algorithms which learn and improve without explicit instruction as more data is provided (39–43). To put more simply, ML is just one application of AI, but other types of AI also exist such as limited memory AI, which is used for the development of chatbots or giving cars the ability to drive autonomously (39–43).

Two main types of ML classifiers are used when AI is applied in the healthcare setting, which include supervised, or inductive classifiers, and unsupervised, which each have their own merits (**Figure 1**) (33, 36, 37). Supervised ML is used when the data has a labelled or identified outcome of interest. When using supervised ML in the neonatal healthcare setting, the dataset will contain features that are thought to influence an outcome (often EMR data including treatments, feeding types, gestational age, etcetera) as well as representation from the potential outcomes or labels of interest (disease vs. no disease; improvement, worsening, or no change following treatment; clinical disease scores; and so forth) (33, 36, 37). Within supervised ML, there are three subcategories depending on the data type including classification, regression, and forecasting (**Figure 1**). Classification supervised ML occurs when the output is categorical/discrete, whereas regression supervised ML uses continuous numerical values as output (33, 36, 37). The final type of supervised ML is forecasting, which is when both past and present data types are used as input to inform the model (33, 36, 37).

The other major type of ML is unsupervised ML, where unlabeled data is used as input and the ML model will identify patterns or structures within the data that would otherwise not be detectable to the human eye (33, 36, 37). Like supervised ML, unsupervised ML can also be divided into subcategories including association, clustering, and dimensionality reduction (**Figure 1**). Association models can be used to identify/predict comorbidities. In contrast, clustering models will group similar datasets together, but distinctly from others. For example, a clustering model would likely group patients with a disease condition together, but distinctly from patients without the disease (33, 36, 37). Finally, dimensionality reduction involves scaling down the data through the process of feature optimization. The process of dimensionality reduction is of particular importance when using EMRs and "omics" datasets because they house a wealth of information. However, because of the volume of data in these datasets, only a fraction of that information is useful when identifying/predicting disease (33, 36–38). Through dimensionality reduction, unsupervised ML models can identify what features best represent an outcome of interest vs. those that are superfluous. Thus, dimensionality reduction through feature optimization as well as feature engineering can be used for biomarker discovery. Dimensionality reduction can also aid in establishing a hybrid ML model (**Figure 1**). In this case, ideal features will be identified using an unsupervised ML model and then those features can be used as input into a supervised ML model to then predict a disease of interest. An additional approach to handling large data sets such as EMRs as well as "omics" data is using deep learning (DL). Deep learning can be used in the context of both supervised and unsupervised ML. DL uses higher complexity algorithms like neural networks and greater computational power to process large or high dimensionality datasets that some of the simpler ML models would have difficulty fitting (33, 36, 37).

Supervised and unsupervised ML models have similarities and differences in the required inputs. To create a supervised ML model, the dataset is first split into a training set, which will contain the majority or roughly 70%–80% of the data, and a test set, which will contain the remaining 20%–30% (33, 36, 37). If sufficient data is available, the 30% of data allocated for the test set can be split into both a test set (10%–15% of data) and a validation set (10%–15% of data). The validation set is utilized for

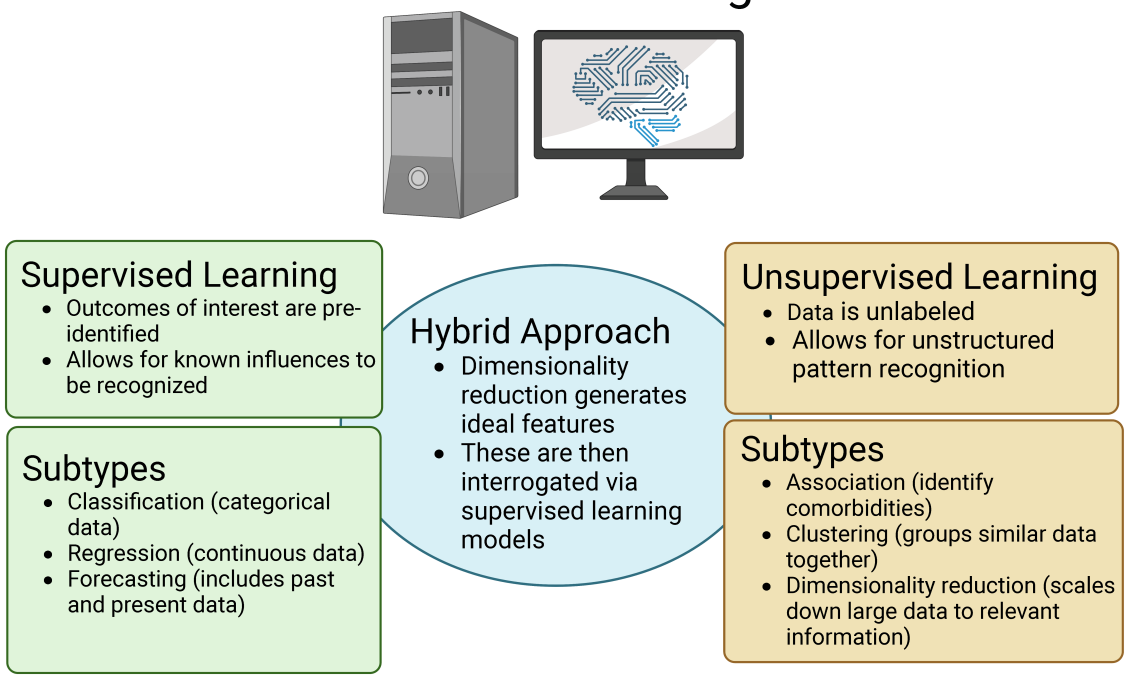

FIGURE 1
Overview of the three major types of machine learning (ML) that are applied in the healthcare setting as well as the respective subtypes. Figure created with Biorender.com.

parameter tuning within the various ML models, so that when the model reaches the testing/evaluation phase, the model is being tested on data it has never seen. Although ideal, if the overall dataset is relatively small and will not require a great deal of parameter tuning, the validation set may not be necessary (44). The training set will be provided to the ML algorithm of choice, ideally multiple different algorithms, and will include both the features as well as the labelled diagnoses/outcomes of interest. The ML algorithm will make a model based on the training data and then will apply the model created on the validation or test set. During the validation/testing stage, the model will use the features from the validation/test set that the model was originally trained on and attempt to predict the diagnosis/outcome. The model can then assess its own efficacy through accuracy scores (both training and test set accuracy), area under the receiver operator characteristics curve (AUROC), sensitivity, specificity, and other evaluation metrics (33, 36, 37, 44). The model developer can then fine tune the algorithm(s) parameters to improve upon the various evaluation metrics using the validation dataset. On the other hand, when using unsupervised ML models, there is no need to split the data into training and test sets because the data is unlabeled resulting in no way to formally evaluate the accuracy of the output. Instead, all the features of interest are used as input for each sample and then the algorithm(s) of choice is/are used to process the data before the model provides the desired output (33, 36, 37). While unsupervised machine learning models do not have the same degree of evaluation metrics, model developers can split data into a training and validation dataset. For unsupervised ML validation sets, it is important to have similar patterns and sample distribution as is present in the training set otherwise the ML model may have a false poor performance. If the ML model and the datasets were developed appropriately, similar output would be anticipated after running either set. For example, when using a clustering unsupervised ML model, samples would cluster similarly, and the same number of clusters would be found in both the training and validation set. Ultimately, while the input in supervised and unsupervised ML is different, using a validation set in both can help to ensure the model is being trained using the correct algorithm and is behaving in the way intended.

3. AI and ML in NEC

ML and AI studies and publications applied to the healthcare setting have rapidly increased in recent years, but most instances have been applied to common and more well-defined diseases such as sepsis or cancer (25–31). In comparison, relatively few studies have been published applying AI and ML to NEC (**Table 1**). While not formally described as ML, one of the earliest applications of computer science in the NEC literature came from the use of univariate and multivariate linear regressions, which was first documented in 1991 by Uauy et al. (61). In this publication, the modified Bell staging definition was used to distinguish suspected NEC (infants in stages IA and IB), proven NEC (infants in stage IIA), advanced NEC (infants in stage IIIA), and perforated NEC (infants in stage IIIB) (61).

TABLE 1 Studies applying artificial intelligence (AI) and machine learning (ML) to NEC including a description of the cohort, type of ML, intended model purpose, and major findings from the model(s).

[Citation]	Cohort (#)	Type of ML Used	Purpose(s) of Model(s) Developed	Findings
Mueller et al. (45)	Multicenter; 197 patients (1) Control (130) (2) NEC (67)	Supervised ML [Artificial neural networks (ANN)]	(1) Determine risk factors for NEC	2 risk factors out of 57 were considered important in distinguishing NEC infants from controls 0.15 mean prediction error value from ANN model using 5 features
Sylvester et al. (46)	Multicenter; 485 patients (1) Medical NEC (345) (2) Surgical NEC (140) Multicenter; 65 patients	Supervised ML [Linear discriminant analysis (LDA)] Unsupervised ML (hierarchical clustering (UHC)	(1) Cluster analysis of urine biomarkers to see if peptide abundance will distinguish medical and surgical NEC infants (2) Distinguish medical NEC from surgical NEC infants using demographics and urine biomarker data	LDA model with clinical parameters: AUROC: 0.817 4 candidate urine peptides were identified in the clustering to best distinguish medical and surgical NEC Model with urine peptide biomarkers: AUROC: 0.86 LDA model with clinical features and urine peptide biomarkers: 100% correct outcome prediction
Doheny et al. (47)	Single Center; 70 preterm infants (1) Control (61) (2) NEC (9)	Supervised ML [Two step multiple logistic regression (LR)]	(1) Predict NEC based on the high frequency component of heart rate variability (HF-HRV)	Model sensitivity: 0.89; specificity: 0.87 Cutoff of 4.68 ms^2 with NEC infants being below the cutoff was a non-invasive biomarker
Ji et al. (16)	Multicenter; 520 patients Clinical Concern for NEC (Bell stage IA-IIIA) (1) Confirmed/Medical NEC (344) (2) Surgical NEC (140) (3) Incomplete data (36)	Supervised ML [Hybrid generalized linear mixed effects models (GLMMs)] Hybrid (LDA)	(1) Objectively score NEC on a severity scale of I-III (2) Predict infants at low, intermediate or high risk for NEC progression	9 features were important for NEC severity scoring Severity score model agreement 100% for Bell stage 1, 94% for Bell stage 2; 83% for Bell stage 3 Outcome model AUROC: 0.84 2 features were important for outcome prediction
Irles et al. (48)	Single Center; 76 patients (1) Control (No NEC or IP) (27) (2) NEC without IP (23) (3) NEC with IP (26)	Supervised ML (ANN)	(1) Predict intestinal perforation (IP) associated with NEC from data available at birth (2) Predict IP associated with NEC from data available at birth and during hospitalization	Predicting with data available at birth R^2: 0.976 Predicting with data available at birth and hospitalization R^2: 0.98 11 features were important for prediction
Rusconi et al. (49)	Single Center; 96 patients (1) Control (67) (2) NEC Bell Stage ≥II (24) (3) NEC Bell Stage I (5)	Supervised ML (K nearest neighbors (KNN), Partial least squares (PLS), Random Forest (RF), Naïve Bayes (NB), Support Vector Machine (SVM)) Unsupervised ML (UHC)	(1) Predict NEC vs. non-NEC based on altered sphingolipid profiles from metabolomics data and demographic/clinical features	KNN model had the best performance: accuracy: 0.73 Clustering analysis suggested sphingolipid differences were important for distinguishing a subset, but not all NEC patients After including the sphingolipid clustering profile, better accuracy was achieved by the ML models: accuracy: 0.9–0.96
Olm et al. (50)	Single Center; 160 preterm infants (1) Control (126) (2) NEC (34)	Supervised ML [RF, Gradient Boosted Classifier (GBM)]	(1) Distinguish NEC from control infants based on clinical features and stool microbiome data (2) Determine the important features for model decision making	The GBM classifier performed better than RF: accuracy: 0.84 4 features categories were important for prediction
Hooven et al. (51)	Multicenter; 161 patients (1) Control (116) (2) NEC (45)	Unsupervised [Hierarchical Feature Engineering (HFE)] Supervised [Multi-layer neural network (MIL)]	(1) Predict risk for NEC based on serial stool microbiome taxonomy and demographic metadata	MIL: AUROC: 0.9 Prediction can take place over 24 h before disease onset
Gao et al. (52)	Single Center; 827 patients (1) Control (485) (2) NEC (342) Single Center; 379 NEC patients	Deep learning (DL) split attention networks, squeeze and excitation (SE) networks with/without the residual (Res) network (ResNest, SENet, SE-ResNet) Supervised ML (Light GBM)	(1) Predict NEC diagnosis based on 58 clinical features and radiomics data (2) Determine whether surgical intervention will be necessary using 49 clinical features and radiomics data (3) Determine what features were important for model decision making	LightGBM for NEC prediction: AUROC: 0.93; sensitivity: 0.94; specificity: 0.82 18 clinical features were important for prediction 9 clinical features were important for surgery prediction LightGBM model for surgery prediction: AUROC: 0.94; sensitivity: 0.95; specificity: 0.95
Pantalone et al. (53)	Single Center; 246 patients (1) Control (69) (2) Medical NEC (116) (3) Surgical NEC (61)	Supervised ML (LDA, RF, SVM)	(1) Distinguish the three groups based on clinical features and blood count data collected at birth, at baseline, at NEC diagnosis, and 3 days following antibiotic completion	Models had poor performance trying to classify all three together RF model performed the best distinguishing surgical NEC from controls: AUROC: 0.88; accuracy: 0.8; sensitivity: 0.8; specificity: 0.79 RF model performed the best distinguishing surgical NEC from medical NEC: AUROC: 0.76; accuracy: 0.67; sensitivity: 0.37; specificity: 0.82 4 features were important between the two models

(*continued*)

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TABLE 1 Continued

[Citation]	Cohort (#)	Type of ML Used	Purpose(s) of Model(s) Developed	Findings
Casaburi et al. (54)	Multicenter; 1,603 shotgun metagenomic datasets (1) 245 NEC positive (2) 1,358 non-NEC	Supervised ML (RF, GBM)	(1) Predict NEC based on taxonomic relative abundance data (2) Predict NEC or non-NEC based on clinical features and metagenomic taxonomy data (3) Calculate feature importance scores	RF model with species level taxonomy data and samples >29 weeks: test accuracy: 0.9 RF and GBM models with different PMA ≥ 29 or <29; balanced or unbalanced between NEC and non-NEC samples; stratified or unstratified: sensitivity: 0.24–0.92; specificity: 0.91–1.0 NEC associated Enterobacteriaceae spp. were the important features
Cho et al. (55)	Multicenter; 10,353 very low birth weight infants (1) Control (9,649) (2) NEC (704)	Supervised ML [LR, Decision Tree (DT), NB, RF, SVM, ANN]	(1) Predict risk for NEC based on 74 clinical features	LR and RF performed the best: accuracy: 0.93; AUROC: 0.73 and 0.72 respectively 10 clinical features were important for NEC prediction
Lin et al. (56)	Multicenter; 261 patients (1) Control (186) (2) NEC (75)	Supervised ML [Multiple instance neural network (MIL), RF]	(1) Predict NEC based on readily available clinical data and stool microbiome collected through onset of NEC or the first ~60 days of life (2) Predict NEC based on stool microbiome instances the MIL model weighted as important	MIL: AUROC: 0.86–0.92; sensitivity: 0.86; specificity: 0.90 The microbiome data was important for the MIL model and based on the RF model, certain taxa (Firmicutes, Protobacteria, Enterobacteriaceae) drove the decision-making process The MIL model could predict NEC an average of 8.3 days prior to disease onset RF: AUROC: 0.79–0.86
Lueschow et al. (22)	Single Center; 219 patients (1) Control (117) (2) NEC (102)	Supervised ML [KNN, Simple neural network (SNN), NB, RF, SVM, DT]	(1) Predict NEC or non-NEC based on the features required for the NEC definitions (2) Determine important features based on the DT classifier (3) Develop a DT model using important features	DT model had the best performance: sensitivity: 0.83; specificity: 0.96; accuracy: 0.8; AUROC: 0.8 9 features were identified as important for the DT model decision making The most important feature definition DT model: sensitivity: 0.4; specificity: 0.77; accuracy: 0.62; AUROC: 0.62
Lure et al. (57)	Single Center; 40 patients undergoing surgical intervention (1) NEC (29) (2) Spontaneous intestinal perforation (SIP) (11)	Supervised ML [Ridge logistic regression (RLR), RF]	(1) Predict NEC or SIP	RLR: AUROC: 0.93; sensitivity: 0.89; specificity: 0.91 RF: AUROC: 0.98; sensitivity: 0.96; specificity: 0.96 4 variables were important for prediction with 3 associated with NEC and 1 with SIP
Qi et al. (58)	Single Center; 45 patients with NEC	Supervised ML (RF, SVM, LR)	(1) Predict whether NEC patients will need surgery or not based on clinical and radiomic features	(1) The RF model had the best performance with AUROC ranging from 0.68–0.8
Son et al. (59)	Multi-Center; 12,555 very low birth weight infants (1) Control (11, 703 Non-NEC) (2) NEC Non-IP (852) (3) NEC with IP (521) (4) SIP (208)	Supervised ML [ANN/multilayer perceptron (MLP), SVM (linear and radial), LR, KNN, DT, GBM (Light and extreme), RF]	(1) Predict NEC, NEC-IP or SIP (2) Predict NEC vs. Non-NEC then NEC-IP vs. SIP	The ANN/MLP had the best performance: AUROC: 0.81–0.87 depending on which condition it was predicting Applying the ANN/MLP model to a different dataset: AUROC: 0.67–1.0
Song et al. (60)	Single Center; 447 patients (1) Feeding intolerance (FI) (151) (2) NEC (296) Single Center; 296 NEC infants (1) Medical (205) (2) Surgical (91)	Supervised ML [Ridge regression and Q-learning strategy-based bee swarm optimization (RQBSO), SVM]	(1) Predict NEC vs. FI using 119 features (2) Predict the prognosis of NEC patients and whether they will require surgery using 119 features	NEC diagnosis compared to FI: AUROC: 0.94; accuracy: 0.91 7 features were notably important for NEC diagnosis NEC prognosis: AUROC: 0.92; accuracy: 0.84 5 Features were most important for NEC prognosis prediction

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Demographic and clinical features of NEC were used as variables to determine statistical significance in the model distinguishing the various infant groups (61). Medical center, race, gender, birth weight, maternal hemorrhage, duration of ruptured membranes, and cesarean section were all identified as significant risk factors using this multicenter population and methodology (61). Since this publication, univariate and multivariate linear regressions continue to be utilized and seen in over 200 PubMed publications related to NEC to determine what risk factors are associated with NEC as was seen in the Uauy et al. publication or determining the prognosis of a patient with NEC based on treatment strategy. While linear regression is a form of classification ML, many debate whether univariate and multivariate linear regressions are considered true ML. Thus, these publications will not be discussed in detail in this review.

3.1. ML methods for NEC biomarker discovery

Biomarker discovery, particularly non-invasive biomarkers, and determining risk factors for NEC have been a topic of interest for researchers applying ML to NEC (**Table 1**). The first publication to formally apply ML to NEC was by Mueller et al. in 2009 (45). Using artificial neural networks (ANN), Mueller et al. found two risk factors from their set of 57 that were important for distinguishing NEC infants from controls including small for gestational age and being artificially ventilated (45). Additionally, the best scoring metric came from an ANN model using only five features (45). For biomarker discovery, Doheny et al. used the high frequency component of heart rate variability (HF-HRV) to predict NEC with high sensitivity and specificity in a multiple logistic regression model as infants that developed NEC had a much lower HF-HRV than infants that did not develop NEC (47). Pantalone et al. also used ML for biomarker discovery but chose to focus on the predictive ability of complete blood cell count (CBC) data at various time periods before NEC onset to distinguish between controls, patients with surgical NEC, and those with medical NEC (53). Their random forest (RF) model performed the best and while there were high performance scores in all metrics when distinguishing between surgical NEC and controls, the sensitivity was low when the RF model tried to classify surgical NEC compared to medical NEC (53). In both models, absolute bands at NEC and gestational age at birth were important contributors to the model (53). Cho et al. used six different supervised ML models to identify NEC based on 74 clinical features with the goal of understanding, which features may be important for NEC prediction. Two models, logistic regression (LR) and RF, had the best performance with high accuracy and decent AUROC scores (55). They also found 10 of the 74 features to be important for the RF model to distinguish NEC from controls (55).

Hooven et al., Lin et al., and Olm et al. all used stool microbiome data and demographic data to predict risk for NEC (50, 51, 56). In the publication by Hooven et al., following a dimensionality reduction approach through feature engineering, the stool microbiome and demographic data were used as input in a multi-layer neural network (MIL) model that had a high AUROC score (51). Importantly, the model Hooven et al. designed was able to predict NEC over 24 h before disease onset, but due to the complexity of the MIL model, it was difficult to interpret what features were required for the model to make decisions (51). As an extension of the Hooven et al. findings, Lin et al. used a similar hybrid approach with serial stool microbiome data, 10 clinical features, and the overall label of NEC vs. control (56). An unsupervised MIL model was used on each unlabeled stool sample within each patient's labeled set since it is unknown, which stool sample(s) within the set is/are NEC. The stool sample data was used to feed an ANN supervised ML model to predict NEC (56). The model had a high AUROC score and depended more on the microbiome data than it did on the clinical features (56). Interestingly, their model was able to predict NEC an average of 8.3 days before onset and using a RF model they found that certain taxa associated with NEC such as Firmicutes, Proteobacteria, and Enterobacteriaceae within the stool were important for NEC prediction (56). Olm et al. developed ML models using taxonomic data as well as other data that can be gleaned from microbiome data such as secondary metabolite profiles, metabolic pathways, and bacterial replication rates (50). Four feature categories from the original 2,119 features were considered important for prediction and their gradient boosted classifier (GBM) had the best performance in distinguishing NEC infants from controls (50). Casaburi et al. used machine learning to predict NEC vs. control from shotgun metagenomics data collected from several published studies (54). Their RF model had high accuracy and when testing the models under various conditions, it was found that specificity was high, but sensitivity varied greatly (54). Like Lin et al., it was found that NEC associated bacteria such as the Enterobacteriaceae species like *Klebsiella pneumoniae* and *Enterobacter cloacae* were important for the model decision making as well as *Staphylococcus aureus* (54).

Rather than stool microbiome data, Rusconi et al. used stool samples to generate metabolomic data to determine if there were usable biomarkers that could distinguish NEC from non-NEC infants (49). They found that sphingolipid profiles varied between NEC infants and non-NEC infants and used the respective profiles to develop a K nearest neighbors (KNN) model (49). After doing unsupervised ML hierarchical clustering, they determined that sphingolipids were only useful to distinguish a subset of patients, but after including the sphingolipid clustering profile with the other clinical features, much better ML accuracy scores were observed (49). Sylvester et al. used ML methods for biomarker discovery from urine peptides (46). First, unsupervised ML was used to cluster NEC infants with various potential biomarker profiles to distinguish surgical NEC infants from medical NEC infants (46). One cluster of peptides classified as fibrinogen A were most useful and when developing a linear discriminate analysis (LDA) model using both clinical parameters and urine peptide biomarkers, the model was able to correctly classify 100% of the infants as either surgical NEC or medical NEC, while the model using only clinical features was unable to classify 39% of

the patients (46). Song et al. designed an algorithm with the intent of determining features that would be important to distinguish NEC diagnosis from feeding intolerance (FI) and predicting whether infants with NEC will require surgery (60). In their model distinguishing NEC from FI, seven features from their original set of 119 were important for diagnosis and their model achieved a high AUROC score (60). With a similar AUROC score, the model predicting NEC prognosis also had high performance and weighted five of the features as being most important for prediction (60).

3.2. ML used to predict NEC or NEC outcomes

Similarly, many publications have used ML to predict NEC. Ji et al. used generalized linear mixed effects models (GLMMs), on a dataset of 27 clinical features presented by the patients at first suspicion of NEC and historically had been associated with NEC prediction to determine NEC severity (16). Nine of the 27 features were important for the GLMMs to score NEC severity: "abdominal pain, pneumatosis intestinalis, portal venous gas, dilated bowel, air/fluid levels, thickened bowel walls, white blood cell count (WBC), % neutrophils, and neutrophil count" (16). Those nine significant features were used to develop a GLMM (supervised ML) and tested to determine whether it could provide similar scores to the clinician classifications (16). The model classified 100% of stage 1 infants correctly, 94% of stage 2% and 83% of stage 3 (16). Using an LDA algorithm (a dimensionality reduction approach for supervised classification ML), Ji et al. predicted infants at low, intermediate, or high risk for NEC progression (16). In this model, outcome score was most influenced by metabolic acidosis (pH) and portal venous gas (PVG) (16). While the AUROC score was relatively high, the model was unable to predict 18.9% of medical NEC and 57% of surgical NEC subjects and incorrectly predicted 0.6% of medical NEC and 21.4% of surgical NEC infants (16). ML models often struggle when data is missing, which is often the case when considering clinical data/EMRs (33, 36, 37, 44). A further interesting finding from Ji et al., was that their NEC outcome score model still had an AUROC score of roughly 80% when considering as few as five of their 27 features (16). While this groundbreaking study developed two relevant ML models applied to NEC severity diagnosis and prognosis respectively, there were limitations to the models including difficulty in risk stratification particularly of intermediate patients and disagreement in NEC score from the clinician classification in scores $\geq$2 (16).

3.3. ML methods to distinguish NEC with or without IP from spontaneous intestinal perforation (SIP)

While the publication by Ji et al. eliminated all infants with SIP three more recent publications by Irles et al., Lure et al., and Son et al. developed ML models involving SIP and IP (48, 57, 59). Irles et al. used back propagated ANN models on two datasets with one using 23 neonatal and maternal variables collected at birth and the other using 35 variables collected at birth as well as during hospitalization (48). Both models were able to effectively classify the infants (48). They went on to determine which variables were most informative for the model and found several variables associated with predicting IP including neonatal platelet and neutrophil counts, orotracheal intubation, birth weight, sex, arterial blood gas parameters, gestational age, use of fortifier, patent ductus arteriosus (PDA), maternal age, and maternal morbidity (48). Like Irles et al., Lure et al. found gestational age at birth to be associated with NEC as well as post menstrual age (PMA) prior to surgery, and pneumatosis, but found that pneumoperitoneum was associated with SIP (57). Additionally, their ML scoring metric (AUROC) was high with ridge logistic regression and RF models when radiographic findings were included as part of the input variables (57). Finally, Son et al. utilized several different ML algorithms to distinguish NEC infants with or without IP from those with SIP but had the most luck with ANN models/multilayer perceptron (MLP) (59). The first model distinguished between NEC, NEC with IP, and SIP and had reasonably high AUROC scores (59). In the second model, the first layer distinguished between NEC and NEC with IP, while the second layer distinguished between NEC with IP and SIP by utilizing data from the NEC infants from the first layer (59). They also used the models on a new dataset of patients and found an AUROC score of 0.67–1.0 depending on which condition was being predicted, with the highest AUROC score of 1.0 associated with predicting NEC-IP and 0.9 for predicting SIP (59).

3.4. ML methods to evaluate treatment options

Others have used ML to determine what NEC infants may benefit from a treatment such as surgery. For example, Qi et al. utilized LR, SVM, and RF models on a subset of radiographic and clinical features to predict whether surgery would be necessary for infants diagnosed with NEC (58). The RF model had a reasonable AUROC score using a feature engineered subset of 18 radiomic features and 14 clinical features from the original dataset of 79 features (58). Similarly, Gao et al. designed two different models using both clinical data as well as radiomics data (52). Using DL, Gao et al. scaled the radiomics data to use in a light GBM supervised ML classifier (52). The first model predicted NEC depending on 18 clinical features and the radiomics data with a high AUROC score (52). The second model was designed to predict whether surgery would be necessary for infants diagnosed with NEC (52). The second model placed importance on 9 of the clinical features and had also had a high AUROC score (52).

3.5. ML to evaluate currently available NEC definitions

Finally, in a recent publication from our lab, ML has been applied to evaluate the currently available definitions for NEC

with the hope of developing a better definition (22). As mentioned earlier, there are now eight definitions for NEC including the original Bell and the modified Bell staging definitions and the more recent six definitions that have all been described within the last ten years (22). We found that the International Neonatal Consortium (INC) and 2 of 3 definitions had the best overall performance from the definitions and consistently outperformed the Bell and Modified Bell staging definitions (22). Additionally, we found nine features that were important for distinguishing NEC from non-NEC infants, but a model using only those nine features was not able to outperform previously described definitions (22).

4. Limitations and pitfalls for ML and AI

While ML and AI can be powerful tools, there are several pitfalls and limitations that must be taken into consideration when applying ML. First, as mentioned earlier, there is currently no universally accepted definition of NEC, and the Bell and Modified Bell staging definitions that are commonly used suffer from being non-specific to NEC until more severe stages of the disease have been reached. This means there can be discrepancies between what different institutions or even clinicians within an institution classify as NEC or the severity of NEC. Ultimately, this can lead to ML models being provided with subjective labels that may vary between institutions, which can make the model difficult to generalize to infants at other institutions. Along those lines, ML models can suffer from biases based on the input data, which can also make the models difficult to generalize (62). As an example, most studies discussed in this review were single center studies and some had as few as <100 patients. ML models often require 100 s–1,000 s of patients to be sufficiently trained and then additional patients to test/validate the model. Studies using few patients and only from a single center suffer from relatively homogenous populations. ML models trained on small and/or homogenous populations will have more difficulty properly classifying when heterogenous samples are added (62).

An added limitation is differences in EMRs that are often used as input for the clinical/demographic features. EMRs house a plethora of information, but there can be gaps in the data, subjective data, and differences in standard practices between institutions, which may limit its utility for ML purposes, or the generalizability of ML models developed (63). As discussed earlier, ML models struggle to cope with missing data. Thus, scientists developing ML models must make a choice between excluding patients, excluding certain features, imputing the data to fill the gaps, manually deciding for each gap the best way to fill in the feature, or some combination of these. Any decision made can have the potential of skewing the ML model. Other data processing may also be necessary to optimize a ML model's ability to appropriately classify such as scaling or normalizing certain features, which may impact generalizability when adding in different patients (63). Also, data points that are subjective and can vary between clinicians are challenging for a ML model to manage and can result in inaccurate predictions (63). Examples of features that may be subjective in nature are abdominal distension, lethargy, or radiologic findings as well as features that can have cutoffs that may vary between institutions such neutropenia, thrombocytopenia, or acidosis. Differences in standard practices between institutions can also skew the availability of EMR data points (63). For example, performing certain tests at birth may be standard at one hospital, but not at another, or the frequency at which certain tests are performed may vary between institutions which leads to gaps in the data available.

Finally, interpretability of developed ML models can be challenging (45, 64–66). One challenge in interpretability occurs when using feature engineering as it combines multiple different features into one. Hooven et al. used this approach to help scale down the metagenomics data, but they commented that although they knew the model depended on the metagenomics data since removing it resulted in lower performance metrics, it was hard to determine exactly what features within that dataset were important (51). Another challenge in interpretability that arises is when combining EMRs with omics data such as in Hooven et al., Lin et al., and Rusconi et al. (46, 54, 56). Omics datasets have massive numbers of features and require more complicated models to appropriately handle the data (30, 45). To understand more about complex models' decision-making process, separate ML models can be developed like Lin et al. who used a RF model to determine what taxonomic features from the microbiome data were important for the MIL model (54). Others used unsupervised ML through hierarchical clustering to narrow down the features that were used in the final model such as in Sylvester et al. and Rusconi et al. (46, 60). Requiring a secondary model to understand the model being developed adds another layer of complexity to the ML process and can make interpretability difficult for the eventual end users, the clinicians, who have varying levels of understanding of ML (65, 66).

5. Conclusions

While ML and AI have been utilized in the healthcare realm for decades with over 11,000 publications relating to cancer since 1985 and over 500 publications relating to sepsis since 1990, publications applying ML and AI to NEC have been far sparser. Nevertheless, the publications that have applied ML to NEC have covered a breadth of topics such as biomarker discovery, predicting NEC before onset, distinguishing NEC from other conditions, determining prognosis, or evaluating the current definitions of NEC. These studies have all provided promising data to aid in improving diagnosis and/or prognosis of infants with NEC, but there is plenty more that can be done in the future. As mentioned, many of the studies to date have been single center, used small patient sizes, and/or been rife with limitations. ML and AI models are only as good as the input they are provided (33). This reinforces the necessity to foster collaborations between researchers, clinicians, data scientists, biostatisticians, and bio-informaticists to provide future studies with clean, more widely generalizable datasets and overcome the many pitfalls and limitations that come with ML and AI. NEC as a disease has

historically been difficult to diagnose and treat, but, if used effectively, ML and AI offer the potential to more quickly identify and diagnose NEC, help to predict the severity of the case, help optimize treatment strategies, and in summation provide an overall better prognosis for infants with NEC.

Author contributions

SRL and SJM: contributed to the conception, drafting, and critical revisions of this manuscript. SRL and SJM: approve this manuscript for publication. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Identification of serum biomarkers for necrotizing enterocolitis using aptamer-based proteomics

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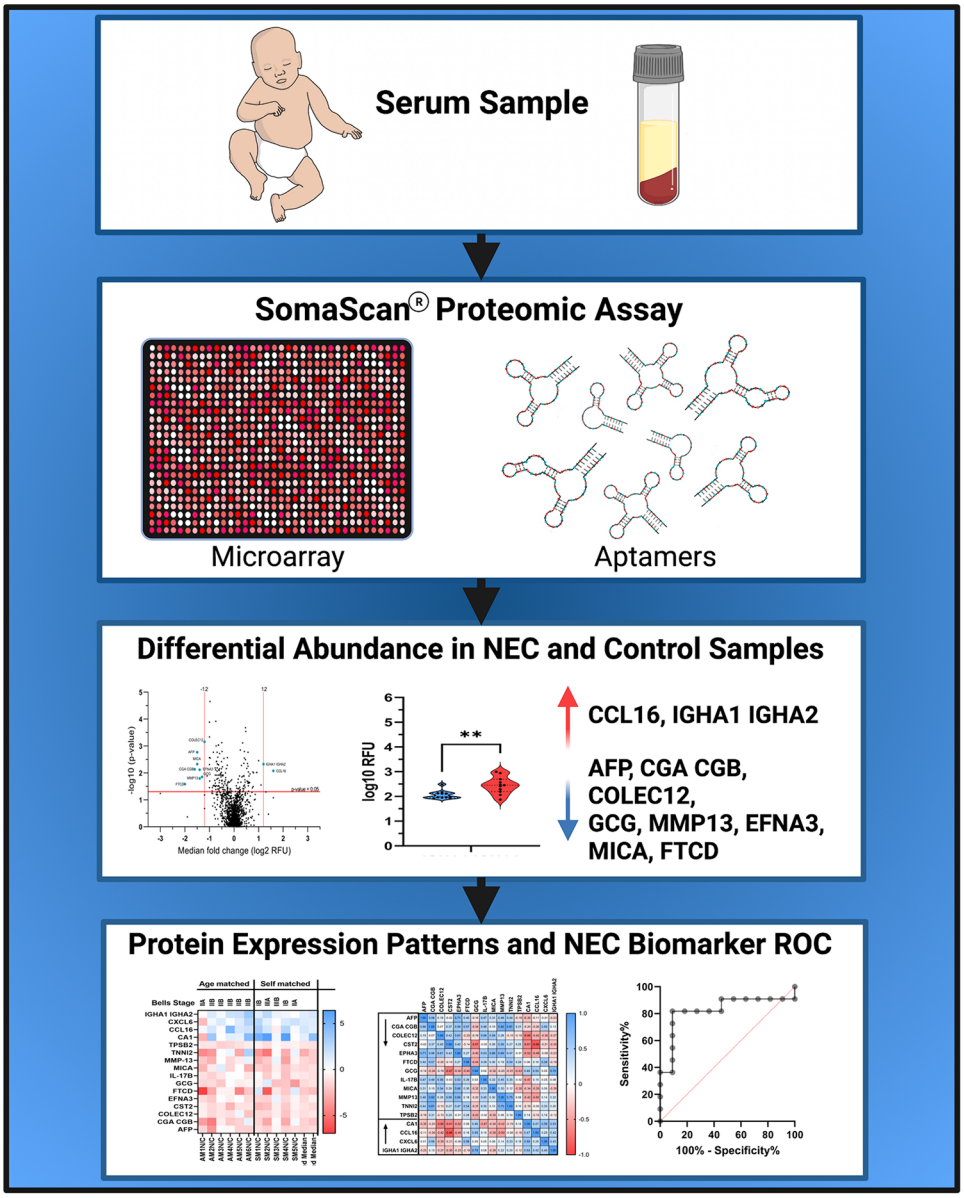
Introduction: Necrotizing enterocolitis (NEC) is a potentially fatal intestinal disease primarily affecting preterm infants. Early diagnosis of neonates with NEC is crucial to improving outcomes; however, traditional diagnostic tools remain inadequate. Biomarkers represent an opportunity to improve the speed and accuracy of diagnosis, but they are not routinely used in clinical practice.
Methods: In this study, we utilized an aptamer-based proteomic discovery assay to identify new serum biomarkers of NEC. We compared levels of serum proteins in neonates with and without NEC and identified ten differentially expressed serum proteins between these groups.
Results: We detected two proteins, C-C motif chemokine ligand 16 (CCL16) and immunoglobulin heavy constant alpha 1 and 2 heterodimer (IGHA1 IGHA2), that were significantly increased during NEC and eight that were significantly decreased. Generation of receiver operating characteristic (ROC) curves revealed that alpha-fetoprotein (AUC = 0.926), glucagon (AUC = 0.860), and IGHA1 IGHA2 (AUC = 0.826) were the proteins that best differentiated patients with and without NEC.
Discussion: These findings indicate that further investigation into these serum proteins as a biomarker for NEC is warranted. In the future, laboratory tests incorporating these differentially expressed proteins may improve the ability of clinicians to diagnose infants with NEC rapidly and accurately.

KEYWORDS

necrotizing enterocolitis, prematurity, aptamer, SomaScan, serum, biomarker



GRAPHICAL ABSTRACT
Overview of the study. Figure made with Biorender.com.

Introduction

Premature and low birthweight infants are at risk for necrotizing enterocolitis (NEC), a severe inflammatory intestinal disease. The incidence of NEC is as high as 7% in preterm infants born at <32 weeks and 5%–22% in extremely low birth weight (ELBW, <1,000 g) infants (1). The symptoms of NEC are often nonspecific and subtle; however, neonates who develop NEC can rapidly worsen and progress to requiring emergency surgery or death within hours of diagnosis. Thus, accurately diagnosing NEC early in the disease course is crucial for initiating potentially life-saving clinical interventions (2). Unfortunately, diagnostic tools and treatment options for NEC have not improved despite decades of intensive research (3).

During NEC, intestinal barrier dysfunction resulting from epithelial injury and inadequate repair mechanisms can lead to bacterial translocation across the gut barrier, systemic inflammation, and potentially sepsis (4–8). Due to this systemic inflammatory response, symptoms of NEC can be nonspecific and difficult to distinguish from other disease processes. Identification of biomarkers for NEC that are both sensitive and specific would be a significant advance in clinical care and facilitate early diagnosis and treatment of neonates with NEC. Serum biomarkers for NEC are a potentially powerful tool that could rapidly differentiate infants with or without disease, but there are currently no effective predictive biomarkers routinely used in clinical practice.

Biomarkers for NEC have previously been investigated using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (9) and enzyme-linked immunosorbent assay (ELISA) (10). This study uses an aptamer-based screening method to determine the relative expression of >1,300 protein targets (SomaScan®). This technology has been used to identify biomarkers in adult and pediatric diseases, including Duchenne's muscular dystrophy, ulcerative colitis, coronary heart disease, and cancer (11–21). Using this assay, we detected ten differentially expressed proteins in the serum of patients with and without NEC. This includes two that were upregulated and eight that were downregulated during NEC. ROC curves indicated that these proteins could effectively discriminate between patients with disease compared to those without. Future studies will focus on validating the efficacy of these potential NEC biomarkers in a larger patient population.

Materials and methods

Study design

In this prospective study, infants admitted to the St. Louis Children's Hospital Neonatal Intensive Care Unit (NICU) in St. Louis, Missouri, USA, were enrolled according to protocols approved by the Washington University in St. Louis School of Medicine Institutional Review Board (IRB protocol numbers 201706182 and 201802101). Infants were eligible for enrollment if they were born between 22 and 42 weeks gestation and either developed NEC or were age-matched controls who did not develop NEC. Infants with any major congenital anomalies were excluded. Clinical information from the infant's medical record was collected from admission until discharge. For the present study, the cohort consisted of infants ($n = 18$) born between 24 and 36 weeks gestation diagnosed with NEC ($n = 12$) and age-matched controls ($n = 6$). Clinical information, demographic information, and NEC severity are summarized in **Table 1**. Modified Bell's Staging for NEC (7, 22, 23) was used to determine NEC severity.

Sample collection

Serum samples were collected once at enrollment for all participants ($n = 18$). A second serum sample was collected at the time of diagnosis if the infant developed NEC after enrollment ($n = 6$ self-matched infants). Age-matching was performed based on the post-menstrual age (PMA) of infants at the time of NEC diagnosis. There were six age-matched pairs included in this study ($n = 12$ infants). After collection, serum samples were centrifuged at 3,000 r.p.m. for 10 min, then subsequently aliquoted and stored at −80°C until analysis.

Proteomics assay

An aptamer-based SomaScan® (24) 1,300 serum protein microarray kit was used by the Genome Technology Access Center at the McDonnell Genome Institute at Washington University School of Medicine to identify biomarkers for each of the serum samples and respective controls according to the manufacturer's guidelines (SOMAlogic®, Boulder, CO, USA). Aptamers are 40 base pair oligonucleotides consisting of natural and modified nucleotides. These aptamers, called SOMAmers®, were immobilized on streptavidin beads. Proteins from serum were tagged with biotin, captured as a SOMAmer® reagent/protein pair, cleaved, denatured, and eluted prior to hybridization on a customized Agilent SureScan DNA microarray. We utilized a resolution of 5 µm and detected Cy3 fluorescence expressed as relative fluorescence units (RFU). Off-scanner raw signal values were calibrated, standardized, scaled at 40%, 1%, and 0.005%, and normalized. The RFU readout intensities are directly proportional to the amount of target protein, performed using Agilent Feature Extraction v10.7.3.1. Differential abundance was calculated using the SomaScan® statistical analysis tool v4.1 (SOMAlogic®) and subjected to a linear model fitting of the signal data and an empirical Bayesian statistical test for group comparisons. Samples were screened by row check intensity scaling and target biomarkers by column check quality control intensity scaling, where aberrant intensities are flagged for exclusion during data analysis. One self-matched sample pair (SM6N and SM6C) was excluded from further analysis due to aberrant scaling in the quality control row check. The raw data file is available in **Supplementary Table S1**.

Statistical analysis

RFU data was normalized by log transformation. Log2 transformations were used to generate volcano plots and heat maps for median and individual sample and target comparisons. A median fold change cut-off value of ≥1.2 and a *P*-value cut-off of ≤0.05 were used for differentially abundant biomarker selection based on the proteomics data using unmatched and matched sampling. Log2 median fold change transformations of significant proteins were used to create Pearson's correlation matrices and calculate the area under the curve (AUC) for Receiver Operating Characteristics (ROC). Confidence intervals of 95% were calculated by Wilson/Brown method. Log10 transformations, of case and control samples, were used to generate violin plots using the Tukey method and a paired parametric one tailed *t*-test. A *z*-score heat map was used to test for variation in case and control samples for each of the differentially abundant targets. All figures and statistics were generated using GraphPad Prism 9.3.1. Gene classification was standardized using the DAVID functional annotation tool (25).

Results

The clinical characteristics of the infants in this study ($n = 18$) are summarized in **Table 1**. Patients ($n = 6$) enrolled at the time of NEC diagnosis were paired with age-matched ($n = 6$) controls based on post-menstrual age. In addition, patients that were enrolled in our prospective study as controls and subsequently developed NEC ($n = 6$) were grouped in a self-matched cohort.

TABLE 1 Description of patient cohort.

Infant data		Pregnancy and delivery details						Disease severity				
Sample ID	NEC (N) Control (C)	Gestational age	Sex	Birth weight (g)	Race (maternal)	Delivery route	Enrollment	NEC	Highest Bell's stage	Surgical NEC	Radiographic Findings	Final disposition
Age-matched												
AM1N	N	34 0/7	Female	2360	White	CS	Case	Yes	IIA	No	PN	Discharged
AM1C	C	34 6/7	Female	1740	Black	CS	Control	No				Discharged
AM2N	N	26 0/7	Male	480	White	CS	Case	Yes	IIB	No	PN	Death
AM2C	C	33 4/7	Female	1940	Unknown	V	Control	No				Discharged
AM3N	N	36 2/7	Female	2330	White	CS	Case	Yes	IIB	No	PN, PVG	Discharged
AM3C	C	36 3/7	Male	3161	White	V	Control	No				Discharged
AM4N	N	24 5/7	Male	640	White	CS	Case	Yes	IIB	No	PN	Discharged
AM4C	C	26 3/7	Female	710	Black	CS	Control	No				Discharged
AM5N	N	31 0/7	Female	1190	White	CS	Case	Yes	IIB	No	PN, PVG	Discharged
AM5C	C	34 0/7	Male	2030	White	V	Control	No				Discharged
AM6N	N	24 2/7	Male	700	White	CS	Case	Yes	IIIB	PD, PR	#	Death
AM6C	C	27 0/7	Male	1150	White	V	Control	No				Discharged
Self-matched												
SM1N/C	C+N	26 0/7	Female	830	Black	CS	Control/Case	Yes	IB	No	N/A	Discharged
SM2N/C	C+N	28 6/7	Male	1220	Black	CS	Control/Case	Yes	IIIA	No	PN, PVG	Discharged
SM3N/C	C+N	25 4/7	Female	760	White	V	Control/Case	Yes	IIIB	PD	PN, PVG, P	Death
SM4N/C	C+N	25 1/7	Male	1620	Black	CS	Control/Case	Yes	IB	No	N/A	Discharged
SM5N/C	C+N	25 0/7	Male	760	Black	CS	Control/Case	Yes	IIA	No	PN	Discharged
SM6N/C*	C+N	24 4/7	Female	630	White	CS	Control/Case	Yes	IIIA	PD	PN, PVG	Death

SM6N/C* failed the assay quality control checks and was not included in the study analysis.

*N = NEC, C = Control, CS = cesarean section, V = Vaginal delivery, PR = Laparotomy, partial resection, PD = Peritoneal drain, PN = Pneumatosis, PVG = Portal Venous Gas, P = Pneumoperitoneum, # = gasless abdomen, bowel perforation, multiple surgeries for NEC. N/A = Not applicable

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For the self-matched cohort, we analyzed protein levels in samples obtained at the time of enrollment and upon a diagnosis of NEC. One self-matched pair, patient SM6N/C, was excluded from further analysis due to failed quality control measures, as delineated in the methods section. Thus, 17 infants in total, including 12 in the age-matched and 5 in the self-matched cohort, were included in further analysis.

In this study, we measured the relative abundance of over 1,300 serum proteins using an aptamer-based proteomic assay. Of the over 1,300 analyzed proteins, ten proteins (two increased and eight decreased in relative abundance) were significantly different between patients with NEC and controls (Figure 1). Proteins of interest were selected based on meeting the criteria of a median fold-change of ±1.2 and *P*-values <0.05 for the log transformed RFU data. Serum proteins that were increased during NEC included C-C motif chemokine ligand 16 (CCL16) and immunoglobulin heavy constant alpha 1 and 2 heterodimer (IGHA1 IGHA2). Proteins that were decreased during NEC included collectin subfamily member 12 (COLEC12), glucagon (GCG), alpha fetoprotein (AFP), formimidoyltransferase cyclodeaminase (FTCD), matrix metallopeptidase 13 (MMP13), glycoprotein hormone alpha polypeptide heterodimer (CGA CGB), MHC class I polypeptide-related sequence A (MICA), and Ephrin A3 (EFNA3). Differentially abundant protein biomarkers are summarized in Table 2.

The relative abundance of the proteins of interest, as determined by Log10 transformations of the RFU values, was compared in serum samples from patients with NEC and controls. We found that all 10 proteins identified in Figure 1 were significantly different between these groups ($P < 0.05$, Figure 2). CXCL6 was not considered statistically significant ($P = 0.051$) but was included due to its potential clinical significance as an inflammatory protein. We next generated a heat map to provide a visual representation of the relative abundance of the proteins of interest across self- and age-matched pairs (Figure 3A). There was a remarkable degree of consistency in the patterns of protein expression across patient pairings. Using a heat map (Figure 3B), we observed that protein expression was similar across control samples and that the greatest variation in the matched pairs was present between patients with NEC.

To determine if there was a statistical correlation between different protein levels, parametric two-tailed Pearson's correlation matrixes were generated. We found that the proteins increased in samples from patients with NEC shared positive correlations with each other and an inverse correlation with

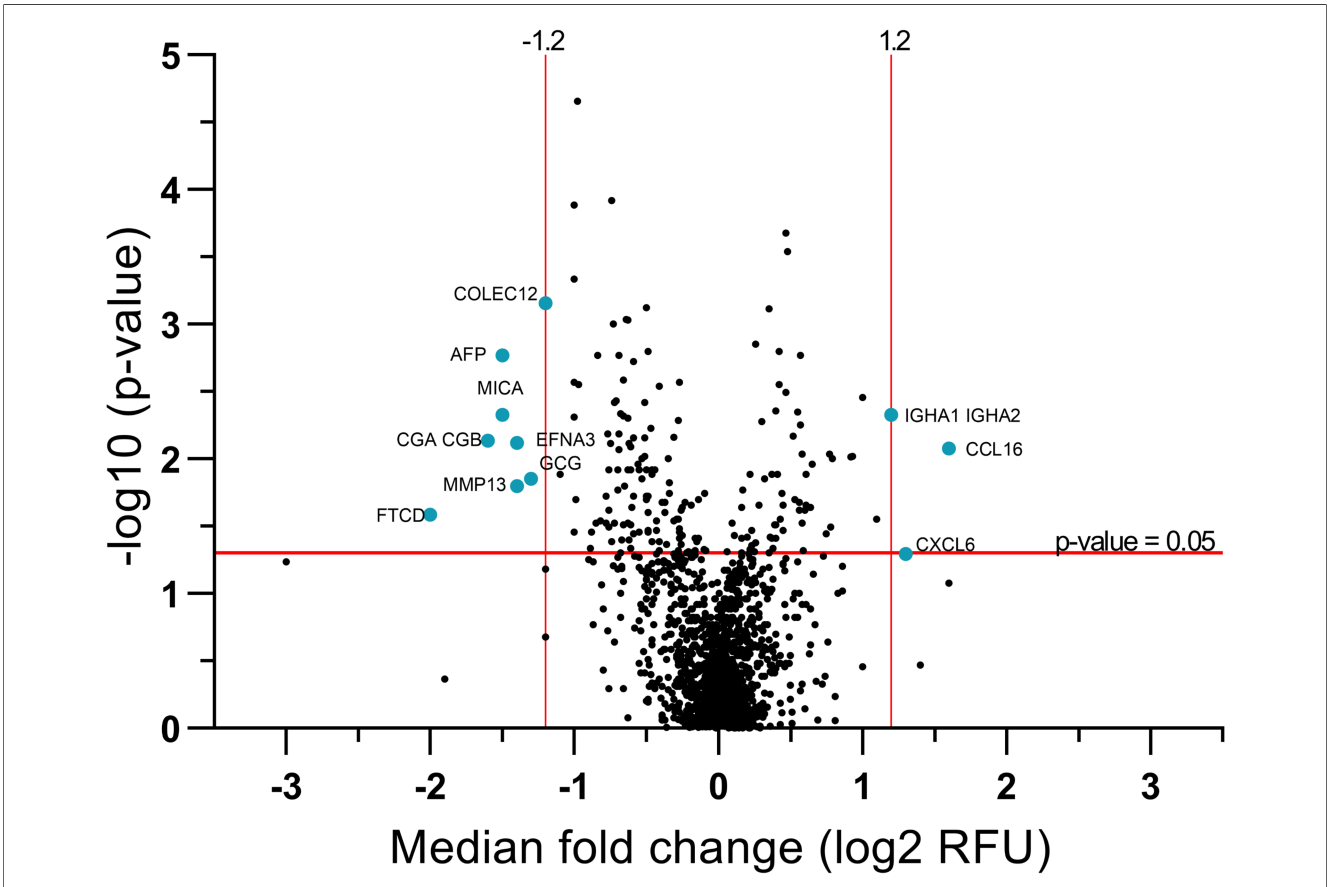

FIGURE 1
Volcano plot of the relative levels of serum proteins in patients with and without NEC identifies 10 differentially expressed proteins. Differential abundant proteins were selected and presented on a volcano plot based on median fold change (log2 RFU) and *P*-value (–log10 *t*-test). Statistically significant proteins of interest (blue dots) were selected based on median fold-change cut-off values (log2 ± 1.2) and $P < 0.05$ indicated as red lines. All selected proteins were statistically significant except CXCL6 ($P = 0.051$), which was considered potentially clinically significant.

TABLE 2 Details of differentially expressed proteins in the serum of neonates with NEC relative to controls.

Protein		Function	Tissue origin	Role in intestinal development, inflammation, and NEC	References
Increased in NEC vs. controls					
C-C motif chemokine 16	CCL16	Chemokine	Neonatal liver, macrophages, and lymphocytes	• Chemotactic toward monocytes and lymphocytes. • Induced expression by IL-10, LPS, and IFN-γ in activated monocytes and lymphocytes. • Ligand for CCR1, CCR2, CCR 5, and CCR8 cell surface and H4 eosinophil and mast cell receptors.	(26) (27) (28)
Immunoglobulin A	IGHA1 IGHA2	Mucosal antibody	Maternal milk in neonates	• IGHA1 IGHA2 is the mucosal specific heterodimer. • Decreased binding of IgA has been shown to correlate with intestinal dysbiosis.	(29) (30–32)
C-X-C motif chemokine 6	CXCL6	Chemokine	Macrophages	• IL17A induced chemokine for neutrophils. • Signal through CXCR5 and CXCR7 receptors.	(33) (34)
Decreased in NEC vs. controls					
Collectin-12	COLEC12	Scavenger receptor	Placenta, small intestine, and colon	• Involved in host defense promoting recognition and phagocytosis of Gram positive and negative bacteria, and yeast.	(35)
Pro-glucagon	GCG	Intestinal barrier development	Enteroendocrine cells	• Pro-glucagon cleaved into glucagon-like peptide-2 (GLP-2). • GLP-2 decreases enterocyte apoptosis, stimulates intestinal growth, crypt cell proliferation and villus formation. • GLP-2 promotes inflammatory cytokine production, delays NEC onset, and decreases mucosal barrier disruption.	(36) (37)
Ephrin-A3	EFNA3	Epithelial development	Small intestine and peripheral leukocytes	• GPI-anchored ligand of Eph receptors involved in signaling during migration and adhesion of epithelial development.	(38)
Collagenase 3	MMP13	Intestinal barrier function	Chondrocytes, connective and soft tissues	• Metalloprotease involved in the regulation of the intestinal barrier during inflammation by TNF signaling. • Reduced MMP-13 expression is a protective response to LPS induced inflammation. Involved in wound healing.	(39)
MHC class I polypeptide-related sequence A	MICA	Intestinal stress signaling	Gastric epithelium, endothelium, and monocytes	• MICA is specifically expressed in enterocytes as a stress induced-antigen recognized by intestinal epithelial $\gamma\Delta$ T-cells. • Over expression of MICA is associated with dysregulation of mucosal homeostasis.	(40)
Alpha-Fetoprotein	AFP	Plasma transport protein	Fetal liver	• Neonatal functional analog of serum albumin.	(41)
Human chorionic gonadotropin	CGA CGB	Developmental hormone	Placenta	• Heterodimer hormone. • Low CGA CGB expression is associated with poor development and low birth weight.	(42)
Formimidoyltransferase-cyclodeaminase	FTCD	Histidine metabolism	Fetal liver	• Functions as a transferase and a deaminase converting histidine to folate through the histidine degradation pathway. • Low histidine metabolism has been associated with NEC.	(43)

proteins of decreased abundance in patients with NEC (**Figure 4A**). All samples correlated positively with the median (**Figure 4B**) except sample pairs AM1N/C-SM5N/C, SM5N/C-SM2N/C, and SM1N/C-SM3N/C. These sample pairs had significant variations for the following specific proteins (**Figure 3A**); AM1N/C-SM5N/C for FTCD and CXCL6; SN5N/C-SM2N/C for FTCD and GCG, SM1N/C-SM3/NC for FTCD and CCL16.

Individual biomarker sensitivity vs. specificity for identifying patients with NEC was measured by Receiver Operator

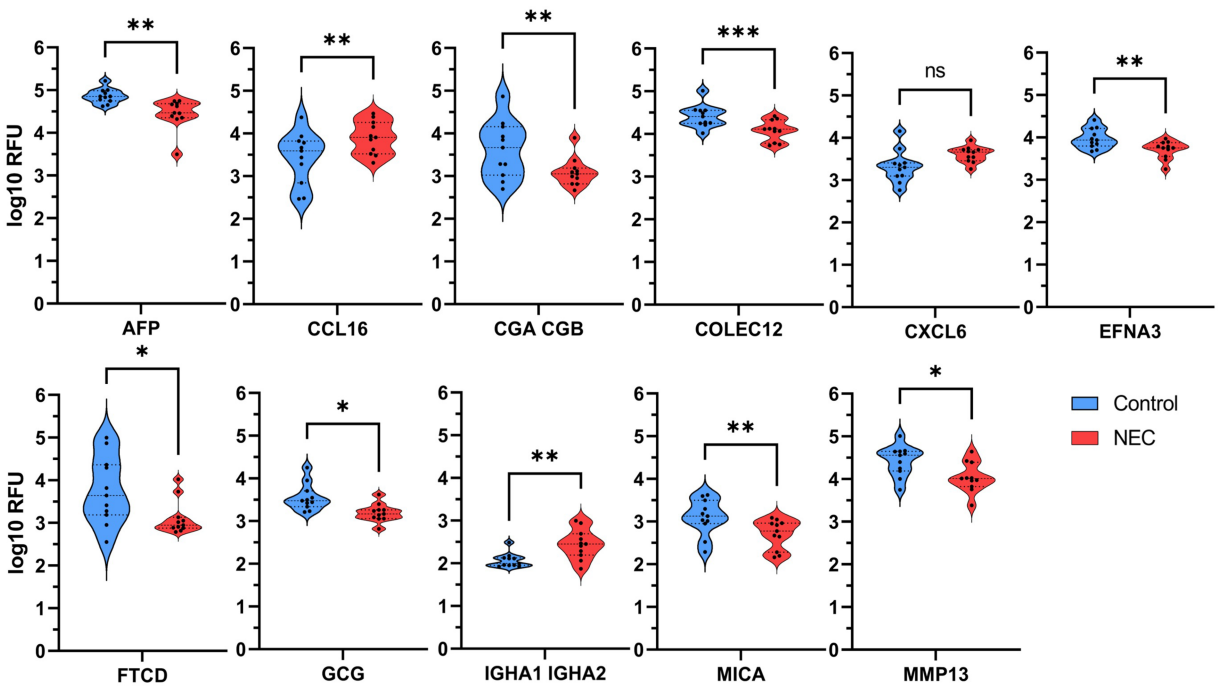

FIGURE 2
Significant differences in individual serum proteins were detected between patients with NEC and controls. Differentially abundant proteins were calculated for median distribution by log10 RFU. The median distribution is represented by the central dotted lines, and outer dotted lines indicate first and third quartiles. All selected proteins were statistically significant except CXCL6 ($P = 0.051$), which was considered potentially clinically significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$ via a paired parametric two-tailed t-test. ns, not significant.

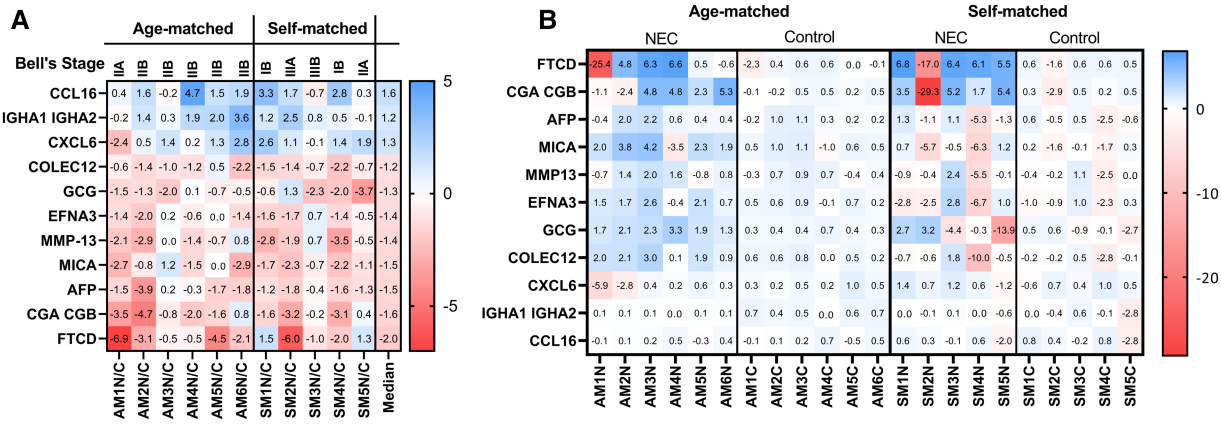

FIGURE 3
Similar relative abundance of serum proteins across the cohort when comparing matched NEC/control patient pairs. Heat maps of the differentially abundant proteins (**A**) in individual age-matched and self-matched pairs and (**B**) by z-score distribution of individual samples ranging between +6.78 to −29.3 where the sum of the z-score across protein targets = 1.

Characteristic (ROC) curves and area under the curve (AUC) (**Figure 5**). ROC curves generated from combined self- and age-matched pairs identify values for given biomarkers where true positive (sensitivity) and percentage of true negative (100% specificity) are the most effective. An AUC value approaching 1 is a perfect diagnostic test. AUC values above 0.7 are considered acceptable, while AUC values above 0.8 are considered good for diagnostic tests. The AUC for proteins increased in the serum of patients with NEC relative to controls were as follows: CCL16 (AUC = 0.744, 95% CI = 0.535–0.953), CXCL6 (AUC = 0.802, 95% CI = 0.587–0.966) and IGHA1 IGHA2 (AUC = 0.826, 95% CI = 0.630–1.00). The AUC for proteins decreased in the serum of patients with NEC relative to controls were: AFP (AUC = 0.926, 95% CI = 0.813–1.00), MMP13 (AUC = 0.777, 95%

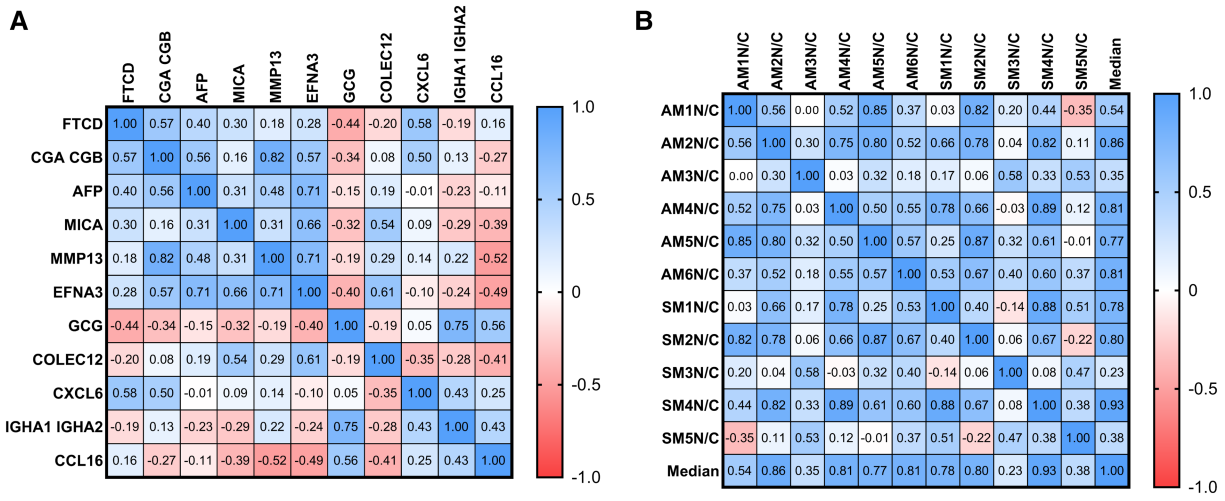

FIGURE 4
Pair-matched samples showed consistent correlations among sample pairs and individual protein biomarkers. Pearson's correlation matrixes between (A) serum proteins and (B) individual patient samples were calculated using median fold change (log2) in a parametric two-tailed test.

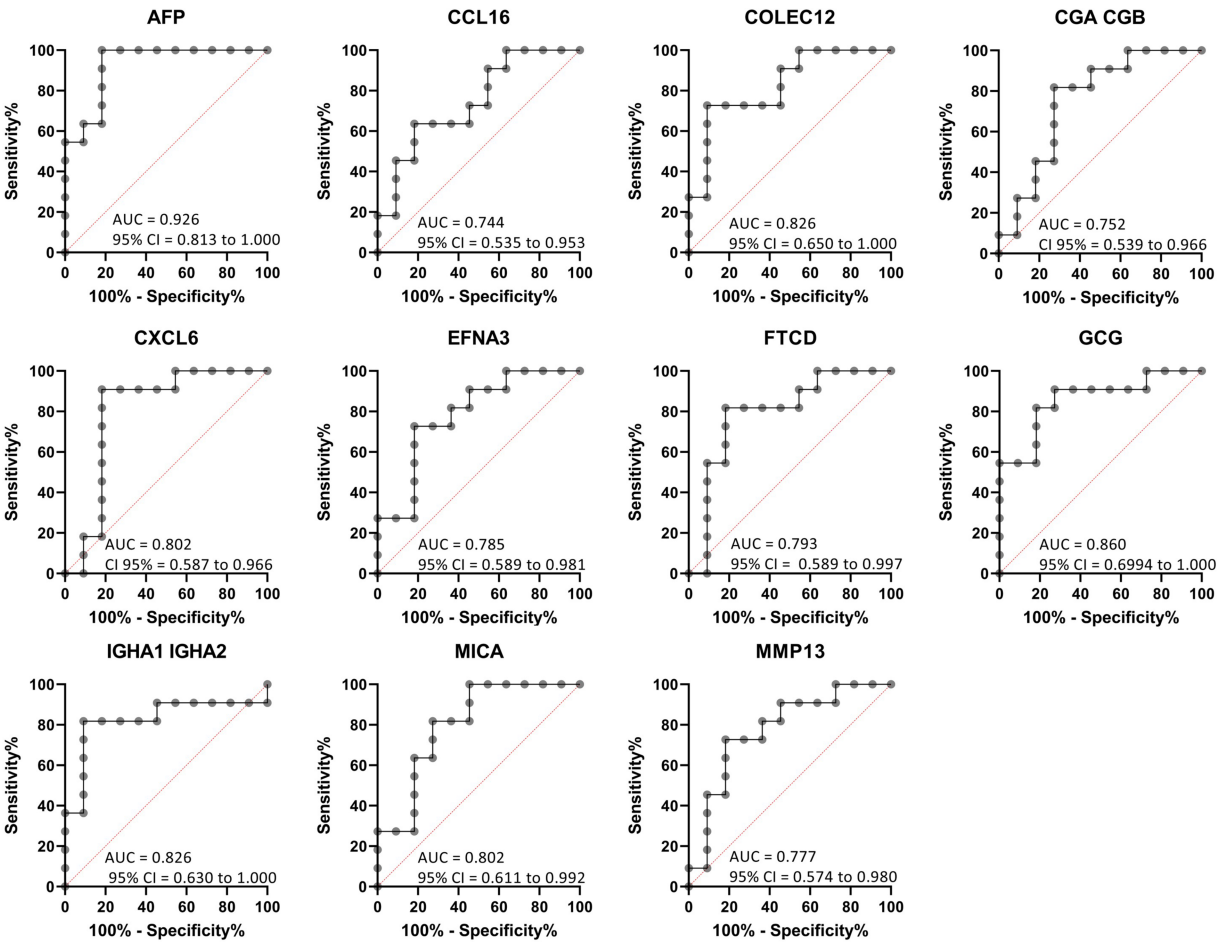

FIGURE 5
Receiver operating curves (ROC) of target proteins indicate that the differentially expressed serum proteins effectively discriminate between patients with and without NEC. Differentially abundant proteins ($n = 10$) were screened for sensitivity vs. specificity based on median log10 transformed RFU data for matched NEC and control samples. ROC curves were calculated using the Wilson/Brown method with a confidence interval (CI) of 95%. AUC values >0.7 are considered valid diagnostic biomarkers.

CI = 0.574–0.980), FTCD (AUC = 0.793, 95% CI = 0.589–0.997), MICA (AUC = 0.802, 95% CI = 0.611–0.992), EFNA3 (AUC = 0.785, 95% CI = 0.589–0.981), GCG (AUC = 0.860, 95% CI = 0.6994–1.00), COLEC12 (AUC = 0.826, 95% CI = 0.650–1.00), and CGA CGB (AUC = 0.752, 95% CI = 0.539–0.966). These values demonstrate high sensitivity vs. specificity for all biomarkers of interest.

Discussion

NEC is a rapidly progressive disease that can be difficult to diagnose using currently available tools. Identification of highly sensitive and specific biomarkers would allow for earlier initiation of potentially lifesaving treatments for neonates with NEC. In this study, we utilized an aptamer-based approach to identify serum proteins that were differentially abundant in samples from infants with NEC relative to controls. Serum proteins that were significantly different between the groups are described in **Table 2**.

Two proteins were upregulated in the serum of patients with NEC compared to controls, CCL16 and IGHA1 IGHA2. CCL16 is a chemokine produced primarily in the liver and secreted into the blood (27). Its production is induced in monocytes by the cytokines interleukin (IL)-10 and interferon-gamma (IFN-γ) as well as by lipopolysaccharide (LPS) expressed by Gram-negative bacteria (27, 28). CCL16 has been shown to induce lymphocyte and monocyte chemotaxis (26). Increased levels of CCL16 in the serum of neonates with NEC may reflect the inflammatory milieu of the intestine, which would support its use as a biomarker of NEC.

Serum IgA is monomeric (~90% IGHA1, 10% IGHA2), whereas IgA derived from maternal milk and present in the intestinal mucosa in the form of secretory IgA (s-IgA), is typically a heterodimer of IGHA1 IGHA2 (44). In infants, IgA is derived solely from maternal milk for the first four weeks of life, until B-lymphocytes populate the intestine (30). The increased abundance of IGHA1 IGHA2 in the serum from neonates with NEC may be reflective of the gut barrier dysfunction observed during NEC (45), which would result in increased circulating levels of this primarily intestinal antibody. The level of IgA bound to the Gram-negative *Enterobacteriaceae* in the stool of preterm neonates is inversely correlated with the risk of NEC (30); however, how serum levels of IGHA1 IGHA2 correlate with NEC has not been previously explored.

We also detected eight proteins that were decreased in the serum of patients with NEC relative to controls. The two proteins that were the most effective at discriminating between patients with and without NEC included AFP (AUC = 0.926) and GCG (AUC = 0.860). AFP is elevated in preterm infants (<37 weeks) and normally decreases rapidly after birth (by 50% in the first 5 days of life in term infants) (41). AFP has been associated with the downregulation of inflammation (46); thus, decreased levels may contribute to the exaggerated inflammatory response observed in neonates with NEC.

GCG regulates blood glucose levels by promoting gluconeogenesis and glycogenolysis. Pro-glucagon is cleaved into several peptides involved in glucose metabolism and gastric function. Importantly, one of the peptide products, glucagon-like peptide 2 (GLP-2), decreases enterocyte apoptosis and stimulates intestinal growth, crypt cell proliferation, and villus formation (36). GLP-2 was also shown to have a protective and anti-inflammatory role in a rat model of NEC (37). It is possible that reduced levels of GCG found in the serum of patients with NEC may indicate that decreased GCG-mediated intestinal protection was associated with increased intestinal injury.

To our knowledge, this is the first study to analyze serum proteins using an aptamer-based assay on samples derived from infants with or without NEC. The traditional technique for differential analysis and quantitative proteomics is liquid chromatography coupled with mass spectrometry (LC-MS/MS). However, improvements in affinity capture and quantitation methods have allowed for alternative methods, which can address biases and limitations in LC-MS/MS and other platforms. A comparative analysis of LC-MS/MS, RNA sequencing, and SomaScan® analysis of mesenchymal and human embryonic stem cells showed a greater identification of unique markers using SomaScan® than LC-MS/MS and RNA sequencing. The benefits of this aptamer-based technology include a high dynamic range, low sample requirements (20 µg protein to 50 µl serum), and high sensitivity with improved detection of small molecule targets (47). This improvement in technology facilitated our detection of new potential serum biomarkers for NEC.

Limitations of this study included the relatively small sample size, the inability to match patients based on factors other than age, and the inclusion of two patients with Stage 1B NEC. This is a pilot study that will be expanded upon in future studies involving larger patient cohorts, which will allow for more detailed matching of patient characteristics and stratification of patients based on disease severity.

Studies analyzing serum biomarkers in preterm infants are complicated by several factors, including limited sample volumes, inflammatory proteins not specific for NEC, and age-specific changes in protein levels (48). We attempted to overcome these challenges by using an assay with high sensitivity, which allowed the detection of protein levels with a small volume of blood. In addition, we utilized age matching to limit confounding in our comparison of serum protein levels. We also found similar patterns of protein abundance in the age-matched and self-matched cohorts, which pointed to differences in protein levels being related to NEC and not post-menstrual age in the self-matched group. Finally, this study employed an unbiased screening approach, which provided the highest likelihood of identifying new biomarkers for NEC.

In conclusion, serum protein levels from infants with NEC were compared to controls using an aptamer-based proteomic assay with the successful identification of 10 proteins that were able to differentiate between the groups. Future studies will focus on the validation of these results in a larger patient cohort. The overarching goal is to improve the speed and accuracy in which

clinicians can diagnose NEC to improve outcomes for critically ill neonates.

Data availability statement

The original data presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Washington University in St. Louis School of Medicine Institutional Review Board (IRB protocol numbers 201706182 and 201802101). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

SM, LF, GB, and MG: contributed to the writing—original draft preparation, review, and editing all versions of the manuscript. SM and DS: analyzed data. GB, QG, OD, and MG: consented patients and/or obtained samples. CM, LF, and MG: created/edited the graphical abstract. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2023.1184940/full#supplementary-material

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The role of human milk nutrients in preventing necrotizing enterocolitis

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Necrotizing enterocolitis (NEC) is an intestinal disease that primarily impacts preterm infants. The pathophysiology of NEC involves a complex interplay of factors that result in a deleterious immune response, injury to the intestinal mucosa, and in its most severe form, irreversible intestinal necrosis. Treatments for NEC remain limited, but one of the most effective preventative strategies for NEC is the provision of breast milk feeds. In this review, we discuss mechanisms by which bioactive nutrients in breast milk impact neonatal intestinal physiology and the development of NEC. We also review experimental models of NEC that have been used to study the role of breast milk components in disease pathophysiology. These models are necessary to accelerate mechanistic research and improve outcomes for neonates with NEC.

KEYWORDS
breast milk, neonates, prematurity, necrotizing enterocolitis (NEC), intestine, nutrients

Introduction

Necrotizing enterocolitis (NEC) is a severe gastrointestinal disease that impacts 2%–7% of preterm infants (1). Risk factors for NEC include prematurity, low birth weight, delivery via cesarean section, lack of breast milk feeds, microbial dysbiosis, inadequate intestinal perfusion, and exposure to medications such as antibiotics and acid blockers (2). Disease pathogenesis is characterized by unrestrained inflammation, injury to the intestinal epithelium, and bowel ischemia, which can rapidly progress to bowel necrosis, sepsis, and death (3). Treatment options for NEC include the discontinuation of enteral nutrition, gastric decompression, broad-spectrum antibiotics, and surgical removal of necrotic bowel (3). There are no targeted therapies available due to our incomplete understanding of disease pathogenesis; however, it has been well described that breast milk feedings are a protective factor against the development of NEC (4–7). Bioactive components in human milk have been demonstrated to reduce intestinal inflammation, enhance stem cell proliferation, decrease enterocyte apoptosis, and promote the development of a healthy microbiome (5–11).

In this review, we discuss important components of breast milk and their role in intestinal immune homeostasis, barrier function, and the prevention of NEC (**Figure 1**). Finally, we outline models of NEC that can be utilized for mechanistic studies into the impact of breast milk components on intestinal physiology.

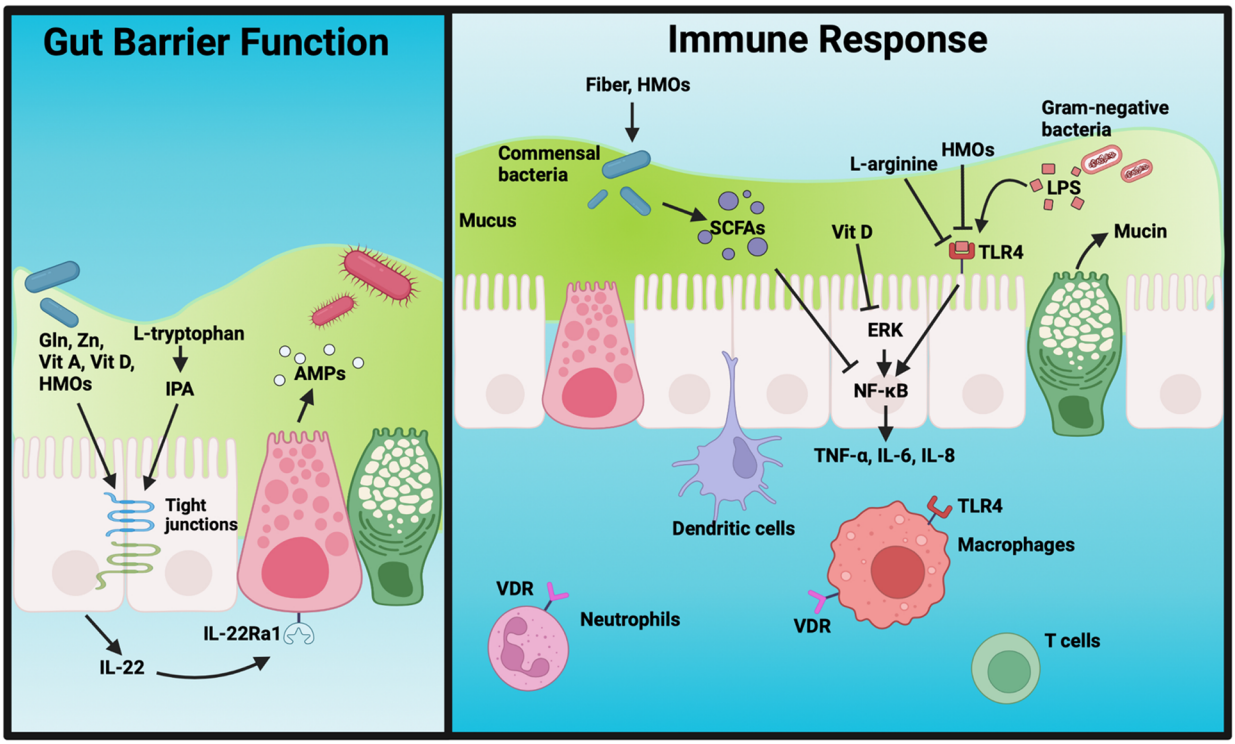

FIGURE 1
Summary of the impact of nutritional factors on gut barrier integrity and the mucosal immune response. Nutritional components improve the intestinal barrier by enhancing the expression of tight junctions, increasing IL-22 production, promoting mucus secretion, and inducing Paneth cell AMP release. They also have diverse effects on the immune response via modulation of the microbiome, downregulation of inflammatory signaling pathways, and prevention of potentially deleterious immune cell activation. Gln, glutamine; Zn, zinc; Vit A, vitamin A; Vit D, vitamin D; HMOs, human milk oligosaccharides; IPA, 3-indole propionic acid; AMP, antimicrobial peptides; SCFAs, short-chain fatty acids; LPS, lipopolysaccharide; VDR, vitamin D receptor; TLR4, toll-like receptor 4; ERK, extracellular signal-regulated protein kinase; ROS, reactive oxygen species. Figure created with Biorender.com.

Lipids

Breast milk lipids are important in supporting a diverse array of physiologic functions in early life, such as organogenesis, lipid membrane development, and signaling molecule synthesis (12). Long-chain polyunsaturated fatty acids (LC-PUFAs) are a class of bioactive lipids that are predominately acquired during the third trimester of pregnancy (13). This translates into inadequate LC-PUFA stores in preterm neonates and rapid declines in LC-PUFA levels after birth (14). The impact of these deficiencies on intestinal health remains an area of active research. In a study of preterm piglets, enteral provision of a lipid emulsion containing varying ratios of the LC-PUFAs arachidonic acid (ARA, C20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) found greater villus height in the ileum of piglets that were adequately supplemented with ARA (15). In a rat model of NEC, supplementation of formula with ARA and DHA led to reduced disease severity relative to controls (16). Finally, *in vitro* studies using human fetal intestinal epithelial cells found that treatment with ARA and/or DHA reduced cytokine production in response to an inflammatory stimulus (17). Additional research is needed in the form of both preclinical models and clinical trials to determine the optimal dose and ratio of LC-PUFA supplementation to support intestinal development and reduce the risk of NEC in preterm infants.

Lactoferrin

Lactoferrin is an abundant component of the whey protein fraction of breast milk that has a diverse array of potentially beneficial functions, including enhancing immunity, controlling inflammation, and promoting intestinal epithelial cell growth (18–21). Host defense properties of lactoferrin arise from iron binding properties as well as direct interactions with microbes and immune cells (22). Clinical trials and a 2020 Cochrane Review have thus far not detected a significant benefit for lactoferrin supplementation in the risk of NEC or mortality for preterm neonates (23–25). Additional studies, such as the Lactoferrin Infant Feeding Trial (LIFT_Canada), are needed to examine the impact of lactoferrin supplementation on the health of preterm neonates (26).

Human milk oligosaccharides (HMOs)

Human milk oligosaccharides (HMOs) are a family of over 150 structurally complex glycans that are abundant in human milk, with concentrations varying based on the stage of lactation (27–30). HMOs are metabolized by intestinal bacteria such as *Bifidobacteria* and *Lactobacilli* spp., and thus shape the development of the intestinal microbiome (31). Additionally,

HMOs serve a diverse array of potentially beneficial roles in the intestine, including augmenting host defense, modulating immune cell function, and enhancing intestinal barrier integrity (32–34). For example, HMOs act as soluble adhesion receptor decoys, blocking the attachment of viral and bacterial pathogens to intestinal epithelial cells (35, 36). HMOs also possess bacteriostatic and bactericidal properties and can modulate intestinal inflammatory responses (34). In addition, maternal breast milk HMO levels have been associated with an infant's risk of developing NEC (37).

The role of HMOs in attenuating inflammatory immune responses in the gut is well described in preclinical models. In a recent study by Suligoj et al., the effects of HMOs on intestinal barrier function were explored using Caco-2 cell monolayers (38). A combination of 2'-O-fucosyllactose (2'FL), the most abundant oligosaccharide in human milk, and lacto-N-neotetraose (LNnT) was shown to significantly decrease paracellular permeability while increasing tight junction protein (claudin-8) expression (38). In an *ex vivo* model of human intestinal tissue, galactosyloligosaccharides (GOs) were shown to downregulate TNF-α and interleukin (IL)-1β production (39). In addition, colostrum HMOs, particularly GOs, attenuated Toll-like receptor (TLR) 3 and TLR5 signaling (32). Lastly, the HMO α-3 sialyllactose was shown to downregulate the expression of the inflammatory cytokines IL-8 and IL-12 in Caco-2 cells by inhibiting nuclear factor-κB (NF-κB) signaling and stimulating peroxisome proliferator-activated receptor gamma (PPAR-γ) expression (40).

Similar anti-inflammatory properties of HMOs have been described in animal models of NEC. For example, in a rat model of NEC, the HMO disialyllacto-N-tetraose (DSLNT) increased survival rates from 73.1% to 95% ($P < 0.001$) and led to a reduction in intestinal pathology (41). A human study found that significantly decreased levels DSLNT in maternal breastmilk were detectable for infants who developed NEC relative to controls (42). In addition, in a mouse model of NEC, administration of 2'FL resulted in a decreased severity of intestinal injury that was associated with improved intestinal perfusion (43). Lastly, the HMOs 2'FL and 6'-sialyllactose (6'-SL) decreased intestinal injury in mouse and piglet models of NEC, which was associated with reductions in TLR4 activation (44). These findings support further investigation into the role of HMO supplementation in the development of a healthy microbiome and prevention of NEC in preterm neonates.

Dietary amino acids

Dietary amino acids (AA) are a primary energy source for intestinal epithelial cells (45). AA in human milk are predominantly protein-bound, with approximately 5%–10% present in free form (46). Free AA are more readily absorbed into the intestinal circulation than their protein-bound counterparts and contribute significantly to the initial rise in AA serum levels in infants following a feed (47). These free AA support intestinal health and may contribute to preventing NEC in preterm infants (45, 48–51). We will discuss amino acids that have been studied in relationship to NEC.

Glutamine

Glutamine (Gln) is the most abundant essential free AA in human milk, particularly in the first three months of lactation (52), and a deficiency in circulating Gln is associated with an increased risk of NEC in neonates (53). The beneficial effects of Gln include promoting intestinal epithelial growth, improving barrier function, reducing oxidative stress, and downregulating inflammation.

Gln promotes intestinal growth by providing energy for intestinal epithelial cell proliferation as well as regulating signaling pathways, including the mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK), and extracellular signal-regulated protein kinase (ERK) pathways (54). Additionally, Gln enhances the effects of growth factors such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα), and insulin-like growth factor-1 (IGF-1) (54).

Gln is critical in preventing epithelial cell atrophy in catabolic states and improves barrier function by regulating the expression of tight junction proteins, including claudin-1, occludin, and zonula occludens (ZO-1) (55, 56). In a randomized clinical trial, improved intestinal barrier integrity was observed for preterm neonates receiving enteral Gln (57).

Gln exerts anti-oxidative properties by acting as a substrate for glutathione (GSH) biosynthesis (58). GSH is a tripeptide composed of Gln, glycine, and cysteine that scavenges potentially damaging reactive oxidants and free radicals (58). In a study involving breastfed newborn rats, enteral Gln supplementation reduced markers of oxidative stress in intestinal tissue (59). In another study examining intestinal epithelial cells (IECs) in the setting of oxidative and non-oxidative stress, Gln exerted anti-apoptotic properties by decreasing the level of cleaved caspase-3 and increasing the expression of heat shock proteins (53).

Gln has also been shown to downregulate inflammation. In an *in vitro* study using healthy human intestinal tissue, Gln supplementation downregulated the production of the inflammatory cytokine interleukin-1 beta and upregulated the level of the anti-inflammatory cytokines IL-4 and IL-10 (60). In a rat model of NEC, Gln supplementation was associated with decreased mucosal injury, reduced inflammation, and downregulated expression of the innate immune receptors Toll-like receptor-2 and TLR4 in ileal and colonic tissue (61). Although these studies indicate that Gln may have a beneficial role in intestinal health, a 2016 Cochrane review found that glutamine supplementation was unlikely to significantly improve outcomes for preterm neonates (62).

L-arginine

L-arginine is a semi-essential amino acid exclusively synthesized by intestinal epithelial cells (63). It is a substrate for nitric oxide (NO) production via the arginine-nitric oxide synthase (NOS) pathway, which plays a vital role in regulating intestinal blood flow and maintaining intestinal integrity (64–67).

The role of L-arginine in NEC has been examined in animal models. In a neonatal piglet model of NEC, reduced arginine levels were detected for preterm piglets prior to NEC onset (68).

In addition, supplementation of L-arginine attenuated intestinal injury in another study using this model (69). Mechanistically, this was attributed to enhanced NOS activity and NO production in the intestine (69). In a murine model of NEC, endothelial cell TLR4 activation was associated with increased tissue damage and reduced endothelial NOS (eNOS) activity (70). NEC severity was also found to be increased in eNOS-deficient mice (70). In addition, enteral L-arginine supplementation attenuated hypoxia-reoxygenation-induced bowel injury in a murine model of NEC (71).

In neonates, low levels of circulating L-arginine have been associated with an increased risk of NEC (72). Data from animal studies and RCTs support a potential role for L-arginine supplementation in NEC prevention (59, 63, 68, 69, 71–73). However, a 2017 Cochrane review determined that L-arginine supplementation was associated with a significant reduction in the risk of Bell's Stage 1 but not Stage 2 or 3 NEC (74). A large high-quality study is needed before the routine arginine supplementation for preterm neonates can be implemented.

L-Tryptophan

L-tryptophan is an essential amino acid found in human milk (75). It is metabolized by tryptophanase expressed by the gut microbiota leading to the production of tryptamine and indole derivatives such as 3-indole propionic acid (IPA) (76). IPA and other tryptophan metabolites have important roles in gut immunity and intestinal barrier integrity.

IPA regulates intestinal barrier function and inflammation by activating the xenobiotic sensor pregnane-X receptor (PXR) (77). PXR activation upregulates the expression of tight junction proteins and downregulates the expression of the inflammatory cytokine tumor necrosis factor-alpha (TNF-α) (78). In epithelial cell-specific PXR-deficient mouse models, enhanced TLR4 signaling results in significant inflammation and loss of intestinal barrier integrity (79).

Indole derivatives also activate the aryl hydrocarbon receptor (AhR) (80, 81). Decreased AhR expression has been associated with the development of NEC, with reduced levels detected in the intestine of neonates, mice, and piglets with NEC (82). Recent evidence from a murine model of NEC found that administration of the AhR proligand indole-3-carbinol (I3C) resulted in reduced severity of NEC (81). Mechanistically, this was associated with downregulated expression of inflammatory cytokines and increased expression of the polyfunctional cytokine IL-22, which has been shown to be an effective therapeutic against NEC (27, 81, 82). Further investigation is needed to determine the protective mechanisms induced by tryptophan metabolites in both animal models and human studies.

Vitamins

Vitamin D

Vitamin D is important in immunoregulation and enhancement of intestinal barrier function. Vitamin D exerts diverse immunomodulatory effects by binding to vitamin D receptors (VDR) expressed on immune cells (83, 84). For example, vitamin D inhibits Th17 differentiation and decreases IL-17 production (85). VDR activation also inhibits IL-17 expression in the intestine and reduces IEC apoptosis by blocking NF-κB activation (86). Moreover, activation of VDR signaling reduces tissue damage by promoting T-cell differentiation into Th2 cells rather than inflammatory Th1 cells (87). T-cell phenotype is important in the pathogenesis of NEC, with a role for increased Th17 cells and IL-17-related inflammatory signaling in disease development (88, 89).

Vitamin D deficiency is prevalent in preterm infants, particularly in those below 32 weeks of gestation, and decreased levels of vitamin D have been associated with NEC (90). The role of Vitamin D in supporting intestinal health has been supported by findings in animal models. In a rat model of NEC, vitamin D downregulated TLR4 expression and attenuated apoptosis of intestinal epithelial cells (91). Moreover, vitamin D protected against intestinal barrier disruption and the loss of tight junction proteins by increasing occludin expression (91). In another study, supplementation of vitamin D to lipopolysaccharide (LPS)-treated cells improved cell viability, increased proliferation and growth, and decreased expression of IL-6, IL-1β, and TNF-α (92). Although the protective role of vitamin D is documented using human cell lines and mouse models, there is limited data available on the impact of vitamin D supplementation in NEC prevention.

Vitamin A

Vitamin A is present in human milk, but concentrations are significantly lower in milk from mothers of preterm infants (93). Vitamin A levels also vary by lactational stage with higher levels found in colostrum relative to mature milk (94). In addition, serum levels of vitamin A in patients with NEC are decreased relative to healthy controls (95). It is possible that Vitamin A is involved in improving intestinal health in preterm neonates, as it has been previously implicated in regulating intestinal immunity and in maintaining intestinal barrier function (96).

Studies in mice found that the intestinal mucosa of vitamin A deficient mice contains a reduced number of immune cells, including macrophages, B- and T-cells (97, 98). Vitamin A deficiency in rats is associated with an increased abundance of *Escherichia coli*, decreased mucin-2 (MUC2) and defensin-6, and upregulation of TLR2 and TLR5 expression in the intestine (99). In a study using a mouse model of NEC, vitamin A supplementation reduced TNF-α and IL-6 mRNA levels relative to controls (100). Vitamin A supplementation also increased the expression levels of claudin-1, occludin, and ZO-1, indicating vitamin A's role in improving intestinal barrier function (95). In another study using murine epithelial cells cultured with retinoic acid (RA), the expression of several tight junction proteins, including occludin, claudin-6, and ZO-1 were induced (101). Finally, decreased permeability and increased transepithelial electrical resistance were noted in another study using intestinal epithelial monolayers grown with all-trans RA (102). These

findings support the role of vitamin A in supporting intestinal homeostasis.

Trace elements

Trace elements are micronutrients present in variable concentrations in human milk (103). Essential trace elements such as zinc (Zn), selenium (Se), and calcium (Ca) improve intestinal barrier integrity, modulate the immune response, and interact with the gut microbiota (104–106).

Zinc

Zinc (Zn) is involved in essential metabolic functions such as immunoregulation, reduction of oxidative stress, and development of the intestinal tract (107, 108). Zn is primarily acquired in the third trimester of pregnancy leading to low stores in preterm infants (100). Zn content in human milk is dependent on the stage of lactation, while absorption is correlated with the maturity of the infant's gut and bioavailability (109–112).

Zn plays an important role in maintaining intestinal barrier integrity. In a study using Caco-2 cells, induced Zn deficiency led to increased intestinal epithelial permeability and decreased expression of tight junction proteins (113). Similarly, Zn depletion led to the downregulation of occludin and claudin-3 in another study using intestinal Caco-2 cells and *ex vivo* mouse colons (104). Zn has also been shown to directly enhance the production of intestinal epithelial cells in crypts and promote IEC differentiation, particularly in disease states with increased mucosal turnover (110, 114, 115). Lastly, Zn deficiency decreases mucin synthesis through disturbances in the goblet cell homeostasis (116). Taken together, these data suggest the importance of Zn in maintaining intestinal barrier function.

Several studies highlight Zn's regulation of intestinal immune function. In an *in vitro* study using chicken intestinal tissue, Zn supplementation (Zn-Gly) increased the expression of secretory immunoglobulin A (IgA), promoted a Th1 and Th2 balance, and reduced the expression of inflammatory cytokines such as TNF-α and IL-1β (117). Zn is also critical for the normal function and morphology of Paneth cells in animal models (118). Similarly, decreased Paneth cell function occurs in human intestinal tissue in response to low levels of Zn (119).

In addition to its immunomodulatory effects, Zn directly affects the composition of the gut microbiota (120). Zn deficiency reduces gut microbial diversity by indirectly promoting the growth of bacteria adapted to low Zn environments, such as Proteobacteria spp. (120). Several studies have associated Gammaproteobacteria, a class of Proteobacteria, with an increased risk for NEC (121–123). Conversely, Zn excess may also lead to gut dysbiosis. Excess levels of Zn in mice colonized with *Clostridium difficile* were found to exacerbate inflammation and intestinal disease by increasing toxin activity (124). Understanding the interplay between Zn deficiency and the intestinal microbiome could provide new insights into NEC pathophysiology.

Interaction between nutrients and the gut microbiota in NEC

One of the central roles of human breast milk feeds in neonatal health is shaping the development of the neonatal microbiome. Breast milk contains its own microbiome, and these bacteria directly colonize the neonatal intestine (125, 126). In addition, breast milk components directly influence the composition of the gut microbiome. For example, HMOs can facilitate *Bifidobacteria* and *Lactobacilli* spp. growth (31), and breast milk IgA supports the growth of *Bifidobacteria* spp. (127).

There is a complex interplay between the intestinal microbiome and the developing intestine. For example, commensal bacteria, including *Bifidobacterium* spp. and *Clostridium leptum* as well as *Faecealbacterium prausnitzii*, *Eubacterium rectale*, and *Roseburia* spp. produce short-chain fatty acids (SCFAs) (128–130). SCFAs such as butyrate, acetate, and propionate regulate inflammation (131–133). Specifically, butyrate inhibits LPS-induced inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (134). Butyrate also enhances regulatory T-cell development and production of the anti-inflammatory cytokine IL-10 (135). In addition to producing SCFAs, these commensal bacteria occupy a niche in the intestine that prevents the overgrowth of potentially pathogenic bacteria. In preterm neonates, the growth of these harmful bacteria can have devastating consequences, and intestinal microbial dysbiosis has been repeatedly associated with the development of NEC (121–123, 136).

Numerous studies have investigated if increasing the abundance of commensal bacteria in the neonatal intestine with probiotics impacts the incidence of NEC. Although data point to a potential benefit of probiotics (137), this remains an area of controversy within the field of neonatology (138). There is a lack of consistency among probiotics used in clinical trials and the lack of regulation of available commercial products. Further research is needed before probiotics become a standard of care in preventing NEC.

Milk composition by stage of lactation

Human milk composition by stage of lactation has been previously reviewed in detail (139–141). Colostrum is the first stage of milk production and consists of a high concentration of potentially beneficial and immunomodulatory components, including secretory IgA, lactoferrin, growth factors, cytokines, and HMOs (139, 141, 142). Although colostrum contains a high concentration of factors that are protective against NEC such as IgA (143), EGF (5) and HMOs (43), studies investigating provision of an extended course of exclusive colostrum feeding on the risk of NEC are limited by the

volume of maternal colostrum available. Over the course of lactation, milk content shifts to a composition that promotes infant growth and development with higher concentrations of lactose and fat in mature milk relative to colostrum, although the composition is influenced by a variety of maternal factors (141).

Donor milk

Donor milk is an alternative source of human milk feeds when maternal milk is not available in adequate quantities. The composition of donor milk is significantly impacted by pasteurization and storage (144–147), and it is generally derived from a pool of high-producing donors, which can also lead to significant differences in milk composition from maternal milk. Meta-analyses point to a reduced risk of NEC for donor milk feeds, although it remains to be determined if there is a significant impact on death or neurodevelopmental impairment (148). The Milk trial is a recently completed randomized control trial that will address these questions by investigating the impact of donor milk vs. formula on neurodevelopmental outcomes 22–26 months.

Breast milk fortification and risk of NEC

The caloric density of human milk feeds is commonly increased with the addition of fortifiers to enhance the growth of preterm neonates. Comparison of human milk-based and bovine milk-based fortifiers has not demonstrated a significant difference in either mortality or morbidity, including in NEC rates, between these types of fortification (149, 150). This remains an area of active research.

Models for studying the roles of nutrients in NEC

Due to the limited availability of human neonatal intestinal samples, mechanistic studies into the pathogenesis of NEC rely upon animal studies and *in vitro* models. NEC-like intestinal inflammation is induced in neonatal rats, mice, rabbits, and piglets through brief periods of hypoxia, feeding formula, LPS, and bacteria isolated from the microbiota of infants with NEC (151, 152). These models have been used to investigate the roles of prebiotics, probiotics, maternal milk constituents (milk proteins, HMOs), vitamins, fatty acid supplementation, and amino acids in the pathophysiology of NEC (81, 82, 91, 95, 153–155).

Numerous *in vitro* models and cell lines have been used in studies investigating the mechanisms involved in NEC (156–159). The human colorectal adenocarcinoma cell line, Caco-2, is often used to study intestinal disease; however, these cells are unable to differentiate into goblet cells leading to a lack of mucus secretion. The human colon adenocarcinoma cell line, HT-29, is also used to study NEC and will differentiate and produce mucus-secreting goblet cells in specific cell culture conditions (160). The benefit of using cell lines for mechanistic studies include abundance, reproducibility, and ease of culture. However, the cellular complexity of the intestine is hard to emulate in these static monoculture cell models. In addition, the relevance of findings in these adult tumor cell lines to neonatal disease is questionable. To overcome these difficulties, an *ex vivo* three-dimensional (3D) human organoid culture was developed to bridge the gap between traditional cell culture and studying primary human samples.

Gastrointestinal organoids are multicellular, 3D structures developed from primary intestinal stem cells (ISCs) or from inducible pluripotent stem cells (iPSCs) (161, 162). Intestinal organoids (also called enteroids) contain multiple intestinal epithelial cell types, which retain their critical structural and functional properties of the intestinal epithelium, such as barrier integrity, mucus and antimicrobial peptide (AMP) secretion, and differentiation capabilities. Therefore, enteroids allow for the study of numerous biologic properties, including barrier function, inflammation, cellular proliferation, therapeutic responses, nutrient effects, and epithelial-microbial interactions (163, 164). Limitations of using enteroids include their polarity and difficulties in co-culturing with immune and endothelial cells (165, 166). These challenges led to the development of novel Gut-on-a-Chip or Intestine-on-a-Chip platforms (167, 168).

The Gut-on-a-Chip platform is a technical advance on enteroid models due to the ability to co-culture multiple cell types, provide a constant flow of media, access the apical side of the epithelium, and mimic intestinal peristalsis via stretch (167). We recently developed a NEC-on-a-Chip model using enteroids cultured from intestinal tissue obtained from neonates undergoing intestinal surgery (168). These enteroids were cultured on a microfluidic device in the presence of an endothelial cell line and the intestinal microbiome of an infant that died from NEC (168). In these culture conditions, we detected cellular and gene expression changes similar to what is observed upon studying samples from neonates with NEC (168). This study highlights the scientific relevance of Gut-on-a-Chip models for mechanistic investigations related to the pathogenesis of NEC.

Conclusions and future directions

The intestine of the preterm neonate faces the difficult task of meeting their nutritional requirements while still undergoing postnatal development and being inundated with microbes and the challenges posed by critical illness. Optimizing the provision of the beneficial components of breast milk is central to supporting neonates through this difficult stage. Disrupted intestinal homeostasis and dysregulated inflammation can lead to NEC. Breast milk provides protection against this dangerous disease, and further research into how modulation of enteral nutrition can prevent NEC and improve outcomes for neonates with NEC remains a priority.

Author contributions

All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Recent advances in our understanding of NEC diagnosis, prognosis and surgical approach

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Necrotising enterocolitis (NEC) remains a devasting condition that has seen limited improvement in outcomes in recent years. The incidence of the disease is increasing as more extremely premature infants survive. NEC is responsible for 1 in 10 neonatal deaths and up to 61% of survivors have significant neurodevelopmental delay. The aim of this review is to highlight recent advances in diagnosis, prognosis and surgical approach in this condition. Many recent studies have reported novel methods of diagnosis of NEC with the aim of earlier and more accurate identification. These include imaging and machine learning techniques. Prognostication of NEC is particularly important to allow earlier escalation of therapy. Around 25% of infants with NEC will require surgery and recent data has shown that time from disease onset to surgery is greater in infants whose indication for surgery is failed medical management, rather than pneumoperitoneum. This indication was also associated with worse outcomes compared to pneumoperitoneum. Ongoing research has highlighted several new methods of disease prognostication which includes differentiating surgical from medical NEC. Finally, recent randomised controlled trials in surgical technique are discussed along with the implications of these for practice. Further, high quality research utilising multi-centre collaborations and high fidelity data from electronic patient records is needed to address the issues discussed and ultimately improve outcomes in NEC.

KEYWORDS
necrotising enterocolitis, decision making, surgery, prognosis, prognostication

Introduction

The incidence of necrotising enterocolitis (NEC) is increasing and outcomes in this condition have shown no improvement in recent years despite advancements in neonatal intensive care and improvements in outcome in a number of other conditions that effect premature infants (1). A recent systematic review and meta-analysis revealed that NEC is responsible for 1 in 10 neonatal deaths whilst 61% of survivors experience significant neurodevelopmental delay (2). Additionally, NEC is the most common cause of intestinal failure in children and parenteral nutrition is required in up to 9% of survivors of NEC at 1 year of age (3, 4). This has significant impact on children and families whilst creating a significant lifelong, financial burden on health and social care systems.

Research into the exact pathophysiology underlying NEC is ongoing and not fully understood however it is felt to be multifactorial involving a number of important molecular signalling mechanisms (5). Toll-like receptor 4 (TLR4) plays a crucial role in the development of NEC and is an immune receptor found in elevated frequency on enterocytes, intestinal stem cells and macrophages of prematurely born infants. TLR4 activation by microbial motifs, such as lipopolysaccharide, triggers a pro-inflammatory response which also induces apoptosis in enterocytes and inhibits enterocyte migration,

contributing to intestinal injury (6, 7). TLR4 also supresses cell proliferation including those of the mucous barrier via the Wnt and Notch signalling pathways (8). Impairment of intestinal perfusion is another critical factor in the pathogenesis of NEC. Prematurely born infants intestinal vascular system demonstrates increased vasoconstriction leading to inadequate vasodilation in response to digestion (9). This leads to ischaemia and intestinal injury following feeding. Further vasoconstriction occurs due to reduced expression of nitric oxide synthase secondary to TLR4 activation. Downregulation of development of a premature infants microvasculature further contributes to ischaemia and necrosis in response to increased postnatal stresses such as feeding and bacterial colonisation moderated by the Vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) pathways (10–12). Additionally, bacterial colonisation stimulates platelet-activating factor (PAF) leading to cell apoptosis. PAF has been shown to be increased in NEC and inhibition has been shown to have protective effects in animal studies (13). It is also clear that the immature gut immune system plays a significant role in development of NEC as lymphocytes and macrophages are pro-inflammatory compared to term infants (14).

Fortunately, there is ongoing research into all aspects of NEC with active research groups across the globe. Prevention of the disease has always been a key focus for researchers and recently there has been great interest in the use of probiotics. The microbiome is implicated in the pathogenesis of NEC (15). Studies have found a bloom of intestinal *Gammaproteobacteria* usually precedes NEC in many preterm infants and protective commensal bacteria such as *Bifidobacterium spp.* are less abundant in infants that develop NEC vs. controls (16, 17). Probiotics have been widely studied to alter the microbiome in infants at risk of NEC and prevent disease (18, 19). This work has culminated in a recent European Society of Paediatric Hepatology, Gastroenterology and Nutrition (ESPHGAN) specialist interest group recommending specific probiotic strains for the prevention of NEC (20). These strains are *L. rhamnosus GG* or a combination of *Bifidobacterium (B) infantis BB-02*, *B lactis BB-12*, and *Streptococcus thermophilus TH-4.* Numerous meta-analyses have been published reporting pooled data from trials of this intervention many of which have shown that probiotics are effective at reducing the incidence of NEC. However a recent Cochrane review of this area concludes that the certainty of evidence is low and the grade of recommendation is weak (21–23). Other techniques that are currently being evaluated for disease prevention include remote ischaemic conditioning. Remote ischaemic conditioning is a technique which has shown promise in animal models of NEC (24). It involves exposing an infant to periods of ischaemia, such as by torniquet of a limb, prior to developing disease which allows greater resilience to ischemia. Ischaemia is known to be a key element in the pathogenesis of NEC. Animal studies have shown that this method is particularly effective and significantly reduces the extent and severity of bowel injury compared to controls (24). At this stage human studies have not progressed beyond safety studies but further clinical research is in progress included a feasibility randomised controlled trial (25, 26). Human breast milk, either maternal or donor, has been shown to almost half the risk of NEC vs. formula feed in meta-analysis (27). The exact mechanisms for this are an area of ongoing research but *in-vitro* studies and animal models suggest that these mechanisms include epidermal growth factor (EGF) mediated inhibition of signalling via the innate immune receptor TLR4, human milk oligosaccharide (HMO) associated enhancement of intestinal perfusion and binding of intestinal bacteria by Immunoglobulin A (IgA) (28, 29). Whilst NEC continues to afflict preterm infants it is important that we can identify and treat NEC as quickly and effectively as possible. There have been recent advancements in diagnosis, management and prognostication which are discussed further in this article along with areas of future research.

Diagnosis

Making an accurate and timely diagnosis of NEC continues to be a significant challenge (30). Other intestinal diseases such as septic ileus and focal intestinal perforation have similar clinical features including abdominal distention and global clinical deterioration. However, early and accurate diagnosis is essential to allow timely treatment for an appropriate duration. Moreover, good quality research in NEC is dependent on accurate differentiation of those with NEC from those with other conditions (31).

Criteria and scoring systems to diagnose NEC, and differentiate it from these other conditions, have been long established and include the Vermont-Oxford Network definition, Bell's criteria and a gestational-age specific scoring system (31–34). Data from a UK based collaboration were used to derive the gestational age specific case definition (31). Clinical and radiological features are assigned a score to give an overall score from 1 to 9. Whether the total score meets the criteria for NEC or not is determined by the gestational age of the infant. If an infant is less than 30 weeks gestational age then 2 points are required whereas 4 are required if the infant has a gestational age of 37 weeks or more. This was effective and using this approach achieved a sensitivity of 63.6% and specificity of 96.8% with a positive predictive value of 85.5%. More recently, machine learning has been employed to differentiate infants with NEC from those with other conditions. One study used these methods to differentiate NEC from focal intestinal perforation at a single centre with remarkable accuracy (35). A random forest model was able to differentiate these two conditions with a sensitivity of 96%, specificity of 96% and an area under the receiver operating characteristic (AUROC) of 0.98. The variables included in the model were pneumatosis intestinalis, pneumoperitoneum, corrected gestational age prior to surgery and gestational age at birth. Another study using machine learning in a modest cohort of infants found that definitions based on Bell were outperformed by novel artificial intelligence methods (36). The most effective model used the presence of apnoea, lethargy, Guaiac-positive gastrointestinal bleed, pneumatosis, gestation age, post-natal age at NEC onset,

volume of feeding at NEC onset, disseminated intravascular coagulation and occult rectal bleeding to differentiate NEC from other conditions. Whether these techniques prove useful in clinical practice remains to be seen.

A metabolomics and proteomics approach to biomarker discovery for the diagnosis of NEC has attracted increased interest in recent years. This approach typically uses liquid chromatography-mass spectrometry to determine the presence of proteins and metabolites in fluids of cases and controls. Various different specimens have been investigated in infants with NEC which include stool, serum, urine, intestinal tissue and buccal swab samples (37). The challenges of this approach are the need for high quality samples, expertise in advanced biochemical techniques and access to specialist equipment. This hypothesis-free approach to biomarker discovery is particularly effective in experimental medicine and has had positive findings in a number of studies (38–42) along with some important reports of negative findings (43–45) mainly limited due to sample sizes. A study which shows particularly potential investigated a multi-centre cohort of infants with confirmed NEC, defined as meeting Bell's criteria, and controls who were healthy or had sepsis (40). Seven urine biomarkers were identified which delineated NEC from sepsis with an AUROC of 0.98. Genomics have also been investigated for the identification of NEC and several associations have been identified between genetic variants and disease (46). Individual genes that increase the risk of NEC include TLRR4, Single immunoglobulin and toll-interleukin 1 receptor (SIGIRR), Nucleotide binding oligomerization domain containing protein 2 (NOD2) and many others (46–49). Genome wide approaches have also been undertaken which found strongest association with a cluster of single nucleotide polymorphisms in chromosome 8 followed by chromosomes 14 and 11 (50). This recent and exciting approach may further uncover the pathogenesis of NEC whilst allowing better identification of those at risk of disease or with early disease.

Another method well known to neonatology but with little implementation with NEC is heart rate variability (51). A study of 245 infants, of which 32 had NEC, calculated heart rate variability using electrocardiogram (ECG) data combined with a panel of blood cytokine levels to diagnose NEC. Decreased heart rate variability was associated with a diagnosis of NEC although the numbers studied were low and the clinical utility of this from this current study is limited (52). Given the ability for heart rate variability to improve detection and outcomes in neonatal sepsis this is certainly an area for further exploration (53, 54).

Abdominal ultrasonography (US) has also gained interest in recent years with many studies exploring the utility of this modality in NEC diagnosis. A recent systematic review and meta-analysis summarised 6 studies which included 462 children evaluating the use of US to diagnose NEC (55). A number of US signs were taken individually including portal venous gas, free air, pneumatosis intestinalis, bowel wall thinning and simple ascites. All these signs were found to have a pooled specificity of between 91% and 99%. The pooled sensitivity however was much lower and between 22% and 48% showing that US is a good modality for excluding NEC however less effective at diagnosing it. The important caveat is that these data are based on individual signs rather than a combined overall impression by an experience paediatric sonographer.

These recent studies all show promise for earlier diagnosis of disease however there are some limitations to overcome prior to incorporation into clinical practice. The majority of which are related to incorporation of these methods into current electronic patient records and real-time monitoring systems. Even the most accurate method of prediction, developed from sophisticated statistical or machine learning methods, requires implementation into bedside systems so that these earlier diagnoses are brought to the attention of clinicians in real time. It is hoped that earlier treatment, including administration of antibiotics, cessation of enteral feeding, advanced monitoring and multi-organ support will limit disease progression. This assumption is yet to be confirmed.

Prognostication

Prognostication in NEC is being recognized as increasingly important. A quarter of babies with NEC undergo acute surgery due to bowel perforation, clinical deterioration with maximal medical therapy or failure to recover (56). After the initial acute episode there is a further cohort of infants that develop stricture formation and may require surgery for this (57). It is anticipated that accurate identification of those with severe NEC early in the disease course will allow earlier surgical intervention. Recent observational data suggest that those infants with NEC that have the longest time from diagnosis to surgery have the worst outcomes. In a secondary analysis of a population-based study infants were grouped depending on indication for surgery as determined by the operating surgeon. Those that underwent surgery on the basis that they were deemed to have failed medical therapy had surgery (adjusted) 30 h later than those with bowel perforation. This same group of infants were 4.5 times as likely to require parenteral nutrition or have died by 28 days following surgery (56). Requirement for parenteral nutrition at 28 days post surgery has previously been shown to be associated with mortality at 1 year follow-up (3). These data suggest that earlier identification of need for surgery in NEC, accompanied by earlier surgery has the potential to improve outcome. These data are however limited by their observational nature and lack of consistent definition regarding whether surgery is indicated or not. For example some infants that underwent surgery may have improved without intervention although reassuringly no intervention at laparotomy was only required in 3% of this cohort (56). Additionally, as many as 20% of infants with NEC die of the disease prior to ever undergoing surgery although it is impossible to know whether surgery would have changed this outcome (58). Moreover, in 1 in 20 that do undergo surgery the extent of necrosis is so great that survival is not possible suggesting that earlier intervention would be of benefit (59). Identification of this group of babies earlier may be key to improving survival and outcomes.

Earlier identification of need for escalation of medical treatment and requirement for surgery are also likely to improve

longer term outcomes. Poor neurodevelopmental outcomes in survivors of NEC is thought to be secondary to reduced cerebral perfusion and exposure of the developing brain to prolonged systemic inflammation which occurs in severe NEC (2, 60, 61). Mouse studies have shown that activation of microglial cells in the brain promote cognitive impairment secondary to production of Toll-like receptor 4 endogenous ligands by inflamed intestine (62). Additionally, in this study it was possible to prevent cognitive impairment with administration of microglia-targeting antioxidants (62). This suggests that medical therapies may be key to unlocking better long term outcomes in NEC however human study of this is required. In the meantime, it is hypothesised that earlier removal of diseased intestine reduces cerebral exposure to these harmful substances and hence reduces cerebral tissue damage with the caveat that it is unknown as to whether surgery itself detrimentally impacts cerebral perfusion due to physiological stress and increased exposure to anaesthetic agents. Nevertheless, to test this hypothesis we require accurate and early identification of intestinal necrosis, preferably in a non-invasive manner. Many methods have been derived to differentiate those with medical NEC from those that require surgery, known as surgical NEC. These include various biochemical biomarkers in blood plasma, urine and stool that are not yet readily clinically available (63–68) along with novel machine learning approaches (39). Additionally, conventional biochemical biomarkers that are readily clinically available have also been investigated (69–71) along with the use of scoring systems (72, 73). Novel methods requiring specialised equipment in the form of Near-Infrared Spectroscopy (NIRS) (74) and heart rate variability (75) have both shown promise in small studies. Finally, imaging methods have been extensively explored for this purpose (55, 76).

Biomarkers

There have been many promising studies published in recent years. Firstly, authors of a retrospective UK based study including 191 infants with non-perforated NEC hypothesised that a serum c-reactive protein (CRP) to serum albumin ratio could predict surgery and also mortality (77). It was found that a CRP to albumin ratio of more than or equal to three on day two of NEC diagnosis was most effective at predicting surgical intervention with an AUROC of 0.71 and was slightly less effective at predicting mortality (AUROC = 0.66). This study addresses the group of most interest which is those with non-perforated disease as this is where decision making is most challenging (56) and the results of prospective use of this method are much awaited.

Another recent study focussing on readily available clinical data retrospectively investigated the ability of the coagulation profile, 12 h after disease diagnosis to predict surgical intervention (78). In 114 infants, where the rate of surgical intervention was 40%, the presence of coagulopathy was defined as a platelet count less than 100×10^9/L or an activated partial thrombo-plastin time greater than 45.4 s or a prothrombin time international normalized ratio greater than 1.3. It was found that the presence of coagulopathy at this timepoint was predictive of surgical intervention with AUROC of 0.869 and a specificity of 91.2% which outperformed individual tests from the coagulation profile within the same study. These results are exciting but again require prospective evaluation and consideration of how the effectiveness of this method changes depending on point of definite diagnosis. It is relatively easy to decide retrospectively the point in which NEC was diagnosed but more challenging in real world settings.

A collaborative study involving multiple institutions in the Netherlands investigating biomarkers for NEC detection and late-onset sepsis separately looked at a cohort of infants in this study with medical NEC and compared these, to those that underwent surgical intervention for NEC (79, 80). Rather than explore the ability of patient characteristics, clinical features or laboratory results to predict those who underwent surgery and those who didn't, associations between these two groups were sought. Multivariable regression was used to adjust for confounding and it was found that surgical NEC was associated with lower gestational age, no maternal corticosteroid administration, earlier onset of NEC, lower serum bicarbonate (prior to disease onset) and a hemodynamically significant patent ductus arteriosus for which ibuprofen was administered. These results are interesting and can certainly be incorporated into further work looking at better ways to prognosticate in this condition but arguably cannot be implemented in the neonatal intensive care unit yet. Additionally, it may be challenging to convince clinicians of the importance of a factor such as maternal corticosteroid administration. Despite showing statistical significance it is very unlikely that neonatologists or surgeons consider this in practice.

Imaging

Abdominal US has been investigated as a radiological method of determining surgical from medical NEC. A systematic review by *Cuna* et al. included 11 studies of which 2 were prospective (55). It was found that there were several features that were associated with surgery or death of which a focal fluid collection, complex ascites and absent peristalsis had the highest odds ratios. The authors conclude that further work is needed to assess whether using this technique improves outcome and when it should be undertaken. A practical limitation of US is that it requires a sonographer with experience of using US in NEC and results in a snapshot of abdominal signs at the time of study. As this is not routine practice it can be difficult and slow to arrange in reality (81).

An alternative radiological method that has for the first time been investigated to differentiate medical from surgical NEC is computed tomography (CT) imaging. Abdominal CT imaging is frequently used in adults to accurately identify ischaemic or necrotic bowel in conditions such as small bowel obstruction or mesenteric ischaemia. It is highly effective in these settings but is rarely undertaken for any indication in premature infants. However, in a study of 34 infants with clinical and radiological features of NEC, 21 participants underwent abdominal dual

energy CT scan (76). The mean weight of infants at time of imaging was just over 1.3 kg with a standard deviation of +/− 0.53 kg. Bowel ischaemia was identified in 9 infants whom subsequently had a laparotomy where ischaemic bowel was found and confirmed histologically. The sensitivity, specificity, positive predictive value and negative predictive values in this study were all 100%. This highly effective approach has similar limitations to US, it requires a highly skilled paediatric radiologist to interpret findings and provides a snapshot of intra-abdominal signs at the time the scan was undertaken. The challenge of moving a critically unwell infant to the CT scanner may also contraindicate this method in real world settings. More detail regarding logistics and timing of these studies is needed to further inform clinicians about the true feasibility of this method.

Summary

The studies discussed here clearly highlight the wealth of research currently being undertaken in this area which has significant importance to all stakeholders. Each method has its advantages but most need further investigation or development before they can be implemented into routine clinical practice. Moreover, incorporation of these, non-radiological, methods into electronic real-time monitoring systems is an essential prerequisite. Most studies into this problem are from single centres and hence only include a handful of patients with NEC. This is a problem for most studies, but particularly those using machine learning where large numbers of participants are required to effectively train models. Multi-centre collaboration is needed to increase the effectiveness of these whilst also ensuring they remain generalisable to populations beyond single neonatal units. These studies are harder to undertake, requiring ethical approval, data sharing agreements and restructuring of data to allow combination into one dataset but these challenges are not insurmountable.

Surgical approach

The principle of surgery for NEC is to reduce contamination and sepsis by control of bowel perforation and resection of non-viable intestine (82, 83). It is also essential to reduce physiological burden on the infant as much as possible by limiting surgical time, ensuring adequate systemic perfusion and avoiding hypothermia which can lead to life threatening coagulopathy (84). Many surgical approaches exist including peritoneal drainage, laparotomy with or without bowel resection, enterostomy formation or primary anastomosis and temporary laparostomy formation (59). The choice of procedure is dependent on extent of disease, surgeon preference and physiological status of the infant, with a significant lack of high quality evidence to guide clinical decisions.

One option for surgical intervention in perforated NEC is insertion of an intra-peritoneal drain rather than undertake a laparotomy. This is less invasive, quicker and reduces the physiological burden on the infant. Randomised controlled trials have explored whether this approach is advantageous in NEC but have shown no difference in outcomes using peritoneal drainage vs. laparotomy (85, 86). However, the most recent trial exploring this question included those with both NEC and focal intestinal perforation and recorded outcomes to 2 years following intervention (87). It was found that rates of death and neurodevelopmental impairment were similar between both treatment modalities when both diseases are pooled together. However, planned subgroup analysis revealed that for infants with a presumed diagnosis of NEC, death or neurodevelopmental impairment was seen more frequently in those with an initial peritoneal drain (85%) than with laparotomy (69%). This difference equates to a 97% likelihood of reducing mortality or neurodevelopmental impairment at 18–22 months corrected gestational age with initial laparotomy in NEC. This is likely due to NEC causing extensive bowel necrosis requiring resection. If necrotic bowel is removed then systemic inflammatory response will be reduced.

Protocolisation of all areas of medicine has become increasingly popular. This approach allows standardisation and allows evidence based practice even in infrequently encountered conditions such as NEC. A recent multi-centre study from the United States has described their protocol for determining surgical approach in NEC or focal intestinal perforation and the outcomes associated with this (88). The authors report that peritoneal drainage or laparotomy is undertaken in those determined to have surgical NEC or focal intestinal perforation depending on weight, age and abdominal radiograph findings. If an infant weighed less than 750 grams, was less than or equal to 14 days old and had either a normal or gasless or pneumoperitoneum on radiograph they underwent peritoneal drainage. All others underwent laparotomy. Those with a drain were monitored closely with planned drain removal at 7 days, but laparotomy if deterioration or no improvement occurred. This protocol meant that only peritoneal drainage, without subsequent laparotomy, was used in 27% of children after implementation compared to 13% prior to implementation. Despite this, no improvement was observed in survival after implementation of the protocol and further reports of this are awaited.

The concept of damage control surgery in NEC was first reported in 2004 (89). More recently a more detailed description of this technique and the potential benefits has been reported (84). In Birmingham Children's Hospital (Birmingham, UK), neonates who were severely unwell with presumed abdominal pathology underwent laparotomy on the paediatric intensive care unit. This took place as soon as possible with ongoing resuscitation during surgery. The aim of the initial procedure was to excise obviously dead or perforated bowel and then leave a laparostomy for planned relook surgery 48 h later. Surgery was undertaken as promptly as possible to limit physiological deterioration with a median operative time of 38 min. Only 13% of those with NEC required an enterostomy at relook laparotomy as most underwent delayed anastomosis. Mortality was seen in 18% of those with NEC at 28 days which is lower than most

previously reported series (2). This technique requires coordination between all team members include transfusion laboratories to allow this approach. Other UK centres are currently developing similar approaches for selected infants.

These studies highlight recent developments in regards to surgery for NEC however it is challenging to robustly compare different surgical procedures in such a heterogenous population where there are no set standards for deciding whether surgery is indicated, or not. Providing the principles of surgery for NEC are met then it is likely that all surgical options will be comparable depending on operative findings in these challenging procedures.

Further areas for research

Fortunately, there is plenty of interest in ongoing research of all aspects of NEC as highlighted throughout this review. Multi-centre collaboration is essential in this infrequently encountered condition, particularly when studying sub-groups such as those with surgical NEC. Important areas for further work include earlier detection of disease and better prognostication which includes earlier identification of need for care escalation and requirement for surgery. These questions will be easier to address in the age of technology driven healthcare, electronic patient records and advanced statistical techniques including machine learning. The ability of studies to address these issues is dependent of quality of data collection and it is more important, now than ever, to ensure that those with NEC are correctly identified in datasets. Those with other disease such as focal intestinal perforation should be correctly labelled as such. With coordinated efforts from all clinicians and researchers interested in this devasting condition it is hoped that the currently poor outcomes will improve for generations of future NEC sufferers and their families.

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GB and NH: contributed to conception and design of this review. GB: wrote the first draft of the manuscript. GB and NH: contributed to manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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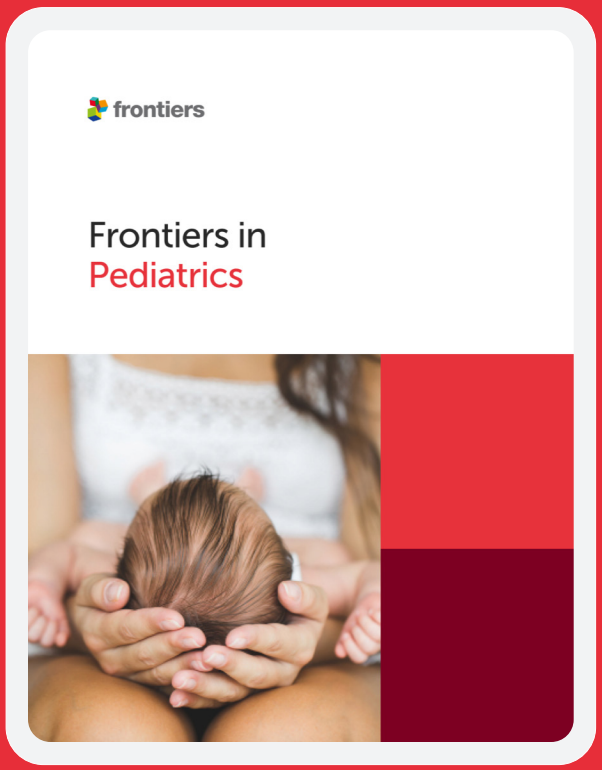

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