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REVIEW ARTICLE

New directions in necrotizing enterocolitis with early-stage investigators

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The 2019 Necrotizing Enterocolitis (NEC) Symposium expanded upon the NEC Society's goals of bringing stakeholders together to discuss cutting-edge science, potential therapeutics and preventative measures, as well as the patient-family perspectives of NEC. The Symposium facilitated discussions and shared knowledge with the overarching goal of creating "A World Without NEC." To accomplish this goal, new research to advance the state of the science is necessary. Over the last decade, several established investigators have significantly improved our understanding of the pathophysiology of NEC and they have paved the way for the next generation of clinician-scientists funded to perform NEC research. This article will serve to highlight the contributions of these young clinician-scientists that seek to elucidate how immune, microbial and nervous system dysregulation contributes to the pathophysiology of NEC.

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a catastrophic intestinal disease that can result in sepsis, multiorgan failure, short gut syndrome and requires long-term intravenous nutrition, subsequent liver damage, and death.[1] While premature neonates are the most susceptible to NEC, it is impossible to predict which infants will develop the disease and the severity of their disease process. Accordingly, families and clinicians are often blindsided by the diagnosis, as tools for early detection and strategies for prevention are nonexistent or underdeveloped. Without sufficient research to improve the standards of care on how to prevent and treat NEC, patient-families receive variable care and often feel disempowered.

Many established investigators have contributed immensely to the understanding of NEC pathophysiology; however, several knowledge gaps still remain. Studies surrounding the importance of the innate immune receptor toll-like receptor 4 (TLR4),[2] nitric oxide,[3] and bacteria[4] in NEC pathogenesis have been crucial to advancing our understanding of the disease. Furthermore, established investigators have developed several novel therapies, including heparin-binding epidermal growth factor,[5] next-generation probiotics,[6] and the use of stem cells and exosomes,[7,8] which have paved the road for young investigators who are now beginning to make an impact in the field. This article reviews the work of these young clinician-scientists with a focus on how maternal stress impacts intestinal development and immunity, the dysregulated signaling pathways during NEC, the microbiome, gut barrier dysfunction and enteric nervous system dysregulation contribute to the pathophysiology of NEC; how paracrine signals in stem cell therapy may protect against NEC; and how tool kits can assist in NEC prevention and diagnosis (Fig. 1).

BASIC AND TRANSLATIONAL SCIENCE

It has long been thought NEC results from prematurity, systemic stress (i.e., sepsis, hypoxia, etc.), formula feeding, and an aberrant microbiome.[9] Together these factors result in an exaggerated immune response, intestinal ischemia and necrosis, and gut barrier disruption, leading to fulminant organ failure[10] (Fig. 2). Understanding how these predisposing factors trigger NEC onset can allow for a deeper understanding of NEC pathophysiology, which may open the door to novel treatment options.

Maternal stress

The Martin lab has focused on how the external environment can shape the neonatal immune system.[11] Their work has recently been expanded to better understand the effects of maternal psychological stress on the developing immune system. Stress can be defined as emotional tension or strain resulting from adverse circumstances. Some examples of stress during pregnancy are financial hardship, emotional and physical abuse, or lack of prenatal care. Stress has a major impact on biological and immune defense mechanisms. A tightly regulated and homeostatic intrauterine environment is needed for fetal and newborn immune development. Excessive psychological stress during pregnancy is harmful to the fetus and increases the incidence of poor neuropsychological outcomes.[12] Children subjected to gestational stress have higher rates of depression, ADHD, autism, and bipolar disorder.[13] Goodman and Emory[14] described the link between maternal psychopathology and neonatal outcomes by showing that low birth weight infants and infants with low APGAR scores more likely had mothers with emotional and psychological disturbances when they were pregnant.[14] Early fetal cortisol exposure is termed fetal programming and results in reduced blood flow and impaired delivery of oxygen and vital nutrients to

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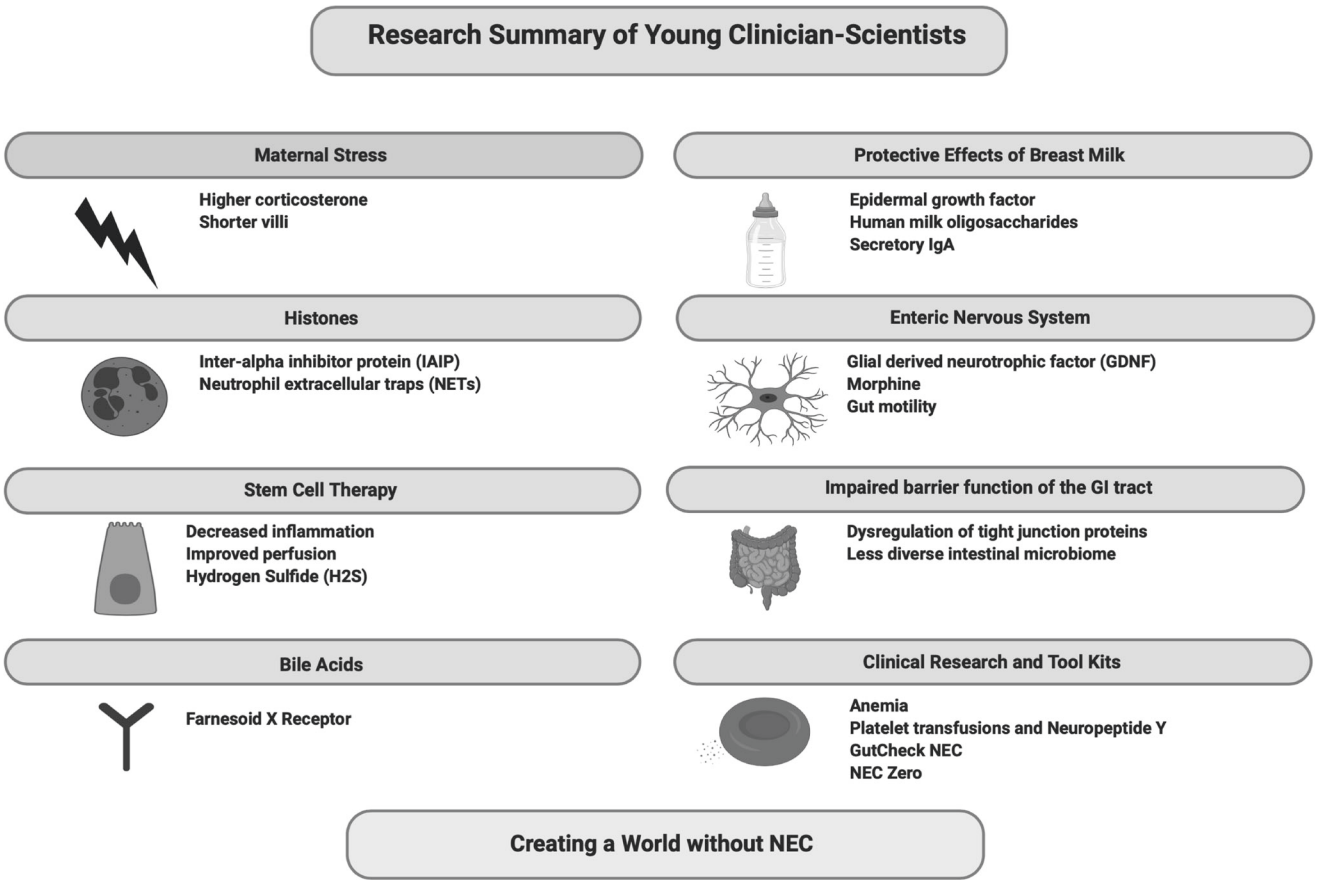

Fig. 1 Research summary of young clinician-scientists. Young clinician-scientists are eager to create "A World Without NEC." The primary focus of these investigators surrounds maternal stress, the protective ingredients in breast milk, histones, the enteric nervous system's response to injury, stem cell therapy, the microbiome, intestinal barrier function, bile acids, transfusions, and patient-centered tool kits. PKA protein-kinase A, ROCK Rho kinase, IAIP inter-alpha inhibitor protein, GDNF glial-derived neurotrophic factor, NPY neuropeptide Y, H_2S hydrogen sulfide. Figure created with Biorender.com.

the fetus.[15] Intriguingly, maternal cortisol levels are inversely proportionate to gestational age and birth weight,[16] all of which could predispose infants to NEC development. However, these effects of early fetal cortisol exposure on the neonatal immune system and subsequent mechanisms are not clear. To understand these effects on the developing immune system, Martin et al. utilized a well-established restraint stress model, in which pregnant mice were placed in a ventilated 50 ml conical tube for 1 h daily. Using this model, they observed that serum levels of the stress hormone corticosterone are significantly higher in 2-week-old newborn mice and their intestines have a phenotype of shorter intestinal villi and crypts. In addition, pups from stressed mothers had decreased levels of the intestinal stem cell marker LGR5.[17] Taken together, this suggests that in the setting of maternal stress, offspring develop altered intestinal architecture and growth, which can play a significant role in the development of intestinal immunity.

Sentinel work from Warner et al. demonstrated that premature infants that develop NEC have a relative abundance of *Gammaproteobacteria* (Gram-negative facultative bacilli) and a decrease in the anaerobic bacteria *Negativicutes* prior to disease onset.[18] These data suggest that prenatal maternal and environmental factors may shape the developing fetal and neonatal immune systems. To begin to understand the mechanisms involved, investigators have demonstrated that 2-week-old pups from stressed mothers had intestinal dysbiosis and complement dysfunction (unpublished data). The complement pathway is a critical component of the innate immune system and is activated by antigen/antibody binding.[19] Complement activation within the intestine results after intestinal immunoglobulins bind luminal pathogens. Ultimately, complement function allows the host to neutralize pathogenic bacteria by forming holes in the cell membrane.[20] Understanding the mechanisms by which maternal prenatal psychological stress alters the immunity of her offspring may allow for the development of novel therapies that could prevent neonatal intestinal dysfunction.

Signaling pathways involved in necrotizing enterocolitis

Elucidating the signaling pathways that regulate the uncontrolled innate and adaptive immune responses in NEC is of particular importance.[21] Previous studies by Good et al. demonstrated that activation of epidermal growth factor (EGF) receptor signaling with the EGF in amniotic fluid attenuated NEC-like intestinal injury by inhibiting the signaling of the innate immune receptor toll-like receptor 4 (TLR4).[22] They also demonstrated that the EGF,[23] human milk oligosaccharides (HMOs),[24] and probiotics[25] found in breast milk[26] are essential in protecting against intestinal inflammation in animal models of NEC. Notably, HMOs are an intense area of investigation from several laboratories and consistently demonstrate protection against NEC.[27–29]

The translational significance of these findings is also being pursued through the development of a large multicenter NEC biorepository in the United States, similar to the U.K. Biobank[30] for the dedicated pursuit of molecular indicators of disease.[31] Using a personalized medicine approach, young investigators are evaluating the susceptibility of infants to

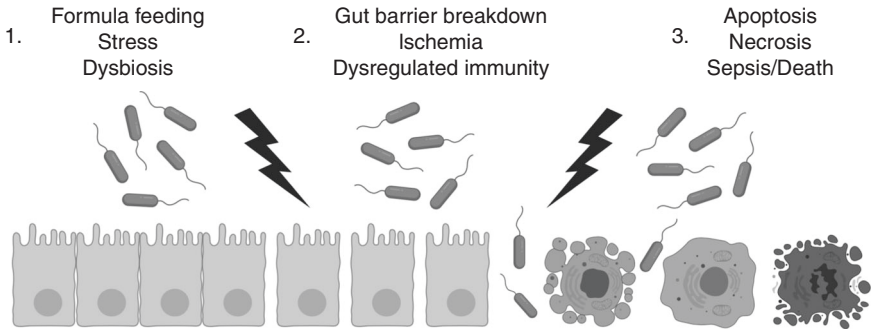

Fig. 2 Pathogenesis of necrotizing enterocolitis (NEC). NEC is thought to result from a combination of prematurity, formula feeding, and dysbiosis. Together, these stressors eventually lead to a dysregulated immune response, gut barrier failure, and intestinal ischemia. This results in intestinal epithelial cell apoptosis and necrosis as well as sepsis, multiorgan failure, and death. Figure created with Biorender.com.

NEC,[32] and in particular, seek to advance the knowledge with regards to the biological signatures[33] and genetic predisposition[34,35] of infants with NEC. Precision medicine approaches performed include "NEC-on-a chip," which utilizes a microfluidic device for the in vitro study of 3D cellular environments to test various therapeutics on multiple cell types derived from the human intestine.[36] Towards this end, investigators have discovered that by manipulating the cytokine environment within the intestine, the NEC-mediated inflammatory response can be attenuated. Current pursuits with the Food and Drug Administration are underway to pursue a clinical trial related to these findings to provide a novel therapy for NEC.

Other molecular mechanisms, such as that of extracellular histones, are major mediators and therapeutic targets in sepsis, the systemic inflammatory response, and thrombosis.[37–39] Histones are released into the extracellular environment actively, through neutrophil extracellular traps (NETs), and passively, during cell death.[40] While extracellular histones play an important role in innate immune defense, they also induce extensive "collateral damage." Neutralizing histones with specific antibodies prevented thrombocytopenia, platelet activation, and improved survival in mice administered lipopolysaccharide or *E. coli*.[38,39] Investigators showed that an endogenous serine protease inhibitor, known as inter-alpha inhibitor protein (IAIP), binds to extracellular histones and neutralizes their toxic effects through high molecular weight hyaluronic acid and chondroitin sulfate.[41] Notably, levels of IAIP are significantly decreased in infants with NEC[42,43] and sepsis.[42] Ongoing studies to investigate the role of histones and NETs in NEC will determine whether histone levels could serve as biomarkers or prognostic indicators for NEC. Additional studies are planned to investigate the effects of histone inhibitors, such as IAIP, as well as NET formation in NEC.

Microbiome and gut barrier function

The intestinal microbiome, and the balance between beneficial and harmful bacteria, play a significant role in neonatal intestinal health.[44] Studies have investigated the impact that bacterial administration has on enterocyte apoptosis and the signaling mechanisms involved.[45] Investigators have appreciated that the cyclic adenosine monophosphate pathway (cAMP) is an important mediator of the inflammasome and promotion of apoptosis. In addition, protein-kinase-A (PKA) and Rho-kinase (ROCK) are critical in the pathogenesis of NEC.[45,46] PKA, a serine/threonine kinase, is the best characterized downstream target of cAMP,[45] while ROCK, also a serine/threonine kinase, affects apoptosis and tight junctional integrity.[46] By inhibiting these pathways, investigators have been able to reduce cellular apoptosis as well as the severity of NEC in animal models.

The intestinal barrier prevents the passage of microorganisms, xenobiotics, and various antigens into the host circulation via tight junctions between the epithelial cells.[47] The interplay of the ROCK and PKA pathways on intestinal tight junctions appears to be significant.[48] Studies have suggested that inhibition of the Rho-kinase pathway improves gut barrier integrity and increases the tight junction occludin in the small intestine.[46]

Of particular interest is defining the role of bile acids and their metabolites on intestinal integrity, as well as how the microbiome alters the bile acid pool. Golden et al. recently demonstrated that ursodeoxycholic acid was able to protect against intestinal injury. This specific bile acid was able to promote intestinal restitution in both in vitro and in vivo models through COX-2 and EGFR signaling pathways.[49] This group has also shown that the toxic metabolites of bile acids, specifically deoxycholic acid, inhibit intestinal cell proliferation via blockade of the EGFR/ERK pathway.[50] Given that these secondary bile acids formed as a result of interaction with microbiota in the intestinal lumen, these studies may provide a mechanism through which an altered microbiota contributes to normal or abnormal intestinal epithelial cell proliferation.

Additional work using global and tissue-specific knockouts, as well as various animal models of intestinal injury, is planned to investigate the roles of the farnesoid X receptor and the G-protein-coupled bile receptor (TGR5) in maintaining gut homeostasis. By using natural and pharmacological agonists and antagonists, the mechanism of how these receptors control macrophage inflammatory states and intestinal barrier integrity can be elucidated.

Enteric nervous system

Understanding the effect of the enteric nervous system on intestinal physiology and pathology is also essential to the study of NEC. Enteric glial cells are an essential component of the enteric nervous system and are responsible for intestinal motility, endocrine secretion, and immune development. The enteric nervous system displays significant injury in both human and animal specimens following NEC, and in humans, these abnormalities often persisted after the acute injury.[51] Segura et al. discovered an essential mechanistic understanding of the behavior of enteric glia. They demonstrated that the effect of lysophosphatidic acid mediated the stimulation of calcium secretion on enteric glial cells and endothelial cell differentiation gene expression.[52] They also observed that lysophosphatidylcholine alters enteric monolayer permeability,[53] which may have implications on NEC pathogenesis.

Enteric glial cells also produce multiple different growth factors, including glial-derived neurotrophic factor (GDNF), which is needed for proper intestinal function. Investigators have shown that morphine, a common medication given to neonates, activates the mu-opioid receptor and subsequently decreases GDNF function and epithelial barrier integrity.[54] This is important as epithelial barrier integrity is disrupted during NEC and other neonatal intestinal pathologies.

Mesenchymal stem cell therapy

Mesenchymal stem cells have become a popular experimental treatment option in several diseases including myocardial infarction and stroke.[55,56] The use of stem cells for the prevention of NEC has been proposed by several investigators.[57,58] Mesenchymal stem cells, which can differentiate into cartilage, bone, fat, and other tissues of mesenchymal origin, are immunogenic, exhibit antioxidant properties,[59] enhance neovascularization,[60] reduce inflammation,[61] and improve functional recovery of ischemic tissues.[62] Administering mesenchymal stem cells to animals with intestinal ischemia or NEC has been shown to improve survival and mesenteric perfusion while limiting histological injury.[63] The tissue source of the stem cells (bone marrow, adipose, umbilical cord) also does not appear to alter their protective power.[57,64]

The exact mechanism by which stem cells provide their protection is not well understood. Multiple mechanisms of action have been postulated, including incorporation and differentiation, as well as heterotopic cell fusion. Still, it is likely that the stem cells release paracrine factors that decrease cellular apoptosis, limit inflammation, and improve functional recovery after injury.[65,66] Hydrogen sulfide gas, previously considered as a toxic agent, could be a viable paracrine factor with potent biological properties. This gasotransmitter can increase mesenteric perfusion and limit inflammation in models of intestinal ischemia and NEC.[67] Moreover, endothelial nitric oxide synthase (eNOS) is a critical mediator of H_2S protection.[68,69] Previous studies have shown that H_2S interacts with an important cysteine moiety on eNOS, that when persulfidated, allows eNOS to dimerize and increase nitric oxide production.[69] Understanding how stem cells mediate intestinal protection is critical prior to use in clinical applications.

CLINICAL RESEARCH ADVANCES IN NECROTIZING ENTEROCOLITIS

GutCheckNEC and NEC Zero

In addition to multiple basic science advancements by young investigators, there have been significant advances in clinical and outcomes-related research on NEC. Technical advances and parent-engaged solutions to decrease the burden of NEC in the neonatal intensive care unit (NICU) are of utmost importance. Specifically, the development of the diagnostic strategy called GutCheckNEC for the timely recognition of NEC has been quite advantageous.[70] This tool kit creates a weighted composite risk score for NEC and was designed using a cohort of over 58,000 infants. It found that there were nine independent risk factors for developing NEC (gestational age, history of packed red blood cell (RBC) transfusion, unit NEC rate, late-onset sepsis, multiple infections, hypotension treated with inotropic medications, Black or Hispanic race, outborn status, and metabolic acidosis), as well as two protective factors (human milk feeding and probiotics). The highest contributing factor to the GutCheckNEC score was the unit's NEC rate, which carried a risk three times higher than the infant's gestational age. GutCheckNEC scores (0–58) were very good in their ability to predict surgical NEC (area under the curve (AUC) = 0.84, 95% confidence interval (CI) 0.82−0.84) and NEC leading to death (AUC = 0.83, 95% CI 0.81−0.85). The ability to predict medical NEC was good, but not as dependable as that for surgical NEC (AUC = 0.72, 95% CI 0.70−0.74). The GutCheckNEC platform is currently being developed for implementation into electronic medical records.[71]

An additional tool kit that has been developed by this same research team is designed to decrease NEC incidence to zero and is appropriately called the NEC-Zero project.[72] This tool kit integrates parent engagement and promotes the administration of mother's milk to their infants, utilizes the tool GutCheckNEC that provides structured communication when deterioration is anticipated or expected, limits the duration of antibiotic courses, and promotes strict adherence to standardized feeding regimens. The NEC-zero initiative performed a meta-analysis and found a significant decrease in NEC in the premature population when mother's own milk was used, a 64% lower odds of NEC when donor human fortifiers were used over bovine fortifiers, and a 67% reduction in NEC when standard feeding protocols were used. Furthermore, the NEC-zero team reviewed other collective studies and noted an increased risk of NEC when empiric antibiotics were used beyond 4 days of negative cultures, as well as increased risk of NEC when histamine blockers were used (OR = 1.78, 95% CI 1.4, 2.27, $p < 0.00001$).[72]

Together, this combination of critical measures in the NICU will hopefully improve the early recognition of deterioration related to NEC. These studies provide clinical decision-making support guided by an implementation science framework to prevent NEC and to improve timely diagnosis.

Transfusions

The relationship between common exposures in the NICU, specifically RBC, platelet transfusion, and probiotic use, may also play a role in NEC risk. Studies provide conflicting evidence on the effect of RBC transfusion and anemia on NEC.[73–75] However, Patel et al.[74] showed in a prospective observational study of 598 very low birth infants that severe anemia, not RBC transfusion, was associated with an increased risk of NEC. They observed that the potential mechanism by which anemia increases NEC risk is through the promotion of intestinal inflammation and barrier disruption via macrophage activation.[76]

Investigators have also observed that platelet suspensions in storage contain a substantial amount of proinflammatory mediators such as neuropeptide Y (NPY).[77] Neuropeptide Y is a potent vasoconstrictor, enhances neutrophil adhesion to endothelial cells, and stimulates macrophage adhesion, chemotaxis, phagocytosis, and superoxide anion production. In addition, NPY has detrimental gastrointestinal effects,[78] and may partially explain the increased risk of adverse outcomes in infants with NEC who receive platelet transfusions. Further studies are underway to assess other transfusion-related effects in the NICU.

CONCLUSION

Although many great scientific discoveries have occurred in the field of NEC research over the last several years, their ability to make a clinical impact in reducing the incidence or severity of NEC remains largely unknown. The next generation of basic science and clinical investigators are eager to make an impact on NEC incidence through novel diagnostic tools, therapies, and clinical risk score calculators. We are hopeful that as a result of their diligent studies, the care of infants with NEC will improve over the next decade, and we can build "A World Without NEC" together.

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ADDITIONAL INFORMATION

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