Necrotizing enterocolitis: new insights into pathogenesis and mechanisms

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Abstract

Necrotizing enterocolitis (NEC) is the most frequent and lethal disease of the gastrointestinal tract of preterm infants. At present, NEC is thought to develop in the premature host in the setting of bacterial colonization, often after administration of non-breast milk feeds, and disease onset is thought to be due in part to a baseline increased reactivity of the premature intestinal mucosa to microbial ligands as compared with the full-term intestinal mucosa. The increased reactivity leads to mucosal destruction and impaired mesenteric perfusion and partly reflects an increased expression of the bacterial receptor Toll-like receptor 4 (TLR4) in the premature gut, as well as other factors that predispose the intestine to a hyper-reactive state in response to colonizing microorganisms. The increased expression of TLR4 in the premature gut reflects a surprising role for this molecule in the regulation of normal intestinal development through its effects on the Notch signalling pathway. This Review will examine the current approach to the diagnosis and treatment of NEC, provide an overview of our current knowledge regarding its molecular underpinnings and highlight advances made within the past decade towards the development of specific preventive and treatment strategies for this devastating disease.

Graphical abstract

Necrotizing enterocolitis is the most frequent and lethal gastrointestinal disease in premature infants. This Review outlines current approaches for the treatment and diagnosis of necrotizing

enterocolitis and examines the progress made in our understanding of the molecular mechanisms of this disease as well as potential avenues for future treatment development.

Necrotizing enterocolitis (NEC) is the leading cause of death from gastrointestinal disease in premature infants, affecting newborn babies at a rate of 1–3 per 1000 births per year in North America[1,2], with an average total treatment cost of US$500,000 per patient in the USA in current charges[3,4]. Importantly, the mechanisms leading to the development of NEC in premature infants and the lessons learned from management of patients with NEC could have broad implications to other neonatal inflammatory processes[5,6]. Despite several decades of experience in treating patients with NEC[5,6], the overall mortality and approach to treatment have remained largely unchanged since the initial descriptions of the disease several decades ago[3,7]. Intensive research efforts over the past decade have begun to elucidate the molecular underpinnings of NEC and have identified several promising biologic strategies targeting the specific signalling pathways involved, which could potentially prevent and/or treat this disease in premature infants. Here, we will discuss current approaches to the diagnosis and treatment of NEC, review the current knowledge regarding its molecular pathophysiology and explore the advances made towards the development of specific preventive and treatment strategies.

Definitions and epidemiology of NEC

Epidemiology and trends in the incidence of NEC

The current occurrence of NEC is in fact a manifestation of the tremendous success achieved by neonatologists in their ability to keep premature infants alive at ever earlier gestational ages, with current global estimates of as many as 15 million babies born preterm every year, accounting for 11% of live births worldwide[1,2,8]. In the USA alone, the rate of prematurity is about 10% of all births, with rates as high as 13.23% in black individuals of non-Hispanic origin[9]. Large, population-based and hospital-based multicentre studies coordinated by neonatal research networks in Europe, North America, Australia and New Zealand have determined the incidence of NEC to be up to 13% among infants born ≤33 weeks of gestation or whose birth weight is ≤2,500 g[1,10–18]. Interestingly, the incidence of NEC among extremely preterm neonates in US academic centres has seen either a stabilization or even a decline to about 9% in the past 5 years analysed[1], a trend that might reflect increased vigilance and the implementation of standardized feeding strategies[19]. Despite the fact that no predilection for sex, race or ethnicity has been conclusively established for NEC, a higher disease incidence is observed in male babies of African American decent than in any other single demographic, which could be related to the higher incidence of prematurity in this demographic than in the general US population[9,10,12,17,20]. Modal disease onset occurs between 27–34 weeks after conception, with the highest incidence (13%) among infants with a birth weight <1,000 g[1,7]. Furthermore, overall survival has not changed in the past five decades and the average mortality from NEC is 20–30%, with mortality as high as 50% in those infants requiring surgical management[21]. Although the majority of cases of NEC occur among premature infants, a small subset of babies born at term or shortly before (that is, ≥35 weeks of gestation) develop NEC-like gastrointestinal signs and symptoms, frequently in association with other conditions[22].

Factors affecting the susceptibility for NEC development in premature infants

Despite the complex and multifactorial nature of the pathogenesis of NEC, three major risk factors have been implicated in its development: prematurity, bacterial colonization of the gut and formula-feeding[23]. Although no specific genetic predisposition has been clearly associated with NEC, studies evaluating concordance rates in monozygotic and dizygotic twins have found a familial predisposition for the disease[24]. Moreover, evidence suggests that genetic variants leading to upregulated expression of downstream signalling regulators of Toll-like receptor 4 (TLR4), an innate immune receptor that recognizes lipopolysaccharide found in Gram-negative bacteria, could lead to increased susceptibility to the disease. These signalling regulators include nuclear factor κB1 (REF. 25), single Ig IL-1-related receptor[26], the co-receptor molecule lymphocyte antigen 96 and the small glycolipid transport protein ganglioside GM2 activator[27]. In addition, a single nucleotide polymorphism in the promoter region of *IL18* (REF. 28) and genetic variants encoding proteins linked to the regulation of the immune phenotype shift from type 1 to type 2 T helper cells[29] could all influence the risk of NEC development. Other important clinical factors associated with NEC are summarized in BOX 1.

Diagnosis of NEC

Clinical and radiographic markers

The cornerstone of effective NEC treatment relies on accurately diagnosing the disease, which can usually be established on the basis of readily available clinical, radiographic and laboratory data. The typical neonate with NEC is a premature infant who is thriving, yet suddenly presents with feeding intolerance, abdominal distension, bloody stools and signs of sepsis (that is, changes in heart rate, respiratory rate, temperature and blood pressure)[5,7]. An important consideration in the diagnosis of NEC is the gestational age at which these symptoms present, owing to the existence of an inverse relationship between gestational age and the onset and severity of symptoms in patients with NEC[30,31]. Specifically, an infant born at ~27 weeks of gestation will typically present with NEC at ~4–5 weeks of age and has a substantially higher risk of NEC development than an infant born at closer to 37 weeks of gestation, for whom onset typically occurs within the first 2 weeks after birth[13]. A late onset of NEC in the most premature infants might be related to delayed microbial colonization of the gut and establishment of virulent microbial agents, in part owing to the use of broad-spectrum antibiotics and prolonged hospital stay[32–34]. Signs of sepsis can be associated with high gastric residuals (defined as the volume that remains in the stomach before the next enteral feeding[35]) of ≥ 2 ml/kg or >50% of the previous feeding volume, which could indicate the presence of feeding intolerance[35,36]. Although feeding intolerance is the most common early gastrointestinal symptom associated with NEC[37], some controversy persists as to the use of gastric residuals as an objective measure and their predictive value in the context of the disease progression, owing to the inherent variability in sampling gastric contents through a small nasogastric or orogastric tube, as well as to the lack of standardization in the procedure of obtaining gastric aspirates[36,38].

For descriptive purposes and for disease stratification, the Bell scoring system has been widely utilized, which assesses the degree of NEC severity as mild (Bell stage I), moderate

(Bell stage II) or severe (Bell stage III), as characterized in TABLE 1. The diagnosis of NEC can be established by plain abdominal radiography, which reveals intramural gas (so-called pneumatosis intestinalis) in the early stages of confirmed NEC (Bell stage II), whereas advanced cases of the disease have pneumoperitoneum (Bell stage III)[5,39]. Although no specific laboratory markers have been validated in making the diagnosis of NEC, neutropenia and thrombocytopenia are often present[40–42]. Consideration of alternative diagnoses is critical for infants who present with NEC and in whom overlapping signs and symptoms might be present, including those who have spontaneous intestinal perforation, ileus secondary to sepsis, sensitivity to cow milk, food protein intolerance, ischaemic bowel disease associated with heart disease or haematological disturbances (for example, polycythaemia).

Biomarkers and noninvasive testing for the diagnosis of NEC

The relative nonspecificity of the readily available clinical and radiographic tests described earlier in the article suggest the need for additional molecular markers to improve early diagnosis of NEC in premature infants. In this regard, the presence of several molecules that are detected in the blood have been assessed for their value in establishing the diagnosis of NEC[43–45] and a number of them have shown considerable promise, including acute-phase reactants (such as C-reactive protein) and proinflammatory cytokines (for example, TNFα, IL-6 and IL-8)[45]. In addition, organ-specific biomarkers, such as those that would indicate enterocyte injury or intestinal barrier impairment, include intestinal fatty acid-binding protein, liver fatty acid-binding protein, faecal calprotectin, trefoil factor 3 and claudin-3 (REFS [41,46]). Among these circulating molecules, one of the most promising might be intestinal fatty acid-binding protein, a cytoplasmic protein involved in enterocyte lipid metabolism[47,48] that is released into circulation and secreted into the urine after enterocyte damage, which has been suggested to be useful in the prediction of NEC development[48] and to correlate with the extent of intestinal necrosis[47]. Furthermore, a promising strategy in the identification of progressive NEC that requires surgical intervention has been formulated as a novel algorithm combining 27 clinical parameters and three urine fibrinogen peptide biomarkers, FGA1826, FGA1883 and FGA2659. This algorithm was reported to accurately predict the need for surgery in infants with suspected NEC in 100% of the cases analysed as opposed to only 40.1% when using the clinical parameters alone[49,50], but it remains to be validated independently and in larger population studies. These and other biomarkers, therefore, could be considered for use in association with noninvasive monitoring techniques that assess intestinal tissue perfusion (for example, near-infrared spectroscopy)[51] to identify those infants at risk of developing NEC.

The use of Doppler ultrasonography to assess perfusion of the intestinal wall has been suggested to be an accurate screening tool to determine the need for surgical intervention by identifying the presence of bowel necrosis without perforation[52,53]. Although this strategy has the potential to be more sensitive and specific than detection of the presence of free air in the abdominal cavity by conventional radiology, the presence of intramural as well as intraluminal air might obscure a reliable interpretation of ultrasonographic images. Furthermore, available data regarding the use of Doppler ultrasonography remains confined to small clinical studies with sample sizes in the range of 26–62 patients[52,53]. This

diagnostic strategy and others discussed here are promising; however, they remain incompletely proven, so the development of highly sensitive and specific diagnostic tools for NEC continues to be one of the most crucial areas of need in the field.

Medical and surgical treatment

Despite considerable advances in neonatal care, NEC remains a devastating disease that lacks a cure. Current management is largely nonspecific and includes the administration of broad-spectrum antibiotics, initiation of bowel rest and the provision of fluid and inotropic support to maintain cardiorespiratory function[2,5,39,54–56]. Surgical intervention is required in up to 50% of the NEC cases in large, population-based and hospital-based multicentre studies coordinated by neonatal research networks[3,13,16,17] and typically includes the removal of necrotic intestine. In rare cases, the placement of a peritoneal drain and abdominal irrigation might be sufficient. Although several studies have reported that patients undergoing peritoneal drainage and laparotomy could have similar outcomes[3,56,57], importantly, spontaneous intestinal perforation might resemble the initial presentation of perforated NEC, thus obscuring the interpretation of the findings from these studies. Several surgical guidelines have been published[56,58–60]. Given that up to 74% of infants initially managed with peritoneal drainage will require a subsequent laparotomy[3,59], a commonly accepted approach has been to reserve primary peritoneal drainage for those patients with substantially elevated intra-abdominal pressure that impairs ventilation, or for extremely small infants under 750 g. Additional information based upon the Bell clinical staging criteria is provided in TABLE 1 (REF 39).

Outcomes of infants with NEC

The outcome of children with NEC is characterized by high overall morbidity ranging from 20–50%, as patients experience recurrence, intestinal strictures, short bowel syndrome, growth delay and neurodevelopmental impairment[2,5]. Infants with NEC have longer hospitalization stays, increased risk of death before discharge and accrue higher financial costs compared with premature infants without NEC[3,10]. In the long term, patients who survive NEC are frequently affected by neurodevelopmental impairment, demonstrated by their impaired performance in cognitive and developmental assessments such as the Bayley Scales of Infant Development, the Griffiths Quotient and the Stanford–Binet test[61], underscoring the far-reaching sequelae of this disease[6,62]. A detailed list of complications and outcomes is presented in TABLE 2.

Pathogenesis of NEC

Considerable interest has been shown in advancing our understanding of the molecular mechanisms that lead to the development of NEC, to further the development of more precise diagnostic and treatment modalities for this devastating disease. The following paragraphs will review the current theories within the field that seek to explain how NEC develops, and will in particular highlight opportunities for drug discovery based upon the present understanding of its pathogenesis. On the basis of the work of many investigators, we now propose a unifying hypothesis for the development of NEC: that the intestine of the

premature neonate exists in a hyper-reactive state relative to the full-term intestine, which favours NEC development upon colonization with an appropriate microbial milieu in a patient with a permissive genetic background.

The preterm gut: a susceptible environment for the development of NEC

To understand the reasons for which premature infants are at a particularly high risk of developing NEC compared with full-term infants, investigators have focused their efforts on comprehending the differences between the premature and the full-term intestinal tract. These studies have outlined important differences in bacterial colonization, micro-circulatory perfusion and the maturity of the gastrointestinal immune system[5,63]. Importantly, although none of these factors alone can completely explain the reasons for which NEC develops, taken together they provide a picture that explains the pathogenesis of this disease. These studies also suggest the possibility that a molecular determinant might have a role in distinguishing the premature from the full-term intestine. In this regard, increasing evidence suggests that TLR4 is expressed at higher levels in the premature than the full-term intestine in mice, humans and other species[64]. Activation of TLR4 on the lining of the premature intestine by the Gram-negative bacteria that colonize the premature gut leads to a number of deleterious effects, including increased enterocyte apoptosis, impaired mucosal healing and enhanced proinflammatory cytokine release, which in aggregate lead to the development of NEC[65]. Moreover, the translocation of Gram-negative bacteria through the gut mucosa leads to activation of TLR4 on the lining of the endothelium of the premature bowel mesentery, resulting in a reduction in blood flow and the development of intestinal ischaemia and necrosis[66]. In additional studies, the elevated expression of TLR4 in the premature gut is reflective of the surprising function TLR4 exhibits in the regulation of normal gut development *in utero* via its effects on the Notch signalling pathway and through its expression on intestinal stem cells positive for the leucine-rich repeat-containing G-protein coupled receptor 5 (REF. 64). As a consequence of this critical role in normal gut epithelial development, TLR4 is expressed at high levels in the developing gut; therefore, in the setting of a premature birth, intestinal TLR4 levels remain elevated[67] as a consequence of the gut not having completed its full development, as well as perhaps through the ongoing activation by luminal microorganisms. Upon subsequent colonization of the gut by bacteria in the postnatal period, the deleterious consequences of exaggerated TLR4 signalling occur, leading to the development of NEC. We term this explanation for the pathogenesis of NEC, in which *in utero* TLR4 signalling that is required for gut differentiation becomes deleterious in the postnatal period, 'the cross-switching hypothesis'. This hypothesis partially explains the reasons for which the premature infant is at risk of NEC development and why the disease occurs upon bacterial colonization. In further support of a role for TLR4 in NEC development, pharmacological inhibitors of TLR4 prevent NEC *ex vivo* in mice and human tissue[68] and breast milk — which is long-known to be an effective preventive agent for patients at risk of NEC development[69,70] (discussed in detail later in the article) — is a potent inhibitor of TLR4 signalling[71].

Although TLR4 signalling offers an attractive pathway to explain the development of NEC, additional factors are known to differ between the premature and full-term host that might contribute to this disease. A comprehensive list of factors related to prematurity that increase

the susceptibility and influence the pathogenesis of NEC are presented in BOX 2. The characteristics that predispose the premature intestine to NEC include increased molecular expression and signalling activity of key mediators such as TLR4 (REFS [65,72–75]), immaturity of cellular and physiological processes such as decreased digestion and absorption of nutrients[76,77] and impaired intestinal motility[78–80]. These factors have been identified both in humans with NEC and in experimental animal models of the disease, discussed later in this section. Among the most critical factors is the high baseline level of cellular endoplasmic reticulum stress within the premature intestine, which increases the likelihood of apoptosis in the epithelial lining compared with the intestinal epithelium of full-term infants[81]. In addition, the decreased number of mucus-producing goblet cells in the premature intestine results in deficient mechanical protection that leads to increased exposure of the vulnerable epithelia to pathogenic bacteria and toxic luminal contents[64,82]. The potential for injury resulting from insufficient mucus layer protection is heightened by the impaired clearance of luminal contents, owing to decreased motility[78–80,83] and decreased digestion and absorption as a result of enterocyte immaturity[76,77]. Other important differences in the intestine of premature infants compared with full-term infants include increased microvascular tone within the intestinal mesentery[66,84] and the presence of immature tight junctions[85,86], all of which can render the bowel at risk of proinflammatory signalling, bacterial translocation and NEC development[74,78,84]. Notably, some of these important factors are linked to TLR4 signalling. For instance, TLR4 hyperactivation in the setting of prematurity leads to reduced goblet cell signalling via activated Notch pathways, and TLR4 activation also reduces endothelial nitric oxide synthase protein levels and activity within the vascular endothelium, leading to reduced mesenteric perfusion and potentially reduced motility[66,84]. Furthermore, T lymphocytes have been shown to participate in the adaptation of the premature intestinal mucosa to bacterial colonization and contribute to NEC development[87–89]. NEC is associated with lymphocyte imbalance within the intestinal mucosa, as TLR4 signalling in the intestinal epithelium leads to an upregulation of proinflammatory T helper 17 cells and a reduction in protective T regulatory cells, which can be reversed through the administration of retinoic acid in the diet[89].

Although TLR4 is likely to have a critical role in the pathogenesis of NEC, other pathways have been shown to be important. Specifically, various investigators have identified roles for the increased expression and function of platelet-activating factor in the mucosal injury and barrier dysfunction associated with NEC[74,90,91], whereas inhibitors of platelet-activating factor protect against NEC development in mouse and rat models[74,92]. Furthermore, the expression levels of the receptor for platelet-activating factor were also increased in mouse and rat ileum[90,91]. Infants with NEC have high circulating levels of platelet-activating factor[90,93], associated with the increased expression of this protein as well as with deficient activity of platelet-activating factor acetylhydrolase, the enzyme involved in its degradation[93,94]. Consequently, the presence of this enzyme in human breast milk[95] has been suggested to contribute to the protective effect associated with breastfeeding[90]. Additionally, platelet-activating factor has been demonstrated to induce TLR4 expression and signalling[74,92].

Studies using isolated tissue from infants with NEC as well as from experimental mouse models have implicated a role for intestinal macrophages in the pathogenesis of NEC[96].

Incomplete development of macrophage tolerance to bacterial products within the intestinal lumen, which could breach the barrier as a result of injury to the mucosal epithelia, have been postulated to predispose the preterm human intestine to the development of the disease[96]. Specifically, intestinal macrophages present in the healthy intestinal mucosa of term infants have increased phagocytic and bactericidal activity, but do not produce inflammatory cytokines when challenged by bacterial products, an effect that has been ascribed to the inflammatory downregulation orchestrated by transforming growth factor β2 (REF. 96). On the other hand, macrophages present within NEC injury lesions are characterized by a highly inflammatory phenotype, resulting from increased expression of mothers against decapentaplegic homologue 7, an inhibitor of transforming growth factor β2 signalling[97,98]. Further studies have suggested a role for impaired Paneth cell function in the development of NEC[99]. Paneth cells are highly specialized secretory cells located within the intestinal crypts of Lieberkühn and are key components of the innate immune system of the small intestine through their release of antimicrobial peptides into the intestinal lumen[100]. Evidence from studies in animal models indicates that Paneth cell depletion in the presence of *Klebsiella pneumonia* can induce NEC-like pathology[67,101–103]. However, given that the newborn gut is reasonably deficient in Paneth cells at baseline, it is unclear what the functional relevance of Paneth cells might be in the disease pathogenesis, although additional studies into this potential cellular mechanism of NEC are clearly warranted.

The gut microbiota in NEC pathogenesis

Linking alterations in the intestinal microflora with the development of various gastrointestinal diseases, including NEC, has received tremendous interest[32,104]. An important factor to consider in the context of NEC is that colonization of the gut in the early neonatal period happens in two waves[105]. The first wave, which is similar in both term and preterm infants, is predominantly dependent upon the mode of childbirth[105,106]. The second wave of colonization in term infants is determined by feeding type, namely breastfeeding, which is rich in bifidobacteria and *Bacteroides*, or formula-feeding, which predominantly comprises streptococci, staphylococci and lactobacilli[106]. In the case of preterm infants the second wave of colonization is less influenced by the type of feeding and is characterized by high numbers of Clostridiaceae and Enterobacteriaceae and very low relative numbers of bifidobacteria and Bacteroidetes, in contrast with term infants[105]. In fact, 16S ribosomal RNA gene pyrosequencing has shown that the single most important determinant factor of the composition of the premature gut microbiota is the degree of prematurity[107]. A discernible patterned progression of colonizers from Bacilli to Gammaproteobacteria to *Clostridium* characterizes gut colonization in premature infants but the rate of assembly of the microbial population is dependent upon gestational age: that is, the more premature the infant, the slower the progression of bacterial colonization, yet the same pattern of colonization is followed[107]. These findings demonstrate that host biology is an essential modulator of microbiota composition and equilibrium, rather than a passive culture environment. Several investigators have shown a link between an abnormal gut microbiota in premature infants and the development of NEC[32,85,105,108–111]. Additionally, multiple reports have suggested that functional expression of TLRs is critical in the dynamic interaction between the host epithelium and the microbiota that enables successful intestinal adaptation to the commensal microbiota[112–114]. Furthermore, microbial colonization of the

gut is required for the development of NEC[115,116], as NEC occurs only after this event[5] and can be treated in humans and animal models with broad-spectrum antibiotics that target enteric microorganisms[2]. However, whether abnormal bacteria represent a cause as opposed to a consequence of NEC is yet to be resolved[115,117]. Despite multiple reports of NEC outbreaks associated with certain bacteria, identification of a specific pathogen as the main aetiological factor remains elusive[108,115]. Several studies have shown that there is decreased diversity in the gut microbiota of infants diagnosed with NEC when compared with age-matched controls, although without a unified pattern except for the overabundance of strict anaerobes[108,118,119].

Many of the studies described in the preceding paragraphs have been performed in animal models of NEC and validation of these results in human tissue wherever possible is important. Experimental models of NEC in mice and rats that employ a combination of hypoxia, administration of formula supplemented with bacteria isolated from human NEC stool and exposure to hypothermia have been the mainstay of many such studies and are roughly comparable to the human disease. Other animal model studies, involving clamping of the mesenteric artery or ablation of Paneth cells[101,102], have relevance in certain scientific circumstances and are technically easier to perform. Larger animal models, especially the piglet model, share greater similarity to human NEC, but are technically more demanding and costlier to perform[120]. The benefits, drawbacks and challenges of individual models have been reviewed elsewhere[120].

Strategies for NEC prevention

Given that NEC occurs in a well-defined population of patients — that is, those who are premature — there might be benefit in identifying specific preventive strategies that, if administered successfully to the appropriate patients, could reduce the incidence of NEC. In this regard, there has been tremendous interest in developing specific nutritional and pharmacological strategies to reduce the incidence of NEC. The most relevant and effective will be reviewed here.

Nutritional approaches for NEC prevention: the use of breast milk

Multiple randomized clinical trials have now validated the empirical observation that breast milk statistically significantly reduces the incidence of NEC[69,121]. Human milk contains a variety of beneficial bioactive factors, among which several have been shown to reduce NEC incidence and progression[69,122]. In BOX 3 we present a list of human milk components and the experimental evidence supporting their protective effects. Considerable research efforts have been deployed to identify these critical factors in the hope that new preventive strategies can be developed[121]. Although the precise mechanisms by which breast milk protects against NEC are not yet fully understood, emerging experimental evidence suggests that breast milk inhibits TLR4 signalling by preventing glycogen synthase kinase 3β activity[71]. Consequently, breast-milk-mediated downregulation of TLR4 signalling can reverse the inhibition in intestinal stem cell proliferation and mucosal healing, which are themselves inhibited by TLR4[64,71,123]. Moreover, these effects were shown to be partially dependent upon activation of epidermal growth factor receptor signalling[71]. Whether the

development of NEC in association with formula feeding represents the presence of an injurious component in infant formula, or the deficiency of a protective agent only present in breast milk remains to be determined[37,69,124]. The lack of availability of human breast milk (which can arise for a number of reasons, such as insufficient production by the mother of an infant) remains a major challenge in neonatal care[37,69], and has led to the use of donor breast milk as a potential substitute or supplement to formula-feeding. Multiple reports support the use of donor human milk as a potentially effective strategy for reducing the incidence of NEC[70,125]. For those instances in which no human breast milk is available, emphasis has been placed on determining the best evidence-based strategies for formula-feeding[37]. Although no specific feeding regimen (that is, composition, volume and rate of feeding) has been validated to prevent NEC[37], the use of standardized feeding guidelines (for example, patient-specific orders with set thresholds to manage feeding intolerance)[126] have been implemented in multiple centres and have been proven to be effective to reduce the incidence and severity of the disease[126].

Probiotics in the prevention of NEC

Probiotics are defined as live microorganisms that provide a health benefit to the host[127,128]. These agents have been shown to protect against NEC and reduce disease severity and overall mortality in premature infants[127,128]. The finding that a degree of perturbation in the normal gut microbial flora exists in patients with NEC supports a rationale of using probiotics to treat and prevent this disease[105,115,116]. Considering the vulnerability of premature infants, routine administration of probiotic agents has elicited substantial controversy regarding the type of agent to be used, dosing and timing[128,129]. A systematic review, analysing 24 trials, evaluated the efficacy and safety of probiotics for preventing NEC[130] and suggested that oral administration of probiotics decreases all-cause mortality and incidence of severe NEC in preterm infants; however, the precise probiotic agent, timing and length of therapy still remains to be established[128,130]. Emerging consensus is that the use of probiotics in NEC could be effective in reducing the incidence of the disease without increasing rates of sepsis or other adverse events[56,131,132]. Mechanistic insights supporting the use of probiotics are scarce but are starting to emerge. Administration of the probiotic bacteria *Lactobacillus rhamnosus* was shown to increase enterocyte proliferation and differentiation of Paneth cells in enteroids grown in a 3D bioscaffold[133]. Furthermore, treatment with CpG-containing bacterial DNA, which bypasses the potential adverse effects of live bacteria, is effective against experimental NEC in mice and piglets, and acts by activating TLR9 and inhibiting TLR4 (REF. 134), providing a potential alternative to the use of live probiotics.

Novel pharmacological approaches

Certain biologic agents could have a role in preventing NEC or in treating NEC once the disease is established. Heparin-binding EGF-like growth factor has been identified as a biologic agent capable of preventing NEC in various animal models and of reversing the effects of established NEC, via positive effects on mucosal healing[135], intestinal stem cell function[136] and vascular perfusion[84,137]. A readily absorbed and nontoxic oligosaccharide that inhibits TLR4 was shown to prevent NEC in mice and piglets and to reduce intestinal inflammation in *ex vivo* human intestine obtained during the treatment of NEC[68]. Other

investigators have established an important role for human milk oligosaccharides in NEC prevention and treatment[138,139]. Additionally, we and others showed that the administration of a simulated amniotic fluid might have benefit for the prevention or treatment of NEC, on the basis of the mucosal protection offered by amniotic fluid, which is rich in growth factors, and exerts anti-TLR4 effects[140–142]. A summary of biological approaches for the prevention of NEC is presented in FIG. 1. Randomized clinical trials are underway to determine the potential therapeutic and/or preventive strategies of some of these approaches. In particular, clinical trial NCT00437567 has been designed to evaluate the effect of the prebiotic galacto-oligosaccharide, a component of human milk, in the prevention of NEC[143]. Additionally, clinical trial NCT02405637 aims to evaluate the efficacy of synthetic amniotic fluid in preventing NEC among very-low-birth-weight infants[144]. Emerging evidence also suggests a prophylactic benefit against the development of NEC by oral administration of lactoferrin with or without probiotics to preterm infants at risk of NEC (gestational age <32 weeks or birth weight <1,500 g)[121]. Although these findings are encouraging, widespread use of these therapies cannot be recommended at this point, as the current evidence has been determined to be of moderate-to-low quality[121] awaiting the completion of ongoing clinical trials[145–147].

Conclusions

NEC is the most common and lethal gastrointestinal pathology that afflicts premature infants. Characterized by high morbidity and mortality, complex pathogenesis and devastating short-term and long-term sequelae, it has been dreaded by health-care providers and families for over a century. Only within the past decade have substantial strides been made in the understanding of the molecular mechanisms that determine NEC pathogenesis. These advances undoubtedly hold the promise to improve the development of effective preventive and diagnostic strategies to curtail the devastating consequences of the disease. Although substantial challenges lie ahead to translate the lessons learned at the experimental level, continued translational research efforts will certainly provide avenues to alleviate the healthcare and financial burden attributed to NEC.

Acknowledgments

Biographies

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Box 1

Factors linked to increased NEC incidence

Factors related to the infant

- Prematurity (highest risk with lowest gestational age)[1,16]

- Very low birth weight (<1,500 g)[1,10,148]

- Low Apgar score at 5 min[148]

- Formula feeding[37,125,149,150]

- Mechanical ventilation[148]

- Congenital defects

  - Congenital heart disease[151]

  - Patent ductus arteriosus[152]

  - Gastroschisis[153,154]

- Pharmacological interventions

  - Indomethacin[152,155]

  - Histamine H2 receptor antagonists[11]

  - Prolonged empirical antibiotic use (5 days)[156]

  - Concomitant use of indomethacin and glucocorticoids[148]

  - Indomethacin tocolysis[157]

- Anaemia[158]

Factors related to the mother

- HIV-positive status[159,160]

- Illicit drug abuse (including opiates, cannabinoids and cocaine)[161]

- Chorioamnionitis[162]

- Vaginal delivery[148]

NEC, necrotizing enterocolitis.

Box 2

Factors related to prematurity that increase the susceptibility to NEC

- Increased expression of the innate immune receptor Toll-like receptor 4 (TLR4) in the apical surface of enterocytes[65,72–75] and within intestinal stem cells[81,123]

- Increased baseline levels of endoplasmic reticulum (ER) stress within intestinal crypts[81]

- Decreased number of mucus-producing goblet cells[181]

- Impaired intestinal motility[78–80]

- Decreased digestion and absorption[76,77]

- Enterocyte immaturity[76,77]

- Impaired regulation of microcirculatory perfusion of the gut[66,84]

- Increased bile acid levels and decreased bile acid-binding protein in the intestinal lumen[182]

- Tight junction immaturity and impairment[85,86]

- Inefficient antigen processing and presentation[183]

- Impaired intestinal regeneration and healing[75]

- Discontinuation of gut exposure to amniotic fluid

- Increased levels of platelet-activating factor (increased production and decreased degradation)[74,90,91] and increased expression of its receptor in the ileum[90,91]

- Decreased FOXP3$^+$ regulatory T cell levels in the small intestine[87,89,184]

- Decreased levels of intraepithelial lymphocytes expressing the T cell receptor γδ[88]

- Decreased intestinal expression of transforming growth factor β2[40,96]

> **Box 3**
>
> ### NEC-protective factors in human milk
>
> - Nitrate and/or nitrite and antioxidant factors[66,163]
>
> - L-arginine[164,165]
>
> - Human milk oligosaccharides and prebiotics[138,139,166–168]
>
> - Lactoferrin[121,169–172]
>
> - Secretory IgA[173]
>
> - Platelet-activating factor acetylhydrolase[90,95]
>
> - Growth factors:
>
>   - Epidermal growth factor[174–176]
>
>   - Heparin-binding EGF-like growth factor[177–179]
>
>   - Transforming growth factor $\beta 2$[96]
>
>   - Erythropoietin[180]
>
> NEC, necrotizing enterocolitis.

Key points

- Necrotizing enterocolitis (NEC) is the most common and devastating gastrointestinal disease affecting premature infants; overall NEC mortality remains unchanged over the past 30 years owing to a lack of treatment options

- The main risk factors for the development of NEC are prematurity, bacterial colonization and administration of formula feeds

- The premature intestinal epithelium is predisposed to mounting an exaggerated inflammatory response to colonizing bacteria, leading to mucosal destruction and impaired mesenteric perfusion in the pathogenesis of NEC

- The exaggerated inflammatory response is partially due to the increased expression of Toll-like receptor 4 (TLR4), which is expressed at high levels on the premature newborn intestinal epithelium

- Increased expression of TLR4 in the intestinal epithelium of the premature gut reflects the surprising function that TLR4 plays in the regulation of normal gut development through effects on Notch signalling

- Although no specific treatment for NEC exists, several potential biological targets have been identified, including growth factors, modifiers of perfusion and novel TLR4 inhibitors with potential translational importance
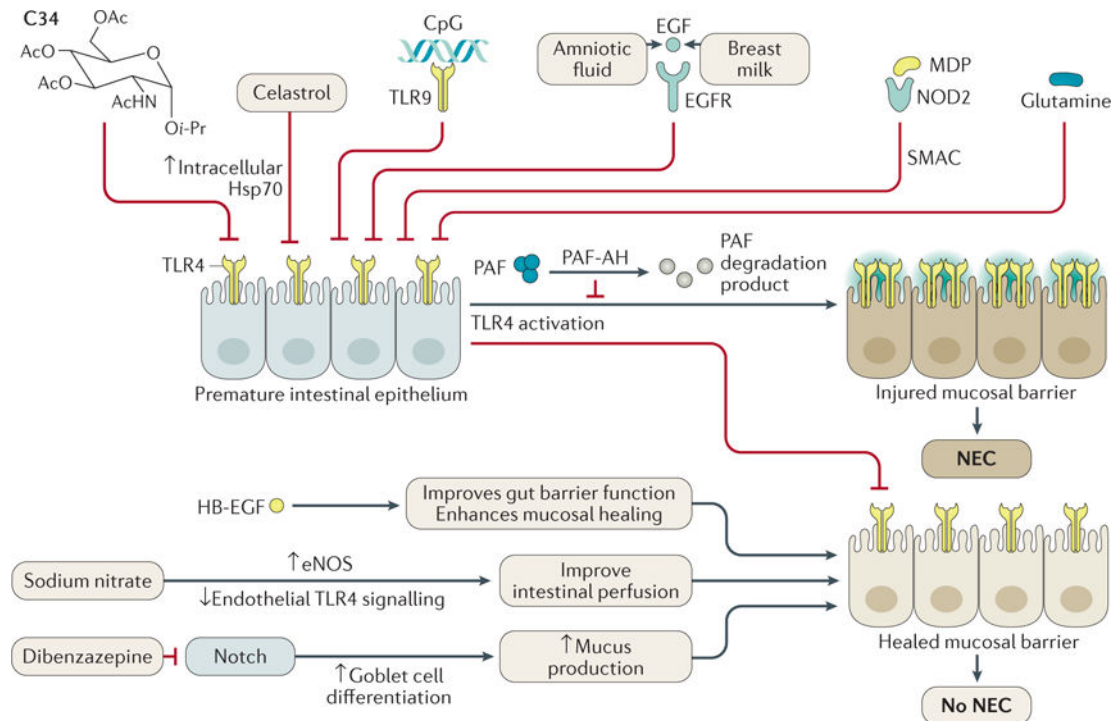
Figure 1. Factors that attenuate or prevent the development of NEC in experimental models
Activation of the innate immune receptor Toll-like receptor 4 (TLR4) plays an essential part in the development of necrotizing enterocolitis (NEC) by increasing enterocyte and intestinal stem cell apoptosis and impairing mucosal healing through decreased restitution and proliferation. These events lead to disruption of the epithelial barrier, which allows luminal bacteria to translocate and trigger a systemic inflammatory response, sepsis, multiple organ failure and death. Counter-regulatory factors can be exploited in order to dampen TLR4 signalling and expression to prevent the development of NEC. Natural factors include: epidermal growth factor (EGF)[71,140,185,186], heparin-binding EGF-like growth factor (HB-EGF)[186–188], nod-like receptor 2 (NOD2)[189], Toll-like receptor 9 (TLR9)[67,190], Platelet-activating factor acetylhydrolase (PAF-AH)[90]. Exogenous factors include: the small-molecule TLR4 inhibitor C34 (REF. 68), bacterial (CpG) DNA[67,190], muramyl dipeptide (MDP)[189], sodium nitrate[66], glutamine[191], celastrol (also known as tripterine)[192] and dibenzazepine[73]. EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; Hsp70, heat shock protein 70; PAF, platelet-activating factor; SMAC, second mitochondria-derived activator of caspase (also known as Diablo homolog, mitochondrial).

Table 1

Bell's staging and suggested management for NEC

Bell's stage	Severity	Clinical signs and symptoms		Radiological	Treatment	
I	Mild NEC, suspected NEC	Mild systemic signs and intestinal signs		Nonspecific	•	Close clinical observation
					•	Discontinuation of enteral feeding
II	Moderate NEC	•	Moderate systemic signs with prominent abdominal distension, abdominal tenderness and wall oedema	Pneumatosis intestinalis, portal venous gas	•	Medical management, such as nasograstric decompression, intravenous fluids and broad-spectrum antibiotics
		•	Thrombocytopenia and metabolic acidosis		•	Close clinical, laboratory and radiographic observation
III	Advanced NEC	•	Worsening stage II signs and symptoms plus hypotension	Pneumoperitoneum	•	Exploratory laparotomy and resection of necrotic bowel
		•	Signs of peritonitis		•	Peritoneal drainage in selected cases (abdominal compartment syndrome or weight <750 g)
		•	Severe metabolic acidosis and shock.			

NEC, necrotizing enterocolitis. Table compiled from REFS [5,39,193].

Table 2

Complications and outcomes in patients with NEC

Type of complication or outcome	Incidence	Associated factors	
Recurrence	4–10%[56,194,195]	Nonoperative management, congenital heart disease[2,56]	
Mortality	15–63%[3,196]	•	Main predictor is gestational age
		•	Patients managed surgically have the highest mortality[3]
Intestinal strictures	12–35%[197]	•	Most frequent in patients managed medically
		•	Affects colon in up to 80%[198]
Stoma complications	50%[199]	•	Most common include: prolapse, stricture and retraction
		•	Proximal jejunostomies can cause substantial electrolyte and fluid losses, impaired weight gain and peristomal skin complications[198]
Short Bowel Syndrome	20–35%[200]	•	Relative risk up to 85.9 (95% CI 45.8–160.9)[201]
		•	Increased risk associated with a residual intestinal length <25% of predicted for gestational age[200]
Neurodevelopmental impairment	30–50%[202,203]	NEC vs. no NEC (OR: 1.82). Surgical NEC versus medical NEC (OR: 2.34)[202–204]	
Growth delay	10%[62,201]	•	Affected children fall below 50th percentile for weight and height
		•	Problem more severe in patients with short bowel syndrome after NEC compared with age-matched controls without NEC[201]

NEC, necrotizing enterocolitis.