

Necrotizing enterocolitis: It's not all in the gut

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Impact statement

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal disease; its high mortality rate mandates the development of non-invasive biomarkers to predict NEC before its onset. This review summarizes the pathogenesis, prevention, unresolved issues, and long-term outcomes of NEC.

Abstract

Necrotizing enterocolitis is the leading cause of death due to gastrointestinal disease in preterm neonates, affecting 5–12% of neonates born at a very-low birth weight. Necrotizing enterocolitis can present with a slow and insidious onset, with some neonates displaying early symptoms such as feeding intolerance. Treatment during the early stages includes bowel rest and careful use of antibiotics, but surgery is required if pneumoperitoneum and intestinal perforation occur. Mortality rates among neonates requiring surgery are estimated to be 20–30%, mandating the development of non-invasive and reliable biomarkers to predict necrotizing enterocolitis before the onset of clinical signs. Such biomarkers would allow at-risk neonates to receive maximal preventative therapies such as careful nutritional consideration, probiotics, and increased skin-to-skin care.

Keywords: Vagus, biomarker, animal models, brainstem

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Epidemiology of necrotizing enterocolitis

In the United States, 9.9% of neonates were born prior to 37 weeks' gestational age in 2017; these births are classified as preterm.¹ Many of these premature neonates require extended hospitalization due to comorbid factors due to low gestational age and weight at birth. Necrotizing enterocolitis (NEC) is the leading cause of death due to gastrointestinal (GI) disease in preterm neonates, affecting 5–12% of neonates born at a very-low birth weight (VLBW; <1500 g).^{2–10} NEC symptoms can be slow and insidious at first, including feeding intolerance, but can quickly progress to fulminant NEC with hallmark signs such as pneumatosis intestinalis and/or portal venous gas.^{5,7,8,11–15} In neonates with NEC who require surgery to resect the perforated portions of bowel, the mortality rate is estimated between 20 and 30%, the highest mortality rate among neonates requiring surgery.¹⁶ On average, neonates not requiring surgery are estimated to be hospitalized in the neonatal intensive care unit (NICU) 20 days longer as compared to unaffected neonates, and neonates requiring surgery are on average hospitalized a further 60 days longer.¹⁷ Therefore, NEC accounts for a large portion of the financial burden associated with preterm birth; indeed, the average total

treatment cost per patient is estimated to be \$500,000, with the total cost per year in the United States estimated between \$500 million and \$1 billion.^{7,8,17,18} Furthermore, the need for bowel resection surgery as a complication of NEC is the primary cause of neonatal short-bowel syndrome (SBS); in these cases, the average cost of care for the first five years of life is estimated to be \$1.5 million per patient.¹⁹

NEC risk factors and pathogenesis

NEC pathophysiology is generally hypothesized to be multi-factorial, common risk factors include low gestational age at birth, low birth weight, chorioamnionitis, mechanical ventilation, and many more.^{2,3,13,14,16,20,21} Research into the pathophysiology of NEC has further uncovered risk factors such as genetic predisposition, intestinal immaturity, changes in microvascular tone, and abnormal microbial colonization.^{7,8,15,22} Although no studies have found a clear genetic phenotype associated strongly with NEC, studies have found a familial predisposition for the disease.²³ Most studies further suggest that genetic variants leading to the upregulation of downstream signaling receptors of Toll-like receptor-4 (TLR-4), an innate immune receptor, may

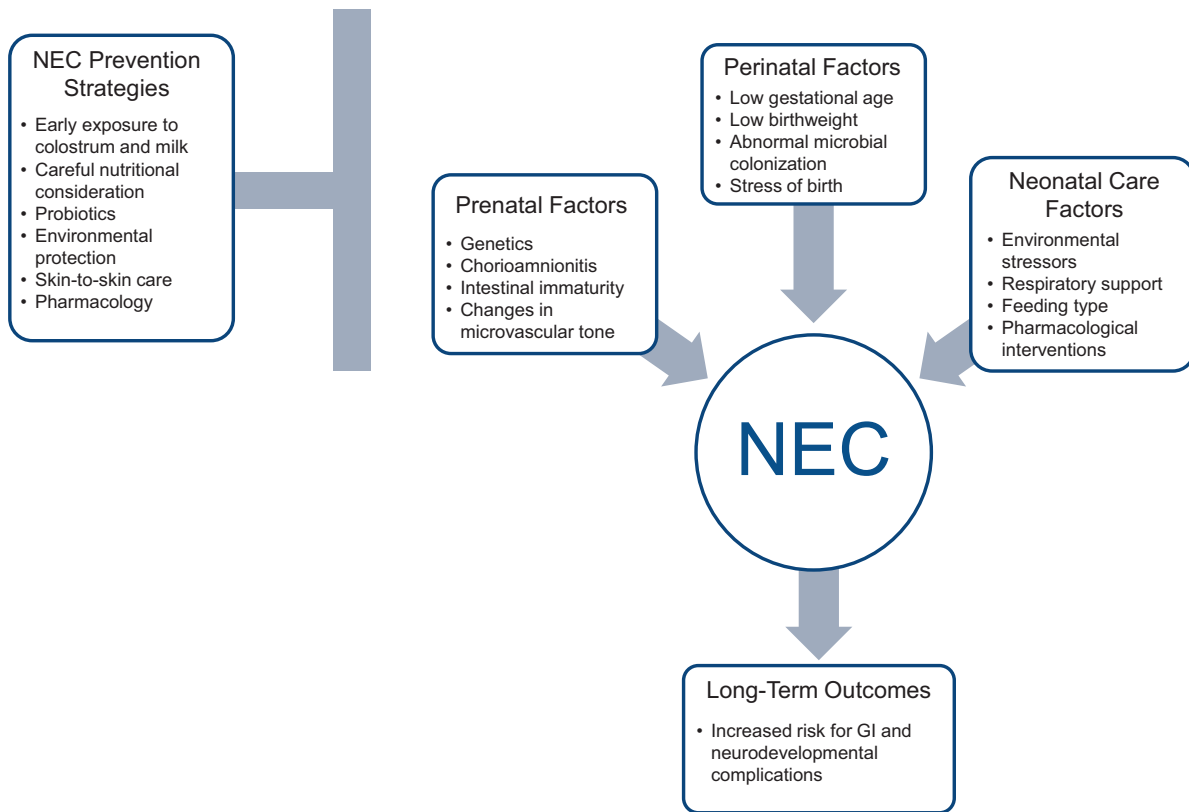


Figure 1. NEC risks, prevention, and outcomes. (A color version of this figure is available in the online journal.)
NEC: necrotizing enterocolitis.

increase the risk of developing NEC.^{24–27} Specifically, these signaling regulators may include nuclear factor κ B1, the small glycolipid transport protein ganglioside GM2 activator, co-receptor molecule lymphocyte antigen 96, and single Ig interleukin (IL)-1 related receptor.⁸ Previous studies have shown that pro-inflammatory cytokines involved in TLR signaling, such as IL-6 and IL-8, are elevated at the time of NEC diagnosis and may also be elevated during the early stages of the disease.^{28,29} Both of these cytokines are increased within the first 2 to 4 h after infection and then decline gradually over the next 24 h.^{30,31} Gender and racial disparities in NEC incidence are most likely due to genetic variations, such as single nucleotide polymorphisms, in these populations.³²

Preterm neonates are more susceptible to intestinal injury due to the underdeveloped nature of their intestine.^{33–37} Specifically, preterm neonates lack several GI defense mechanisms such as gastric acid, digestive enzymes, mucus production, peristalsis, and polymeric immunoglobulin A (IgA).^{34,35,38} In healthy patients, gastric acidity and digestive enzymes eliminate most antigens and ingested pathogens, while mucus inhibits microbial adherence.^{38,39} Active organized peristalsis is required to prevent bacterial immobility and to eliminate antigen–antibody complexes that can be detrimental to the GI tract.^{38–41} Finally, polymeric IgA is required to bind luminal antigens to reduce their risk of penetration.^{38–41} All of these critical defenses are under developed in preterm neonates, increasing their susceptibility to GI injury and disease.^{33–37}

In addition to these defenses, nitric oxide may also play a role in the pathogenesis of NEC.^{8,14,15} Low levels of nitric oxide regulate mucosal blood flow and vascular tone, but high levels may weaken the gut barrier through increased bacteria translocation, impaired mitochondrial function, and decreased leukocyte recruitment to the endothelium.^{42,43}

At birth, neonates are rapidly exposed to environmental bacteria, initially through maternal vaginal flora and enteral feedings. Preterm neonates experience a delayed and often inappropriate colonization, leading to increased inflammatory responses and abnormal bacterial glycosylation patterns.^{38,39,44} Further complications include delayed enteral feedings, early exposure to broad spectrum antibiotics, and formula feeding, which all contribute to the delayed colonization of the gut and an increased risk of pathogenic colonization.^{38,39,44} Studies comparing the microbiota of preterm neonates who develop NEC, as compared to control neonates, have found that NEC leads to unusual intestinal microbial species and an overall reduction in microbiota diversity.^{42,45} This reduction in diversity of the microbiome may leave neonates more susceptible to infectious diseases, especially when harmful bacteria may be introduced via catheterization and enteric feeding.^{46,47}

Other risk factors for NEC include maternal, ex-utero transition, and neonatal care factors.^{2–5,7,8,12–15} Maternal risk factors include illicit drug abuse, infection with chorioamnionitis, and HIV-positive status.^{2,7,8,13,48} Risk factors that occur during the transition to ex-utero life include low

flow and perfusion states due to perinatal events, such as placental abruption, leading to neonatal shock which is characterized by hypovolemia and academia.^{9,10,49} These conditions are reflected in the Score for Neonatal Acute Physiology (SNAP) and/or Apgar score at 5 min of life; these scores have been shown to be reliable and significant predictors of neonatal mortality in preterm neonates, and scores are typically decreased in neonates who later develop NEC.^{21,50} Neonatal care factors include respiratory support, feeding type, and pharmacological interventions. Studies have also shown that neonates who require respiratory support during the early neonatal period are 12.6 times more likely to develop NEC.⁵¹ Formula feeding without supplemental breast milk has also been shown to increase the risk of developing NEC by 6.4 times.^{39,44,51} Furthermore, pharmacological interventions such as histamine H₂ receptor antagonists, indomethacin, indomethacin tocolysis, glucocorticoids, and concomitant use of indomethacin and glucocorticoids also leave the neonate at an increased risk of developing NEC.^{21,52} Finally, congenital abnormalities, especially those affecting the heart and/or GI, such as congenital heart disease, patent ductus arteriosus, and gastroschisis, can increase the risk of NEC.⁵²⁻⁵⁴

NEC diagnosis and treatment

Typically, NEC is diagnosed via the Bell's Modified Staging Criteria, which has three classical stages of NEC: mild (Bell's Stage I), moderate (Bell's Stage II), and severe (Bell's Stage III).¹¹ Mild or suspected NEC (Bell's Stage I) is classified by mild systemic signs such as temperature instability and bradycardia, in addition to mild non-specific intestinal signs such as mild abdominal distension and occult blood in the stool.^{7,8,11} Moderate or definitive NEC (Bell's Stage II) further includes radiological findings of pneumatosis intestinalis and/or portal venous gas with moderate systemic signs such as abdominal tenderness, thrombocytopenia, and metabolic acidosis.^{7,8,11} These systemic and local factors leave the intestine, specifically the distal ileum and proximal colon, susceptible to inflammatory processes and perforation leading to pneumoperitoneum.^{55,56} Finally, advanced NEC (Bell's Stage III) requiring surgical intervention is characterized by bowel perforation with resultant pneumoperitoneum, hypotension, signs of peritonitis, and severe metabolic acidosis.^{7,8,11} Gestational age must also be taken into account when considering the diagnosis of NEC, where gestational age and the onset and severity of NEC symptoms have an inverse correlation.^{10,20,57} Studies have shown that the mean gestational age at birth of peak NEC onset is 32 weeks.⁵⁸

Current treatment strategies of NEC differ based on the severity stage, but generally include broad-spectrum antibiotics, bowel rest, and ionotropic and fluid support.^{3,7,8} Surgical intervention to resect portions of the ischemic bowel is required if the disease progresses to the advanced stages.^{3,5,7,8,12,21} Broad-spectrum antibiotics are typically prescribed where there is concern for sepsis, including antibiotics to cover anaerobic organisms which are also prescribed in cases of suspected or confirmed perforation.^{3,7,8} Depending on the severity stage, treatment may

also include the management of hypotension, metabolic acidosis, and thrombocytopenia.^{3,7,8} Overall, although our knowledge of the pathogenesis of NEC has advanced over the past few decades, treatment strategies have not impacted the frequency or severity of the disorder; therefore, the focus of clinical and basic science has shifted to the prevention of NEC.

NEC prevention

Due to the lack of effective treatments for NEC, research focus has shifted to testing strategies for the prevention of NEC, specifically early exposure to colostrum and mother's own milk, careful nutritional consideration, probiotics, environmental protection, skin-to-skin care (SSC), and pharmacology.^{2-5,14} Colostrum, the first milk produced by mothers in the days after birth, has been shown to contain high concentrations of beneficial immune mediators that provide bacterial and anti-inflammatory protection and stimulate the development of the GI tract.⁵⁹⁻⁶⁴ Our group has shown recently that oropharyngeal administration of colostrum increases salivary secretory IgA levels,⁶⁵ which may be protective against NEC. Other studies have shown that preterm neonates receiving colostrum had a significantly decreased incidence of neonatal sepsis⁶⁶ and shortened time to attain full enteral feeds.⁶⁴ Oropharyngeal colostrum may convey these benefits to the neonates by stimulating the oropharyngeal-associated lymphoid system, providing a mucosal barrier to prevent microbial adhesion, and inducing systemic immune responses.⁶⁷ Additionally, colostrum has a high concentration of growth factors that may stimulate intestinal growth and development, particularly in mothers who have delivered preterm neonates.^{68,69} Although no studies have reported harmful effects of colostrum administration, further randomized clinical trials are needed to evaluate its efficacy and elucidate its mechanism of action.^{64,66}

Many studies have shown the benefits of neonates, especially preterm neonates, receiving either mother's own or banked breast milk.^{2,3,6,8,60-62,70-72} In the preterm neonates, however, there are several challenges that can prevent them from receiving enteral feeds of breast milk. These challenges include an underdeveloped suck-swallow-breathe reflex, motor coordination issues, GI reflux, and low body stores of energy.^{9,20,33,36,37,73} Aggressive enteral feeding in the presence of these issues or respiratory and/or cardiac support, feeding intolerance, or high doses of certain medications can lead to an increase in NEC susceptibility.^{2,3,13,14,21,48,62} Therefore, after birth, preterm neonates often receive parenteral nutrition while progressing to full enteral volume under careful monitoring.^{3,6,62} Trophic, low volume feeds of colostrum and mother's own or banked breast milk are widely recognized as the best means of gut protection by preventing villous atrophy, mucosal injury, and leaky gut.^{2-7,12,13,62,63} Human breast milk contains many factors thought to help prevent NEC including nitrate/nitrite antioxidant factors, L-arginine, human milk oligosaccharides and prebiotics, secretory IgA, platelet-activating factor acetylhydrolase, lactoferrin, and growth factors.^{8,59,62,71,72,74,75}

Administration of probiotics and commensal bacteria may protect the preterm gut against inflammation and injury via a variety of mechanisms.^{47,76-78} These mechanisms are thought to include down-regulation of pro-inflammatory gene expression, upregulation of cytoprotective genes, production of butyrate, and regulation of cellular immunity.^{46,47,77,79} These mechanisms may work to support gut barrier maturation and function, lower the pH of the gut, inhibit other microbes, and nourish colonocytes.^{46,76,77,79} Randomized clinical trials and observational studies have found that probiotics reduce the incidence of NEC and all-cause mortality, and no harmful effects have been reported.^{47,76-84} However, the precise probiotic agent, along with its timing, dose, duration, and most effective formulation in preventing NEC has not yet been established.⁸⁰⁻⁸⁴ In addition to the administration of probiotics, environmental factors may also be effective in the prevention of NEC.

Environmental protective strategies, such as reducing exposure to excessive light and sound, cue driven care, and SSC may also be critical in the prevention of NEC. SSC has been shown to decrease mortality rates, improve short- and long-term developmental outcomes, and strengthen the bond between infant and mother, among other positive effects.^{61,85-87} In this regard, increased frequency of SSC is associated with increased vagal tone during the first week of life, and predicted diminished neonatal morbidity.⁸⁸ Furthermore, SSC decreases the incidence and severity of NEC.^{61,85-87} These studies suggest that SSC enhances stress resiliency, reduces allostatic load, and leads to improved health outcomes.⁸⁸ Animal models suggest that SSC may improve resting vagal tone and promote maturation of vago-vagal circuits; these studies have specifically shown that maternal proximity improves autonomic functioning, arousal regulation, and orienting behavior in newborn rats.^{87,89} In these animal models, isolated components from the dam such as maternal body heat or smell affected the respective body systems in the pups.⁸⁷ This suggests that SSC integrates the thermal, rhythmic, and sensory components of maternal presence to integrate autonomic functions in the newborn.^{87,89} Thus, the widespread use of SSC, beginning soon after preterm birth and continuing daily throughout hospitalization is proposed as a foundational element of care to reduce morbidity, especially in the prevention of NEC.^{61,85-88}

In addition to these environmental protective strategies, several studies have evaluated the potential of numerous pharmacological treatments to prevent the onset of NEC, with varying results. The use of antenatal steroids to reduce the incidence of NEC is controversial, as steroids increase the incidence of spontaneous intestinal perforation, but no studies to date have found a clear correlation between steroid use and NEC.^{21,74} Other pharmacological interventions under consideration in animal models include those that modulate inflammation, specifically those that affect TLR signaling.^{24-27,90-93} Studies in rodent models of NEC and in samples of resected bowel from NEC neonates suggest that there are more TLR-4 surface receptors in NEC cases as compared to controls or full-term neonates.^{24,26,94} High levels of inflammation, like those seen in NEC, lead to differential localization of TLR-4 receptors, making them a

potential target for the treatment of NEC and inflammatory disorders.^{24,26,94} Other pharmacological treatments currently under investigation include, but are not limited to, heparin-binding EGF-like growth factor,⁹⁵⁻⁹⁷ human milk oligosaccharides,^{60,72} and lactoferrin,⁷⁵ but more research is needed to evaluate their efficacy and safety.

Long-term outcomes of NEC

Neonates who survive medical or surgical NEC are at an increased risk for long-term GI and neurodevelopmental complications. Surgical treatment of NEC typically includes resection of ischemic bowel portions; the long-term outcome of these patients is dependent on the length of remaining intestine and its ability to absorb nutrients adequately.^{18,55-57} Specifically, ileal resection may lead to GI dysmotility, abnormal mucosa, bacterial overgrowth, and vitamin B₁₂ or enzyme deficiency, causing malabsorption of nutrients.^{18,55-57} Furthermore, NEC is the most common cause of SBS in neonates, which can lead to GI complications such as gastric acid hyper-secretion, bacterial overgrowth, D-lactic acidosis, translocation of enteric bacteria to the bloodstream, and intestinal failure-associated liver disease.^{18,55-57,98} Clinical management of SBS requires a multi-disciplinary approach to ensure that the neonate receives sufficient nutrition for growth, to maximize intestinal adaptation, and to minimize fluid, electrolyte, and nutritional losses.⁹⁸

In addition to potential GI complications, neonates with NEC are at an increased risk for persistent neurological and cognitive alterations. NEC Stage ≥ 2 is associated with long-term neurodevelopmental impairment, and these neonates have an increased incidence of cerebral palsy, visual, cognitive and psychomotor impairment.^{99,100} These long-term effects are likely due to NEC occurring during a critical developmental time frame, when the developing brain is vulnerable to insults and nutritional deficits.¹⁰⁰⁻¹⁰³ Acute insults as a result of NEC may include systemic inflammation, hypoxia, ischemia, and multisystem organ failure, but long-term nutritional deficits due to SBS may also impact the developing brain.¹⁰⁰⁻¹⁰³ The exact mechanisms underlying these long-term impairments remains unclear; however, the increased levels of local inflammation and circulating pro-inflammatory cytokines suggest that inflammation may play a major role.^{100,101} Some researchers hypothesize that intestinal injury and barrier dysfunction as a result of NEC allows for the translocation of inflammatory mediators into systemic circulation.^{100,101} These inflammatory mediators may then target vulnerable cerebral oligodendrocytes and microglia, both of which are still developing during the peak time of NEC incidence.^{100,101} This theory is supported by studies that report increased NEC severity is associated with adverse neurodevelopment and that increased serum levels of pro-inflammatory cytokines in NEC neonates are associated with increased risk for poor growth and development.^{104,105}

Unresolved issues

The devastating effects of NEC mandate the development of minimally invasive predictive biomarkers to identify

neonates at risk prior to the onset of clinical signs; one of the most promising non-invasive biomarkers currently under investigation is heart rate variability (HRV). HRV measures can be calculated based on electrocardiogram (ECG) recordings, using a fast Fourier transformation of inter-beat-interval (IBI) values for both time- and frequency-domain analysis.¹⁰⁶⁻¹¹² In time-domain analysis, the difference between sequential IBI values and root-mean-square of successive differences (RMSSD) represent vagal tone; similarly, the HF power spectrum (HF-HRV) in frequency-domain analysis assesses indirectly vagal parasympathetic outflow and has been identified as a marker for fetal and neonatal wellbeing.¹¹¹⁻¹¹⁴ Conversely, the low frequency (LF) spectrum of HRV frequency-domain analysis represents a mix of sympathetic and parasympathetic outflow.¹⁰⁶⁻¹¹² Our group has shown that preterm neonates who later develop NEC display a diminished HF-HRV power prior to the onset of clinical signs, as compared to control neonates.¹¹³ We have also shown that a rat model of mild NEC significantly attenuates the typical developmental increases in HF-HRV when combined with the stress of laparotomy or subdiaphragmatic vagotomy.¹¹⁵ The major advantages of HF-HRV include its utility in non-invasively predicting NEC days to weeks before its onset, relatively low cost, and ease of analysis using existing software.

Another non-invasive method that has been evaluated in the prediction of NEC is breath hydrogen monitoring.¹¹⁶⁻¹¹⁸ This test relies on analyzing the various gases present in human breath, including carbon dioxide, carbon monoxide, hydrogen, and nitric oxide.¹¹⁷ Breath hydrogen can be used to indirectly assess stress levels in the GI mucosa; increased concentrations of hydrogen in breath samples indicate bacterial metabolization of luminal substrates.^{116,117} Studies have shown elevated concentrations of hydrogen in the breath of neonates who later develop NEC on average 24 h before NEC diagnosis.^{119,120} Some studies report a high sensitivity and specificity for breath hydrogen monitoring in the detection of NEC, but it has been shown to have a low positive predictive value of only 33%.^{119,120} In addition to this low positive predictive value, there are also technical difficulties and confounders that limit its use as a predictive biomarker. Specifically, the site of breath collection must be tightly sealed and the measure is affected by many covariates; interference can be caused by changes in the microbiome, tidal volume, respiratory rate, breath holding, the patient's cardiopulmonary status, and need for mechanical ventilation.¹¹⁷ Furthermore, calibration can often be an issue as breath hydrogen levels must be standardized to amount of carbohydrates in enteral feeds, which is often changing in preterm neonates.¹¹⁶⁻¹²⁰ Overall, experts in the field agree that the confounders of this technique outweigh its utility, and agree there are practical and theoretical flaws with breath hydrogen monitoring for the detection of NEC.^{117,118}

Other biomarkers under investigation include pro-inflammatory cytokines, C-reactive protein (CRP), and serum amyloid A (SAA).^{22,121} The early rise in cytokine levels has been shown to have high specificity in the diagnosis of neonatal sepsis alone, but combination with additional biomarkers is needed to have specificity

and sensitivity for NEC diagnosis.^{28,29,31,122,123} CRP is a generalized biomarker of inflammation and was previously one of the most widely used predictive biomarkers of NEC and sepsis by clinicians in the NICU.^{31,121-123} However, studies have shown a wide range of sensitivity levels using CRP for the prediction of early onset neonatal sepsis, perhaps due to its wide range of comorbid associations such as meconium aspiration syndrome.^{31,121} Due to this lack of sensitivity and relative high cost for CRP analysis, currently, most clinicians do not utilize CRP as a biomarker of NEC.^{28,29,49,123-125} Alternatively, SAA levels rise earlier in the inflammatory response as compared to CRP, and SAA has a high sensitivity level in the prediction of neonatal sepsis.^{126,127} Currently, clinicians most commonly use advancing thrombocytopenia and elevated neutrophils as a marker of NEC progression and need for surgical intervention.^{3,5,8,18} Overall, serum biomarkers likely have the most clinical relevance when used in combination with one another, and perhaps in addition to a non-invasive marker such as HF-HRV.¹²⁸⁻¹³⁰ However, the collection of serum biomarkers can be invasive and stress-inducing, and results from these tests may not be received quickly enough to ensure neonates receive preventative measures to avert the onset of NEC.

Animal models of NEC

Insights into the pathogenesis and novel treatments of NEC have largely resulted from the study of animal models; currently, the most commonly used animal models for NEC are rats, mice, and piglets.^{4,7,8,15,131,132} In rats, NEC is typically induced in newborn rats through differing combinations of cesarean section, maternal separation, formula feeding, hypoxia, hypothermia, ischemia-reperfusion, and administration of intragastric lipopolysaccharide (LPS) or commensal bacteria.^{4,7,8,15,131,132} Studies relating human development have shown that rats at postnatal day 12/13 are representative of a term human neonate; therefore, newborn rat pups are an excellent model for the study of preterm neonates.^{133,134} The first rat model of NEC was described in 1974,⁷⁰ and consisted of formula feeding and hypoxia. Shortly thereafter, the same group found hypothermia and hypoxia to be the key stressors in inducing moderate NEC.¹³⁵ Our group has since established a mild form of NEC (equivalent to Bell's Stage I) in rats through administration of hypothermia and hypoxia twice daily; this mild NEC is more representative of the early stages of NEC when preventative treatments would have the most efficacy.¹¹⁵ We have also shown that ghrelin, an orexigenic GI peptide, is able to attenuate the effects of mild NEC in rats, in a vagally dependent mechanism.¹³⁶ Other models also include treatments such as intragastric LPS or commensal bacteria from stool samples to determine the roles of IL-18,¹³⁷ IL-12,¹³⁷ intestinal epithelial apoptosis,¹³⁸ maternal milk,^{71,72} probiotics,¹³⁹ NF- κ B,¹⁴⁰ nitric oxide dysregulation,^{42,141} and many more. Some models also include ischemia-reperfusion injury to mimic intestinal injuries occurring in NEC,¹⁴² but these models are more controversial as the direct connection to NEC remains uncertain.¹⁴³ Overall, rat models can be highly effective in the study of

NEC due to their ready availability, relative low cost, and high litter size. However, as few transgenic rat strains related to NEC pathology are commercially available, they do have limitations.

Similar to rat models, NEC in mice is typically induced through formula feeding, hypoxia, hypothermia, commensal bacteria administration, and ischemia-reperfusion, or through transgenic manipulation.^{4,7,8,15,131,132} The most widely used non-transgenic murine model of NEC includes formula feeding, hypoxia, and hypothermia, and induces NEC with gross and microscopic evidence of intestinal necrosis.^{24,144,145} This model has been used to study the role of various genes in the pathogenesis of NEC, including TLR-4,²⁴ IL-18,¹⁴⁶ MUC2,¹⁴⁷ among others. Transgenic NEC mouse studies have evaluated the effect of manipulation to genes including IFN- γ ,¹⁴⁸ TLR-4,^{26,91-93} TLR-9,⁹¹ inducible nitric oxide synthase,¹⁴⁹ 70 kD heat shock proteins (HSP70),⁹⁰ and many more. Such studies have begun to elucidate the role of the innate immune system on the developing GI tract and NEC,¹³¹ but further studies are needed to develop possible therapeutics to treat NEC. In summary, mice are an effective model of NEC due to the widely available transgenic strains, ease of genetic manipulation, high litter size, and relative low cost but can be difficult to handle due to their small size.

Piglets have a GI tract very similar to humans with regard to anatomy, development, nutrition, and physiology; these characteristics make them an ideal model for the study of NEC.^{131,150} Similar to rodent models, NEC is inducible in piglets through different combinations of formula feeding, hypothermia, and hypoxia.^{151,152} One of the most widely accepted models was established in 2006, and

involves cesarean section at 92% gestation and total parenteral nutrition followed by formula feeding, without hypoxia or hypothermia.¹⁵³ This model is sufficient to increase in IL-6,¹⁵⁴ as seen in human neonates with NEC, and was used to study the effects of colostrum,¹⁵³ human milk,¹⁵⁵ amniotic fluid,¹⁵⁶ antibiotics,¹⁵⁷ and changes in microvasculature.¹⁵⁸ Overall, piglets are an excellent model for NEC because of their anatomical and physiological similarity to the GI tract of humans, but are much more difficult and expensive to maintain than rodents.¹³¹

Brainstem control of autonomic function

In the caudal brainstem, vago-vagal circuits are comprised of multiple nuclei, including the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus (DMV), and the nucleus ambiguus (NA).¹⁵⁹ The NTS receives visceral sensory inputs, while the DMV and NA are the origin of vagal motor fibers that form synaptic connections with postganglionic neurons in the target organ to modulate functioning.¹⁵⁹⁻¹⁶¹ Specifically, the NTS integrates brainstem, limbic, and hypothalamic inputs to coordinate, among others, GI reflexes, motility, and emptying by sending signals to adjacent motor nuclei such as the DMV.¹⁵⁹⁻¹⁶² The DMV is comprised of preganglionic parasympathetic neurons that innervate the GI tract from the lower third of the esophagus to the splenic flexure in the transverse colon.^{159,161,163,164} These slow, spontaneously firing pace-making neurons of the DMV are involved in the fine modulation of GI tone, motility, and secretion through the vagus nerve.^{159,161,163,165} Postganglionic neurons of the myenteric plexus, located between the longitudinal and circular smooth muscle of

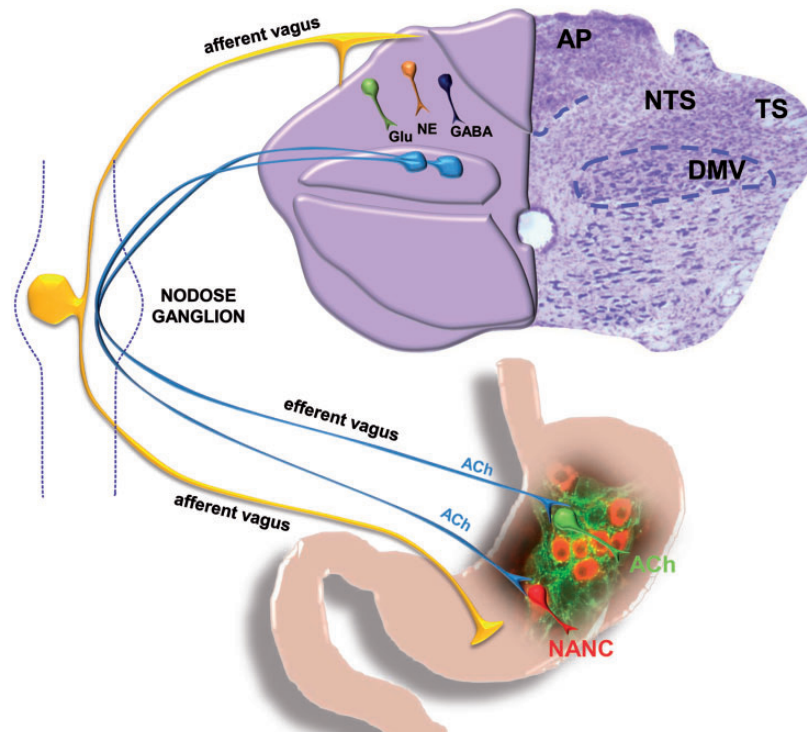


Figure 2. GI vago-vagal reflexes. (A color version of this figure is available in the online journal.)
NANC: non-adrenergic non-cholinergic.

the GI tract, use two distinct pathways to modulate GI functioning: an excitatory cholinergic pathway and an inhibitory non-adrenergic non-cholinergic (NANC) pathway.^{159,161,162,164,166} The excitatory cholinergic pathway uses acetylcholine to induce smooth muscle contraction via activation of muscarinic receptors, while the inhibitory NANC pathway induces relaxation of smooth muscles via release of vasoactive intestinal polypeptide or nitric oxide.^{159,161,166} Studies have shown that the excitatory cholinergic pathway is tonically active and plays a major role in the control of basal gastric tone and motility, while the inhibitory NANC pathway does not appear to be tonically active.^{159,161,165} Overall, bidirectional communication between the gut and the brain regulates homeostasis; these vago-vagal brainstem circuits are neuroplastic and able to respond to internal and external stressors.^{159,163,167–170} A lack of adaptability or resiliency to these stressors or other adverse events frequently results in GI dysfunctions such as delayed gastric emptying and/or accelerated colonic motility.^{171–177}

Heart rate and cardiac function are modulated by pre-ganglionic parasympathetic cardiac neurons in the brainstem, located primarily in the NA.^{178–185} These neurons have a tonic level of parasympathetic firing in conscious and anesthetized animals; this pattern is synchronized to the cardiac pulse.^{178–183} The activation of cardiac vagal neurons is influenced strongly by the activation of NTS pathways modulated by glutamate and γ -aminobutyric acid.^{186,187} Furthermore, the respiratory system can influence cardiovascular reflexes through the modulation of baroreceptor and chemoreceptor inputs to cardiac vagal neurons.^{188,189} Cardiac vagal neurons are also involved in a number of higher order connections with nuclei such as the locus coeruleus and the paraventricular nucleus (PVN) of the hypothalamus.^{184,185,190–192} Specifically, the PVN is involved in the control of autonomic function under both normal conditions and during stress challenges, such as hypoxia.^{184,185,193} Studies have shown that many disease states induce diminished cardiac vagal activity, as measured through both the firing properties of the neurons and through HF-HRV.^{113,194–203} Diseases that decrease cardiac vagal activity include NEC, hypertension, diabetes, hypothyroidism, coronary/peripheral artery disease, and chronic obstructive pulmonary disease, to name a few.^{113,194–203} We have recently demonstrated a positive correlation between HF-HRV and GI motility in rats,²⁰⁴ supporting the hypothesis that the reduction in HF-HRV power observed in preterm neonates prior to the development of NEC is associated with decreasing GI motility in these neonates.

Conclusion

Overall, NEC is a devastating disease that mandates the development of non-invasive methods to predict its onset before clinical signs. The pathogenesis of NEC is still under investigation, but major risk factors are thought to include premature birth, low birth weight, chorioamnionitis, and mechanical ventilation. In addition, preterm neonates may be more susceptible to NEC due to their

underdeveloped intestine and lack of fully developed GI defense mechanisms. Current treatment strategies generally include broad-spectrum antibiotics, bowel rest, ionotropic and fluid support, and surgery if the bowel perforates. There is a critical need for reliable, non-invasive biomarkers to determine which neonates are at an increased risk of developing NEC, before the onset of clinical signs. A biomarker such as the reduced HF-HRV in combination with the detection of pro-inflammatory cytokines would allow at-risk neonates to receive maximal preventative therapies such as early exposure to colostrum and mother's own milk, careful nutritional consideration, probiotics, increased SSC, and pharmacology.

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