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New insights into necrotizing enterocolitis: From laboratory observation to personalized prevention and treatment

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Abstract

Background/purpose: Necrotizing enterocolitis (NEC) is a devastating disease of prematurity that develops after feeding, often without warning, and results in diffuse intestinal necrosis leading to sepsis and death in many cases. The lack of improvement in overall survival is influenced by nonspecific diagnostic modalities as well as inexact and nonpersonalized treatment strategies.

Methods/Results: Recently, we and others have shown that NEC develops in response to exaggerated bacterial signaling in the premature intestine, as a consequence of elevated expression and activity of the bacterial receptor toll-like receptor 4 (TLR4), which is important for normal gut development. Breast milk is a powerful TLR4 inhibitor, while mutations in TLR4 genes lead to increased NEC risk in humans, providing proof-of-concept for its role in NEC. Recently, a drug discovery approach has revealed a novel class of TLR4 inhibitors which are being developed for personalized approaches to NEC treatment.

Conclusion: This review will highlight the current understanding of the role of bacterial signaling in NEC pathogenesis, and will describe advances in diagnosis, prevention and treatment of NEC that may hopefully improve survival for these most fragile patients.

Systematic Review: Level of Evidence: Level II.

Keywords

Necrotizing enterocolitis (NEC); Toll-like receptor 4 (TLR4); Innate immunity; Breast milk; Probiotics; Prematurity

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1. Introduction: Challenges and opportunities in the care of the child with necrotizing enterocolitis

Necrotizing enterocolitis (NEC) is the leading cause of death from gastrointestinal disease in premature infants and affects up to 10% of all premature infants [1]. Despite significant advances in the care of premature infants in general, the overall survival of infants with NEC remains unchanged since the disease was first described over 30 years ago [2]. The presentation of NEC – at least in the most severe form – is well known to pediatric surgeons and neonatologists, i.e. a premature infant, making slow steady progress in the neonatal intensive care unit (NICU), who suddenly becomes ill with abdominal distention and bloody stools, and progresses to the point of peritonitis requiring surgical intervention. The care team is often caught off guard by the rapidity of disease progression, and is frustrated by the typically unsuccessful search for reversible precipitating factors. This scenario reveals the typical barriers in the field of NEC research, namely the fact that the diagnosis in the early stage can be delayed by the lack of specific diagnostic modalities, and treatment is nonspecific and is directed towards removal of dead or dying intestine rather than at reversing the early pathogenic processes which lead to the disease.

These challenges in the management of the child with NEC also offer exciting opportunities to advance the field in several important ways. First, a more nuanced understanding of the risk factors that lead to the development of NEC has resulted in effective disease reduction programs in some NICUs, such that the incidence of NEC has been trending downwards in the last 5 years [1]. Second, a focus on improving diagnostic modalities for NEC, including biomarker discovery and sophisticated imaging techniques, offers the potential for earlier intervention in children with this disease [3,4]. Most excitingly, as will be described below, recent preclinical discoveries based upon an improved understanding of the pathogenesis of NEC have the potential to be translated into novel preventative or treatment strategies, opening up the possibility for targeted and personalized therapies for infants at risk for this disorder. In this review, we will explore in greater detail the advances in our understanding of the pathogenesis of NEC, with an eye to closing the gap in the clinical care of these fragile patients.

2. The role of bacterial signaling in the newborn intestine in the pathogenesis of NEC

Work from the Hackam laboratory and others has advanced a unifying hypothesis to explain the pathogenesis of NEC, while also building on the work of many others in this complex field [recently reviewed by Nino et al. [5] and summarized in Fig. 1]. It should be mentioned that the studies described were performed using a variety of animal models of NEC, in mice, rats and piglets, and while each model has important limitations, each also shares critical similarities with the human disease, and we have recently reviewed the strengths and weaknesses of each of the models [6]. Many studies have revealed a requirement for bacterial colonization of the intestine in order for NEC to occur [7,8], as bacterial endotoxin could be identified in the blood of patients with NEC [9], and NEC has always been treated initially with broad-spectrum antibiotics that target intestinal bacteria [10]. Although NEC is

not typically associated with a single enteric pathogen, the fecal microbiota in infants with NEC is characteristically abnormal and features a predominance of Gram-negative Gammaproteobacteria [11] or a dysbiosis of the phylum Bacteroidetes [12], which have been linked to inflammatory diseases [13,14]. In explaining how the presence of bacteria in the premature intestine can induce the development of NEC, the Hackam laboratory [15,16] and others [7] have demonstrated a critical role for the bacterial receptor toll-like receptor 4 (TLR4) in the intestinal epithelium, which is expressed at significantly higher levels in the premature intestinal tract in mice and humans as compared with the full-term gut [15–17], findings which have also been supported by other labs [7]. The Hackam laboratory has also shown that TLR4 activation in the intestinal epithelium regulates the balance between injury and repair in the newborn mouse intestine [15,16,18,19]. Specifically, the activation of TLR4 by the dysbiotic bacteria in the premature gut leads to death of the intestinal epithelium [15–17,19–27] and a reduction in the capacity of the intestinal mucosa to undergo self-repair [16,18,19,26]. Further, the translocation of bacteria across the disrupted intestinal barrier into the blood stream leads to the activation of TLR4 on the endothelial lining of premature blood vessels in mice, which results in mesenteric vasoconstriction owing to TLR4-mediated reduction in nitric oxide release [27], which contributes to the intestinal ischemia which then leads to NEC [23,27]. In mice, inhibition of TLR4 in the intestinal epithelium protects against experimental NEC development which is achieved in a validated model by subjecting newborn mice to a combination of gavage formula feeds and intermittent hypoxia [16], while the use of small molecule TLR4 inhibitors prevents NEC in mice, piglet and human tissue *ex vivo* [28].

Why would TLR4 expression be higher in the premature gut than the postnatal gut, and why do levels of TLR4 expression fall immediately prior to birth? In a series of additional studies, Hackam, Sodhi and colleagues have determined that TLR4 is *required* for normal gut development in mice, as TLR4 activates the Notch signaling pathway in the intestinal stem cells, which is required for their normal proliferation and differentiation [16]. A premature infant thus has an elevated expression of TLR4 in the intestine as a consequence of TLR4's role in normal gut development [16]. The early delivery of the fetus thus necessarily has elevated TLR4 expression in its intestine, which then becomes activated by bacteria in the postnatal period, leading to barrier injury and NEC [16,18]. It is noteworthy that we showed that amniotic fluid – which is swallowed by the developing fetus – inhibits TLR4 signaling in utero, in part through the presence of epidermal growth factor, which is richly expressed within the amniotic fluid [18].

3. Proof-of-concept studies for a role for TLR4 activation in the pathogenesis of human NEC

Several observations provide proof-of-concept for the role of TLR4 in the pathogenesis of human NEC. First, breast milk – which has long been known to be protective against NEC – significantly reduces TLR4 signaling in neonatal mice, in part through its constituent epidermal growth factor content, as we recently showed [19]. Further, probiotic bacteria – which have been shown in several clinical trials to limit the incidence of NEC – can activate Toll-Like receptor 9 (TLR9), a molecular cousin of TLR4 [29] whose activation reciprocally

limits the degree of TLR4 signaling in a model of experimental NEC in mice [22,24]. In addition, mutations in TLR4 family genes which lead to increased activation of TLR4 signaling have been identified to be genetic risk factors for human NEC, providing further information linking TLR4 signaling to the pathogenesis of NEC [30–32]. Taken together, these findings provide a strong basis to understand the molecular underpinnings which drive the development of NEC. It is noteworthy that while there is no perfect experimental model of NEC, various animal models share important components of the human disease, and have provided useful information, as has been recently reviewed by Lu et al [6]. These findings may be used to understand the clinical features that are associated with NEC, while also providing a platform to develop novel therapeutic approaches for this devastating disease, as will be described below. In this regard, a novel family of TLR4 inhibitors was described by Hackam and colleagues [28,33], and the lead compound, an oligosaccharide, was recently licensed for clinical use as a specific therapy for NEC. This oligosaccharide, named C34, was discovered after we sought to identify novel TLR4 inhibitors utilizing an *in silico* similarity search as well as *in vitro* and *in vivo* screening of compounds that bind to the synthetic lipid A analogue eritoran (E5564) and thus inhibit TLR4 [28]. C34 is a 2-acetamidopyranoside (MW 389) with the formula C₁₇H₂₇NO₉, which inhibited *TLR4 in vitro* in intestinal epithelial cells and macrophages, and reduced TLR4-mediated inflammation in mouse models of endotoxemia and NEC [28], and phase 1 clinical trials are in preparation.

4. Can the TLR4-mediated pathogenesis of NEC explain clinical risk factors for this disease?

The above description of the pathogenesis of NEC provides a framework for understanding the clinical presentation of this disease, while also opening up the possibility of more sensitive diagnostic measures. For instance, the timing of NEC onset is inversely related to the number of weeks of life at birth, i.e. a premature infant who is born at 24 weeks' gestation will typically develop NEC at 6–8 weeks of life, while an infant who is born at 30 weeks' gestation typically will develop NEC much sooner, closer to 2 weeks of life [34]. This finding reflects the pattern of gene expression of TLR4 in the newborn intestine [24], which rises during development, and peaks at the time when NEC develops, such that the more premature the infant, the longer it takes for the rise to occur, and for NEC to develop. These findings may also explain why recurrent NEC is seen in premature infants after surgery, i.e. the expression of TLR4 has not yet fallen to full term levels. The above pathogenesis may explain why formula-fed infants are at risk of NEC, as the presence of formula leads to alterations in the microbiome, and the lack of the salutary effects of breast milk on TLR4 signaling leaves TLR4 activation unabated, leading to barrier injury, and NEC development [19]. The known association of chorioamnionitis with NEC [35] may be explained by the *in utero* activation of TLR4 that occurs in response to bacterial colonization of the amniotic fluid, leading to increased TLR4 signaling and NEC development in the postnatal period. Moreover, the increased risk of NEC that is seen in patients receiving prophylactic gastric acid suppressive therapy [36–39] and long terms antibiotics [40–42] may be attributed to deleterious effects on the intestinal microbiome, potentially resulting in greater TLR4 activation, and NEC development. Additional studies will be required to

determine the varying contributions of environmental factors and genetic factors in the development of NEC, which may be based in part on the above pathophysiologic framework and may provide insights into novel diagnostic approaches.

5. New diagnostic approaches for NEC and the bacterial-enterocyte signaling hypothesis of NEC development

One of the most important challenges in the NEC field is the ability to render an appropriate diagnosis. Unlike other diseases in modern medicine, the diagnosis of NEC remains largely a clinical one, based upon readily available data obtained from the history, physical examination and laboratory findings [43]. It is frustrating that a disease with such an impressively high mortality cannot be diagnosed more accurately, yet it is this very reality that likely contributes to the stubbornly high mortality in patients with this disease. Much has been written about the importance and also the limitation of the physical exam [44,45] and plain abdominal radiographs [44,45] in the diagnosis of NEC, and while biochemical and hematologic evaluations are very useful in the late stages of disease when thrombocytopenia and acidosis may be present, they lack specificity in its earlier stages, when diagnosis is more important yet more difficult to make [43]. This overall lack of an accurate diagnostic test in early stages of NEC has led to a search for novel diagnostic approaches, which often reflect the downstream pathways that are activated in response to the bacterial-enterocyte signaling mechanisms described above. For instance, Abdel-Haie et al have identified that intestinal fatty acid binding protein (iFABP) can be measured in the serum [46] and urine [47] of neonates with NEC, which was found to have a sensitivity of 90% and specificity of 100%, reflective of the fact that enterocyte death is a natural consequence of disease development after TLR4 activation [46]. Fecal calprotectin – a molecule present in both leukocytes and enterocytes – is elevated in NEC, reflective of the inflammatory pathways that are recruited during NEC pathogenesis [48–54]. Further, salivary epidermal growth factor was found to be lower in neonates with NEC compared with those who do not develop NEC [55], which might be expected to lead to exaggerated TLR4 signaling, given that EGF is a significant inhibitor of TLR4 activation [18,19]. Much attention has been given to C-reactive protein [56] and serum amyloid protein A [57,58], two separate acute phase reactive proteins which are increased in response to injury and inflammation, and are each expected to be elevated in inflammatory diseases like NEC [59]. Excitingly, Sylvester and colleagues recently described the impaired activity of blood coagulant factor XIII in patients with NEC [60], and this group has also performed an extensive proteomic approach in order to search for novel biomarkers in the urine or blood to distinguish NEC from other non-NEC causes of sepsis [61–63]. Other groups have focused on assessing the microbiota in the stool of patients with NEC [11], in a search for signature bacterial changes that can be readily assessed and which may provide clues to the onset of NEC development [64,65]. More recently, laboratories have focused on identifying various genetic risk factors for NEC [66], which has led to an understanding that polymorphisms in TLR4 associated genes, as well as others, may be linked to disease progression [30–32]. Finally, investigators are using more advanced diagnostic modalities including near-infrared spectroscopy to predict the course of NEC, and to identify its development at earlier stages based upon patterns of blood flow and tissue perfusion [67,68].

A recent alternative to current diagnostic criteria for NEC has been proposed by Phil Gordon and was recently published by Gephart et al., whereby a “Two out of Three Rule for Bedside Diagnosis of Preterm NEC” was described [4]. The criteria for the “Two out of Three” rule include 1. *Pneumatosis intestinalis*, and/or portal air diagnoses at presentation; 2. Persistent platelet consumption ($<150,000 \times 3$ days after diagnosis); 3. Postmenstrual age at disease onset more consistent with NEC than spontaneous intestinal perforation [4]. These approaches, either in isolation or in combination, offer the potential to significantly advance our ability to diagnose NEC earlier and more accurately than the currently available physical and biochemical tests, and to provide a more personalized and pathogenesis-focused approach to NEC diagnosis.

6. A rational approach to the treatment of NEC in the current era

The treatment of infants who have NEC is guided by a set of accepted principles that include attention to normalizing hemodynamic derangements, minimizing progression of sepsis, and removal of dead intestine in cases in which the infant is clearly not responding to lesser measures. Each of these principles is driven by a goal of reducing the exaggerated bacterial enterocyte signaling that plays a critical role in NEC pathogenesis. For instance, in the early stages of disease, treatment is targeted toward the cessation of feeds and the administration of broad-spectrum antibiotics, which should minimize bacterial translocation and the deleterious effects of feeds on the inflamed intestine [43]. The rationale for holding feeds in NEC has in the past been based on the neonate’s inability to absorb calories and other nutrients across the inflamed gut [69], yet may also reduce the negative effects of feeds on the “stressed” microbiome [70]. Despite these interventions, approximately 50% of patients will proceed to the need for surgical intervention, which may include either a peritoneal drain or an exploration [71,72]. While a comparison between the relative merits of peritoneal drainage versus laparotomy for perforated NEC has been extensively explored by prior authors [72–76], over a third of patients who receive drainage alone will require laparotomy for resection of diseased intestine [77], and many studies that compare peritoneal drainage with laparotomy and intestinal resection do not convincingly exclude those neonates that actually have spontaneous intestinal perforation and not NEC [78]. That said, in the patient with a distended abdomen and abdominal compartment syndrome, placement of a drain even if performed as a temporary measure can be both life-saving and efficient, and can allow for a subsequent laparotomy [79]. Infants who weigh less than 1 kg and who are not fed, and who develop pneumoperitoneum within the first 10 days of life are likely to have spontaneous intestinal perforation, which is a different disease entity than NEC, and which may be definitively treated by a peritoneal drain [78].

There may be additional considerations to perform a laparotomy in infants with NEC, especially those in whom a lack of pneumoperitoneum does not “push” the situation towards surgery. For instance, it is now recognized that premature infants with NEC have more severe cognitive impairment and white matter injury than age-matched premature infants who do not have NEC [80–82]. The presence of ongoing intestinal inflammation may thus drive the release of intestinal-derived molecules which can cross the premature blood–brain barrier, leading to neurological injury. This paradigm would suggest that performing earlier intestinal resection may limit the degree of NEC-associated brain injury. Similarly, infants

with NEC develop more severe and more refractory NEC-induced lung injury, which is more difficult to treat than bronchopulmonary dysplasia that affects premature infants who don't have NEC [80]. We postulate that the decision to perform an intestinal resection, and the timing of any resection, should consider not only factors within the abdominal cavity, but also the effects on organs which are affected by the inflammatory stew to which they are exposed as active intestinal necrosis remains *in situ*, including the brain and the lungs.

7. Towards zero cases of NEC: strategies for prevention

Since treatment of NEC is so often ineffective in improving outcomes [1,2], and since the overall survival of infants with NEC has not improved since the disease was first described 40 years ago [44], there has been a focus in the field of NEC towards an understanding of prevention strategies [83], and indeed several neonatal intensive care units have experienced zero cases of NEC within the time period in which the preventative measures have been undertaken [84]. These measures are listed in Table 1, which also provides information regarding the effectiveness and relative strength of the measure. Specifically, investigators have shown that the administration of breast milk [85–87], avoidance of antibiotics [41], limitation of acid blockers [36], indomethacin by itself and with concomitant corticosteroid use [88], prevention of anemia [89] have been associated with NEC reduction. Recently, a campaign in the NICUs to reduce NEC across the country has shown significant progress and is being rolled out as a standard of care [4,84].

Additional mention should be made of two specific strategies that have been proposed to reduce the incidence of NEC, yet are associated with significant controversy. The first is the strategy of administration of probiotics, in which several randomized controlled trials have been performed in preterm infants including the largest thus far, the Probiotics in Preterm Infants Study (PIPS) [90]. This study demonstrated no difference in NEC, late-onset sepsis, or mortality with the *Bifidobacterium breve* (BBG-001) treatment group compared to the control group [90]. Another large randomized control trial with the probiotic combination of *Bifidobacterium infantis*, *Streptococcus thermophiles* and *Bifidobacterium lactis* identified no difference in late-onset sepsis or mortality; however it found a 2.4% decrease in the incidence of NEC in the probiotic treated group [91]. Although these results differed, a recent meta-analysis from 23 RCTs found a significant decrease in NEC stage 2 in the probiotic group [92], with the most effective probiotic formulations as the *Lactobacillus* and *Bifidobacterium species* [92]. Some studies have been criticized owing to the observation that NEC incidence was low at baseline [91], that during the course of the study the standard of care and other clinical factors may be different, and that the actual probiotic formulations have varying degrees of live bacteria, use different types of bacteria, and vary by dose and amount administered. Probiotics act in various ways including through the activation of TLR9 which prevents TLR4 signaling [24], providing a scientific rationale for their usage [15, 22]. However, important questions regarding the appropriate timing, duration, optimal strain and long-term outcomes have yet to be determined in a large cohort.

A second controversial practice that has been ascribed to NEC reduction includes avoiding blood transfusion administration to prevent the development of NEC [93]. This association was first reported in 1987, where an increased incidence of NEC (30.6%) was noted in one

level III NICU [93]. They found that receipt of a packed red blood cell transfusion was associated with an increased risk of NEC (odds ratio 15.1 [95% CI 2.59–92.51] [93]. A meta-analysis to evaluate this association found an increased risk of NEC in infants that had received a PRBC transfusion in eleven case–control studies (adjusted odds ratio 2.0, [95% CI 1.6–2.5]) [94]. Recent reports from Cunningham et al. demonstrated that administration of PRBCs was associated with increased severity of death and NEC-related disease [95]; however, Le et al. found no association with NEC development and PRBC transfusion prior to NEC in a case–crossover design [96]. In subsequent studies, other authors showed that NEC is not associated with blood transfusions [97, 98], and in fact, Patel et al. demonstrated the link to NEC was with the development of anemia and not the administration of the blood itself [89]. The clinical data thus suggest that careful correction of anemia may be more relevant than the administration of blood, regarding the pathogenesis of NEC. Taken together, these studies suggest a strategy of NEC prevention based upon limiting typical practices in the NICU may have a role in improving outcomes in these fragile patients.

8. Unanswered questions and the future of NEC research and practice

This review has provided insights into how the molecular underpinnings of bacterial enterocyte signaling in the premature intestine through TLR4 may lead to the development of NEC. We have also shared how these insights into the molecular biology of NEC can guide our understanding of the major signs and symptoms of NEC and provide a rationale for novel diagnostic and therapeutic approaches to this disease. It should be mentioned that several investigators have made great strides in recent years regarding the role of additional therapeutic agents that may prevent NEC, including HB-EGF [99–105] (we note that EGF is an effective inhibitor of TLR4 in breast milk [19] and amniotic fluid [18]), neuroregulin [106], and nitrates [23, 27] which promote per-fusion and limit the ischemic changes in the disease. Other molecules, including anti-IL-17 therapy [107] and anti-TNF [108], may also be of benefit, based upon the known impact of these inflammatory agents in disease pathogenesis. The future of NEC research will likely require a more integrated approach towards understanding how the various cells and tissues interact in order to lead to tissue injury, both in the intestine and in the organs affected by NEC. Research will also seek to identify which neonates are at risk for NEC development in the first place, through a study of host genetic, environmental and bacteriological factors, and allow for personalized medicine approaches and perhaps individualized clinical pathways of care. The clinical future of NEC research will likely involve a greater emphasis on specific diagnostic modalities for NEC, including biomarkers but also more sophisticated imaging approaches [4], as described above. It is our hope that the early diagnosis of NEC can lead to prompt intervention to potentially diminish the severity of NEC and minimize the need for operative intervention. Addition emphasis is now being placed on NEC prevention, including the practical use of donor breast milk, probiotics, and the development of an adaptive formula that is endowed with the preventative benefits of breast milk, such as through the incorporation of human milk oligosaccharides [23] and C34 [28], which have been linked to NEC reduction in preclinical models. It is through studies such as these and involving a committed community of NEC researchers and advocates, along with affected family members and NEC-based organizations (such as the NEC Society), that true advancements

in NEC prevention and treatment will be made, and we will finally reach a day when we no longer need to operate on infants with this devastating disease.

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Fig. 1.

Summary of factors that either predispose or protect premature infants from the development of necrotizing enterocolitis. The development of NEC in preclinical models is dependent on elevated expression and signaling of the lipopolysaccharide receptor toll like receptor 4 (TLR4) in the epithelium of the premature intestine. Clinical factors that predispose to NEC in humans are associated with increased TLR4 signaling, while those that protect NEC are linked to decreased TLR4 signaling, suggesting that personalized NEC therapy may be achieved through modulation of intestinal TLR4 in humans. See text for details.

Table 1

Measures associated with the prevention of necrotizing enterocolitis.

Measures associated with NEC prevention	Intervention	Effectiveness (Quality)
Feeding strategy	Prioritizing human milk feeds [¹⁻⁵] Standardized feeding protocols [^{6,7}]	Consists of growth factors, oligosaccharides, immunoglobulins (High quality) Limits interfacility variability among providers (Moderate quality)
Medication stewardship	Limit histamine receptor blocker use [⁸⁻¹¹] Limit unnecessary and prolonged antibiotic administration [^{12,13}]	Acidic gastric pH limits bacterial overgrowth (Moderate quality) Antibiotic stewardship; less than 5 days for negative cultures (Moderate quality)
Prevention of anemia	Delayed cord clamping [^{14,15}] Avoidance of severe anemia [^{16,17}]	Prevention of anemia may maintain adequate gut perfusion (Low quality for delayed cord clamping; moderate quality for avoidance of severe anemia)

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