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Immunological and Hematological Abnormalities in Necrotizing Enterocolitis

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Abstract

Objective—Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in preterm infants born prior to 32 weeks gestation or with a birth weight less than 1500 grams. In this article, we review the immunological and hematological abnormalities associated with NEC.

Methods—A literature search was performed using the databases PubMed, EMBASE, and Scopus. Keywords were shortlisted from PubMed's Medical Subject Heading (MeSH) thesaurus prior to the actual search and combined with text words likely to be used in titles and abstracts.

Results—During NEC, disruption of the gut mucosal barrier results in bacterial translocation that triggers a damaging local and systemic inflammatory response. The premature intestine is at risk of inflammatory injury because of developmental limitations in both the innate and adaptive arms of the mucosal immune system. The systemic inflammatory response during NEC is characterized by elevated circulating cytokine levels and hematological abnormalities such as thrombocytopenia, increased or decreased neutrophil counts, low monocyte counts, and anemia. These findings might convey important diagnostic and prognostic information.

Conclusions—The premature intestine displays a 'pro-inflammatory' bias that increases the risk of NEC. Consistent patterns of hematological changes are frequently encountered in infants with NEC and may provide important diagnostic and prognostic information.

Keywords

NEC; intestinal injury; blood counts; inflammation; macrophages; signaling; neutrophils; platelets; monocytes

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Introduction

Necrotizing enterocolitis (NEC) is a devastating inflammatory condition of the gastrointestinal disease that afflicts 4-11% of very-low-birth-weight (VLBW) infants and is a leading cause of morbidity and mortality in this population.¹⁻³ The pathogenesis of NEC is complex and is not well-understood. Clinical studies associate NEC with diverse pre- and postnatal factors such as placental insufficiency, prolonged/ premature rupture of membranes, chorioamnionitis, gut ischemia, altered bacterial colonization, viral infections of the gastrointestinal tract, bacterial overgrowth, and red blood cell transfusions.^{1,2} Although a unifying mechanism may not be readily evident in all the risk factors of NEC, some of these conditions presumably alter/disrupt the intestinal epithelial barrier to allow bacterial translocation from the lumen into the sub-epithelial *lamina propria*, where these bacteria or their products trigger an exaggerated, damaging mucosal inflammatory reaction.^{1,4} In severe intestinal injury, bacterial products and/or the inflammatory mediators may spill into the bloodstream, causing a systemic inflammatory response and multi-organ dysfunction.⁵ In this article, we review the immunological aspects of the pathogenesis of NEC and its hematological manifestations. A literature search was performed using the databases PubMed, EMBASE, and Scopus. To minimize bias, keywords from PubMed's Medical Subject Heading (MeSH) thesaurus were shortlisted prior to the actual search and combined with text words likely to be used in titles and abstracts.

A. Immunological aspects of NEC

Mucosal sensitivity to bacterial products in the premature intestine—Several lines of evidence indicate that luminal bacteria play a central pathophysiological role in NEC: (1) bacterial overgrowth, and *pneumotosis intestinalis*, the accumulation of gaseous products of bacterial fermentation in the bowel wall, are prominent histopathological findings in NEC; (2) ischemic intestinal injury in the sterile *in utero* microenvironment may cause strictures or atresia, whereas similar insults after postnatal bacterial colonization may increase the risk of NEC;⁶ (3) enteral antibiotics can reduce the incidence of NEC and NEC-related mortality.⁷ Although specific bacterial species have not been causally-associated with NEC, infants who go on to develop NEC often display a microbial imbalance ('dysbiosis') with abnormal abundance of gammaproteobacteria (*Enterobacteriaceae* and *Pseudomonadaceae*) and Clostridia, but with fewer Firmicutes, the dominant Gram-positive bacterial phylum in infants who do not develop NEC.^{8,9} Gammaproteobacteria express lipopolysaccharides and other products that have unique microbial-associated molecular patterns (MAMPs), which engage with the Toll-like receptors on mucosal cells to activate downstream inflammatory signaling. As discussed in the following section, the developing intestine is at risk of inflammatory injury because of two major factors: (1) the epithelium and mucosal immune cells in the developing intestine are uniquely sensitive to bacterial products; and (2) paucity of adaptive mechanisms that normally limit the interaction of luminal bacteria with the mucosa.

Intestinal Epithelium—Fetal intestinal epithelial cells (IECs) express a variety of innate response receptors and display a 'hyperactive' Toll-like receptor (TLR)-activated transcriptional program, which manifests with exaggerated expression of cytokines and

inflammatory mediators.¹⁰⁻¹⁵ This epithelial sensitivity to bacterial products correlates with high levels of expression of TLR2, TLR4, downstream adaptors such as the myeloid differentiation primary response gene 88 (MyD88) and tumor necrosis factor receptor associated factor 6 (TRAF6), and the transcriptional regulator nuclear factor kappa B 1 (NF- κ B1). In addition, fetal IECs are developmentally-deficient in many negative regulators of TLR signaling, such as the single Ig interleukin-1-related receptor (SIGIRR), interleukin-1 receptor-associated kinase (IRAK)-M, tumor necrosis factor- α -induced protein 3 (TNFAIP3), and the Toll-interacting protein (TOLLIP).¹⁶ Other reports implicate low levels of inhibitor of κ B (IkB).¹⁷ NF- κ B signaling may also be dampened after full-term birth due to post-transcriptional down-regulation of IRAK-1, another key intermediate in TLR signaling.¹⁸

Goblet cells start producing mucus by week 12, but this mucus layer contains low amounts of the protective mucin 2 (muc2).¹⁹ Muc2 expression is further compromised during NEC, possibly through bile acid-mediated mucosal injury.²⁰ Paneth cells appear at about the same time as goblet cells and produce antibacterial proteins such as lysozyme and α -defensins. The number of Paneth cells is low in the premature intestine and increases with maturation until adulthood.²¹ In rodents, Paneth cell ablation may trigger NEC-like injury in the developing intestine.²² Developmental differences in secretory IgA are described below.

Intestinal macrophages and dendritic cells—Macrophages first appear in the fetal intestine at 11-12 weeks. The resident macrophage population increases rapidly during the 12-22 week period and then a slower pace through early childhood.²³⁻²⁵ These cells play a critical role in host defense as the first phagocytic cells of the innate immune system to encounter luminal bacteria that breach the epithelium and gain access to the *lamina propria*. In the adult intestine, macrophages display avid phagocytic and bacteriocidal activity but are attenuated in their inflammatory responses,²⁶ a unique adaptation that minimizes inflammation in the gut mucosa despite the close proximity to luminal bacteria. We have shown that macrophage precursors undergo inflammatory downregulation upon recruitment to the intestinal mucosa under the influence of transforming growth factor-beta (TGF- β), particularly the TGF- β ₂ isoform, present in the local extracellular matrix.⁴ In the midgestation intestine, which is developmentally-deficient in TGF- β bioactivity, macrophages are yet to acquire this inflammatory anergy and produce inflammatory cytokines upon exposure to bacterial products.^{4,27} These inflammatory responses of the mucosal macrophages likely add to the risk of mucosal injury during NEC. Transgenic mice with defects in tissue-specific differentiation of gut macrophages due to loss of TGF- β signaling or mutations in the signal transducer and activator of transcription (STAT) pathway are at increased risk of inflammatory mucosal injury.^{4,28}

There are very limited data on fetal/neonatal intestinal DCs.²³ HLA-DR⁺ DC-like cells are detected in both *lamina propria* and the Peyer's patches after 14 weeks, but these cells may have some overlap with *lamina propria* macrophages.²⁹ In rats and non-human primates, DCs have been noted in the fetal lamina propria as well in Peyer's patches.^{30,31} The functional importance of these DCs in NEC remains unclear, although DCs were proposed as a cause of epithelial damage in mice with *Cronobacter sakazakii*-induced NEC-like injury.³²

Mucosa-associated lymphoid tissue (MALT)—Peyer's patches first appear at 11 weeks and develop during mid-late gestation^{33,34} (Table 1). At birth, these lymphoid aggregates are structurally complete but 'naïve', as germinal centers take a few weeks to develop.³⁵ The number of Peyer's patches in the ileum increases as a function of gestational maturation, and premature infants born prior to 32 weeks' gestation may have only half as many Peyer's patches as their full-term counterparts.³⁶ Other MALT structures, such as the lymphoid aggregates in the vermiform appendix, develop after birth following postnatal bacterial colonization.^{37,38}

T-lymphocytes in the *lamina propria* and intra-epithelial compartments—T-cells are first seen in the fetal intestine at 12-14 weeks' gestation.³⁹ Outside the MALT, intestinal T-cells are distributed in the lamina propria and the intra-epithelial compartments. Lamina propria lymphocytes (LPLs) develop in the fetal intestine in utero and reach densities similar to the full-term intestine by 19-27 weeks' gestation.³⁹ In contrast, intra-epithelial lymphocytes (IELs) expand mainly after birth.^{40,41} About 10-30% IELs express the $\gamma\delta$ T-cell receptor³⁴ and may serve specialized roles in epithelial homeostasis, cytotoxic activity, and antimicrobial immunity.⁴²⁻⁴⁴ The fetal intestine also shows some early-lineage T-cell populations, indicating that T-cells may also develop locally in a mucosal, extra-thymic pathway.^{34,39,40,45-49} In premature infants, the T-cell receptor shows a polyclonal repertoire that undergoes gradual restriction to a mature, oligoclonal pattern, possibly due to the emergence of a few dominant clones specific for commensal bacteria.^{50,51} Although the role of T-cell subsets in NEC remains unclear, there is an overall paucity of T-cells in surgically-resected bowel affected by NEC and in murine models of NEC-like injury.⁵²⁻⁵⁴ Consistent with this deficiency in T-cell development, infants who go on to develop NEC had lower circulating levels of T-lymphokines such as IL-2, IL-18, CCL4, and CCL5 in the preceding weeks than other premature infants who did not develop NEC.⁵⁵

FOXP3⁺ T regulatory cells (Treg) can be seen in both the small intestine and colonic mucosa as early as 23 weeks' gestation.⁵⁶ The role of Tregs in mucosal homeostasis is evident from the early development of enteropathy in patients lacking this subset of regulatory immune cells due to FOXP3 mutations (IPEX syndrome).⁵⁷ Interestingly, excessive innate immune activation can suppress Treg function in the preterm intestine.⁵⁸ Tregs can act via several distinct mechanisms, such as expression of anti-inflammatory cytokines (IL-10, IL-35, TGF- β); granzyme- and perforin-mediated cytotoxicity or induction of apoptosis in T-effector cells; and inhibition of dendritic cell maturation.⁵⁹ Compared to gestational-age matched non-NEC controls, infants with surgical NEC show decreased ratios of Tregs to effector T cells in the ileal mucosa.⁶⁰

B-cells and secretory immunoglobulins—The first B-cells are seen in the *lamina propria* at 14 weeks' gestation and display a mature B-cell phenotype similar to the thymic B-cells (CD20⁺ IgM⁺ IgD⁺ light chain⁺).³⁴ Some pre-B-cells (IgM⁺ light chain⁻ CD20⁻) may also be seen, indicating that the mucosa may serve as an alternative site for B-cell development.⁶¹ During the 2nd postnatal week, some B cells in both the *lamina propria* and the MALT⁶² undergo IgA class-switch.^{63,64} The number of IgA⁺ plasma cells reaches adult

levels at 2 years, although serum IgA concentrations may not reach adult levels until the 2nd decade.⁴⁰

Secretory IgA (sIgA) is first detected in mucosal secretions at 1-8 weeks after birth.⁶⁵⁻⁶⁸ In premature infants, sIgA may first appear in secretions at a similar chronological age as in full-term infants, although the concentrations are usually lower as sIgA concentrations rise as a function of post-menstrual age.^{69, 70, 30,31} The IgA responses may also be functionally less robust with a predominance of monomeric (instead of polymeric) sIgA^{71,72} and IgA1 (instead of the sIgA2) sub-class.⁷³ Premature infants also show global abnormalities in their immunoglobulin responses such as reduced antigen affinity, polyreactivity, and autoreactivity.^{74,75} In addition, immunoglobulin heavy chains have short complementarity-determining regions in premature neonates,⁷⁶ which markedly lowers the potential antibody diversity available to these infants.⁷⁶

During the neonatal period, colostrum provides an important alternative source of sIgA.⁷⁷ Milk antibodies may contribute about 0.5-1 g/day throughout lactation (comparable to the 2.5 g/day being produced by a 65 kg adult), and are directed against antigens present in the environment shared by the mother-infant dyad.⁷⁸ Immune cells stimulated by antigens in the maternal intestine and bronchial mucosa have been shown to migrate specifically to the mammary gland.^{77,79-81} Interestingly, sIgA levels in colostrum and milk of mothers of preterm neonates may be higher than in mothers who delivered at full-term.⁸²

Platelet-activating factor (PAF)—PAF, an endogenous phospholipid mediator produced by platelets, leukocytes and endothelial cells, is believed to represent a final, common effector in diverse forms of intestinal injury.⁸³ In rodent models, exogenous administration of PAF induces systemic hypotension, microvascular permeability, and gut mucosal necrosis, which is often most prominent in the ileum.⁸⁴ In NEC, the role of PAF is supported by several lines of evidence: (1) compared to age-matched controls, infants with NEC have elevated plasma PAF levels and decreased plasma activity of PAF-acetylhydrolase (PAF-AH), the enzyme that normally degrades PAF;^{85,86} (2) human milk contains PAF-AH;⁸⁷ (3) NEC-like injury was prevented in rat pups and piglets by PAF-receptor antagonists;^{88,89} (4) enteral administration and intravenous infusion of recombinant PAF-AH prevented NEC in rats;⁹⁰ (5) PAF-AH knock-out mice are more susceptible to NEC-like injury;⁹¹ and (6) ileum, the site of predilection of NEC, shows highest regional expression of PAF receptors in the gastrointestinal tract.⁹²

Nitric oxide (NO)—NO is a key regulator of vascular tone, inflammation, neurotransmission and tissue restitution and repair.⁹³ There are three isoforms of NO synthases (NOS): endothelial NOS (eNOS) and neuronal NOS (nNOS), which generate low (picomolar) concentrations of NO, and inducible NOS (iNOS), which produces high (micromolar) concentrations of NO. Low levels of NO play physiological roles in epithelial homeostasis and water absorption. However, at high concentrations, NO reacts with superoxide to form peroxynitrite, a highly toxic intermediate that disrupts protein conformation, alters cellular metabolism, and can block epithelial restitution and induce apoptosis in diverse cell types.^{94,95} Increased iNOS expression was noted in IECs in

surgically-resected intestinal tissue samples of NEC.⁹⁶ Formula-fed rat pups show increased iNOS expression in the gut mucosa, which is further increased during NEC-like injury.^{97,98}

Reactive oxygen species (ROS)—ROS are believed to play a key pro-inflammatory role in NEC. The xanthine oxidase/dehydrogenase system is one of the main producers of ROS in the intestine. Xanthine dehydrogenase is constitutively expressed in IECs and normally catalyzes the transformation of xanthine into uric acid ($\text{Xanthine} + \text{H}_2\text{O} + \text{NAD} \rightarrow \text{Uric acid} + \text{NADH} + \text{H}^+$). However, during ischemia, xanthine dehydrogenase is converted into xanthine oxidase, which leads to xanthine oxidation into uric acid and superoxide ($\text{Xanthine} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{Uric acid} + 2\text{O}_2^- + 2\text{H}^+$). ROS activates the intestinal mitochondrial apoptotic signaling pathway during oxidative stress and leads to IEC apoptosis *via* the p38 mitogen-activated protein kinase.^{99,100} Xanthine oxidase and superoxide are implicated in intestinal reperfusion injury and PAF-induced bowel necrosis, as allopurinol, a xanthine oxidase inhibitor, has been shown to be protective in these models.^{101,102}

Transforming growth factor- β —We have shown recently that premature infants who developed NEC had lower circulating TGF- β_1 levels than their non-NEC controls since birth.⁵⁵ In this study, blood TGF- β_1 concentrations <1380 pg/mL on the first postnatal day predicted future onset of NEC with 64% accuracy. Although several biomarkers (such as the inter-alpha inhibitor protein, intestinal fatty acid-binding protein, hexosaminidase, proapolipoprotein CII, and *des*-arginine serum amyloid A) have been identified for their ability to discriminate between confirmed NEC and other causes of feeding intolerance,¹⁰³⁻¹⁰⁶ blood TGF- β_1 is the first biomarker to estimate the risk of NEC in a newly-born premature infant. Even though genetic factors do not seem to be a major contributor to the risk of NEC,¹⁰⁷ the ability of blood TGF- β_1 to identify infants at risk of NEC on day 1 is surprising because it indicates that the risk-stratification for NEC may start early, possibly *in utero*, before most hypoxic-ischemic events of the early neonatal period would have occurred or the microbial flora would have been established in the intestinal lumen. These findings are consistent with our previous reports indicating that preterm neonates may be at risk of inflammatory mucosal injury and NEC because of a developmental deficiency of TGF- β bioactivity in the intestine.^{4,27} The reasons for low blood TGF- β levels in infants who developed NEC were unclear, although a few interesting possibilities merit consideration. The first is increased peripheral uptake of TGF- β to compensate for low tissue expression. We have previously shown that TGF- β expression is decreased in healthy margins of tissues resected for NEC.⁴ A second explanation could be based on an assumption that the developing intestine is a major contributor to circulating TGF- β levels. Because TGF- β expression increases in the intestine as a function of gestational maturation,⁴ lower tissue expression of TGF- β in infants who developed NEC could conceivably reflect an underlying state of arrested mucosal development, where low TGF- β expression may indicate persistence of a cytokine profile corresponding to an earlier, less mature developmental epoch. In support of this possibility, we detected lower TGF- β levels in NEC group than in controls at the same post-menstrual age. Finally, infants who developed NEC may constitutively produce less TGF- β than controls due to genetic/epigenetic factors. Premature baboons with spontaneously-occurring NEC-like injury

showed histone methylation in the TGF- β nucleosome, a repressive modification associated with facultative heterochromatin assembly and transcriptional silencing.²⁷

Cytokine responses associated with NEC—Several studies have investigated the cytokine response during NEC. Cytokines are attractive not only as potential biomarkers of the primary disease and the severity of inflammation in NEC, but also for possible pathophysiological roles in intestinal injury. At the tissue level, increased interleukin (IL)-1 β and tumor necrosis factor expression have been detected in surgically-resected tissue specimens of human NEC.¹⁰⁸⁻¹¹⁰ In pre-clinical models, NEC-like intestinal injury induces chemokines such as the CXC-motif ligand 5 (CXCL5), CXCL1, and CXCL2, CC-motif ligand (CCL) 2, CCL3, CCL5, CCL20, IL-1 β , IL-6, IL-12, and IL-18.^{54,109-113} In plasma, the diagnosis of NEC in premature infants was associated with elevated IL-1 β , IL-1 receptor antagonist, IL-6, IL-10, CXCL5, IL-8/CXCL8, CCL2, CCL3, neurotrophin-4, and C-reactive protein.^{55,114-116} In one study, plasma IL-8/CXCL8, epithelial-derived neutrophil chemoattractant-78/CXCL5, and IL-10 levels were higher in infants with NEC than in those with sepsis syndrome.¹¹⁷ IL-6 has a shorter half-life and may be detectable only for short periods after onset of NEC,¹¹⁸ whereas IL-8/CXCL8 levels show sustained elevation¹¹⁶ and correlate with the extent of NEC (NEC-totalis), the need for surgical resection of bowel, and higher 60-day mortality in infants with NEC.^{114,115,117}

B. Hematological manifestations of NEC—Hematological abnormalities occur frequently in infants with NEC, and in many, carry important diagnostic and prognostic information. These abnormalities remain mild and require no intervention in most infants with NEC, although some with severe disease may require blood product transfusions and monitoring for complications such as hemorrhage, infections, anemia, and even death.^{5,119}

Platelet counts—Thrombocytopenia, defined as a platelet count $<150 \times 10^9/L$, is seen at clinical presentation in 50-95% of infants with NEC, and many have platelet counts in the range of $30 \times 10^9/L$ to $60 \times 10^9/L$.¹²⁰⁻¹²² Most neonates with advanced NEC develop thrombocytopenia within 24-72 hours of onset of disease.¹²¹⁻¹²³ The severity of thrombocytopenia correlates with Bell's clinical stage of NEC, and a rapid drop in platelet counts to $<100 \times 10^9/L$ is a sensitive (although not specific) predictor of bowel gangrene or the need for surgical intervention.^{120,124} Ververidis *et al.*¹²⁴ reviewed the clinical course of 58 neonates with advanced NEC (Bell's stages II or III, gestational age 23-41 weeks) and noted platelet counts $<150 \times 10^9/L$ in 54 (93%) infants and counts $<100 \times 10^9/L$ in 51 (88%). Most infants who showed a rapid drop in platelet counts to $<100 \times 10^9/L$ were bacteremic. In patients without intestinal necrosis, platelet counts recovered to normal levels within 24 hours.¹²⁴ These data are similar to previous reports by Hutter *et al.*,¹²¹ Patel *et al.*,¹²² O'Neill *et al.*,¹²⁰ and Kenton *et al.*¹²³

Platelet counts provide important predictive information for the outcome in patients with NEC. In survivors, the median time to recovery to a platelet count greater than $150 \times 10^9/L$ is about 7 to 10 days.^{121,122} Infants who die of NEC tend to have lower nadir platelet counts than survivors. In the cohort described by Ververidis *et al.*,¹²⁴ patients who died of NEC had a lower nadir in platelet counts than the survivors. All patients with a platelet count greater than $100 \times 10^9/L$ during the course of the disease survived. Ragazzi *et al.*¹²⁵ recorded

platelet counts $<150 \times 10^9/L$ in 86% of non-survivors vs. 43.3% of the survivors ($p<0.0001$). Severe thrombocytopenia ($<100 \times 10^9/L$) was noted more frequently in non-survivors (70%) than in survivors (33%; $p<0.0001$). In another study, Kenton *et al.* reported higher median platelet counts in survivors than in non-survivors ($203 \times 10^9/L$ vs. $33 \times 10^9/L$; $p<0.001$). Severe thrombocytopenia was a predictor of mortality (adjusted OR 6.39; $p=0.002$) and NEC-related gastrointestinal complications such as cholestatic liver disease and short bowel syndrome (adjusted OR 5.47; $p=0.006$).

The primary mechanism for thrombocytopenia in NEC is widely believed to be increased platelet destruction. Indirect evidence for platelet consumption is seen in the rapid drop in platelet counts in many patients, and also in the short-lived rise in platelet concentrations following transfusions lasting less than 24-48 hours.¹²⁶ The mechanism(s) of thrombocytopenia in NEC has not been investigated in clinical studies, but existing information from animal models indicates a likely role of platelet-activating factor and circulating bacterial products such as lipopolysaccharide.¹²⁷ These mediators stimulate endothelial cells and macrophages to release inflammatory cytokines and nitric oxide, which, along with thromboplastin released from gangrenous bowel, can increase platelet activation and aggregation in the microvasculature.¹²⁰

Patients with NEC have higher plasma thrombopoietin (Tpo) concentrations than their healthy counterparts.^{128,129} Brown *et al.*¹³⁰ measured plasma Tpo concentrations, circulating megakaryocyte progenitors, reticulated platelet counts, and the percentage of reticulated platelets in the circulating platelet pool (RP%) in 20 neonates with sepsis and/or NEC. They showed elevated concentrations of Tpo and a modest increase in circulating megakaryocyte progenitors and RP% in thrombocytopenic neonates. The authors hypothesized that this dampening of thrombopoietic response may be due to increased levels of platelet factor 4, which is released from activated platelets and is a potent inhibitor of megakaryocytopoiesis.

Coagulopathy—Hutter *et al.*¹²¹ noted signs of disseminated intravascular coagulation (DIC) in 14 of their 40 infants. Many infants showed decreased plasma fibrinogen, tested positive for fibrin split products, and had elevated partial thromboplastin times. These data are similar to Patel *et al.*¹²² and Sonntag *et al.*¹³¹, who described a similar frequency of DIC in their respective cohorts.

Anemia—Patients with NEC may develop anemia due to multiple pathogenetic mechanisms. In the presence of mucosal injury, thrombocytopenia, and coagulation disturbances, occult and obvious blood loss is common. Sites of bleeding may include bloody stools, peritoneal hemorrhage, pulmonary hemorrhage, hemopericardium, myocardial hemorrhage, and intracranial hemorrhage.¹¹⁹ Iatrogenic anemia due to phlebotomy losses is an added problem. In some patients, hemolysis can cause further worsening of anemia. Thrombotic microangiopathy can cause red cell damage, which can often be seen on blood smears as tear drop cells, schistocytes, spherocytes, and acanthocytes. Hutter *et al.*¹²¹ reported red cell fragmentation in 25 of their 40 patients. Another possible cause is activation of the Thomsen-Friedenreich (T) cryptantigen on the RBC surface, a naturally-occurring antigen that is normally concealed by a layer of N-

acetylneuraminic acid but can be exposed in NEC by bacterial or other neuraminidases. T-activation can cause hemolysis and anemia in multi-transfused patients who have previously received adult blood containing anti-T antibodies^{132,133}. Studies show considerable variation in the frequency of T-activation in NEC, ranging from rare occurrences to up to a third of all patients^{119,134}. T-activation is associated with increased morbidity and mortality in NEC. Finally, infants with NEC may be at enhanced risk of inflammatory suppression of erythropoiesis. NEC tends to occur most frequently in extremely premature infants, who show an impaired erythropoietin response for a given level of anemia and are prone to develop severe anemia of prematurity¹¹⁹. The presence of inflammation following onset of NEC in these infants can further suppress erythropoietin and red cell production.

In some situations, anemia may be a risk factor for the development of NEC rather than its effect. Infants with severe anemia related to glucose-6-phosphate dehydrogenase deficiency, hemolytic disease of newborn, and in donor twins in twin-to-twin transfusion syndrome are at increased risk of NEC^{135,136}. Anemia has also been identified as a predisposing factor in patients who develop NEC following RBC transfusions. Singh *et al.*¹³⁷ investigated the association between anemia and transfusion-associated NEC in a cohort of 111 preterm infants with confirmed NEC and 222 matched controls. In a multivariate model, lower hematocrit was associated with increased odds of NEC (odds ratio 1.10, $p=0.01$) after controlling for other factors. They showed that RBC transfusions had a temporal relationship with onset of NEC, where a transfusion within the preceding 24 hours (odds ratio 7.60, $p=0.001$) and 48 hours (odds ratio 5.55, $p=0.001$) was associated with increased odds of developing NEC. Although the mechanisms by which anemia may increase the risk of NEC remain unclear, anemia has been shown to impair splanchnic perfusion and increase oxygen extraction as a compensatory mechanism¹³⁸. Anemia can also impair the normal postnatal changes in splanchnic vascular resistance¹³⁹, predisposing the developing intestine to hypoxemic-ischemic gut mucosal injury, and possibly, to NEC¹⁴⁰.

Neutrophils—Increased neutrophil counts comprise an appropriate inflammatory response in patients with mild-moderately severe disease. In contrast, neutropenia, defined as an absolute neutrophil count (ANC) $<1500/\mu\text{L}$, can be seen in severe NEC and is associated with adverse outcome.^{121,122,124,125} Patel *et al.*¹²² noted neutropenia in 14 of their 23 patients who died of NEC, compared to 6/24 of the survivors. In another study, Ragazzi *et al.*¹²⁵ noted that neutropenia in the initial blood counts at onset of NEC was associated with adverse outcome; there was a trend for a higher frequency of neutropenia in non-survivors (37%) than in survivors (25%; $p=0.136$), and thrombocytopenia and neutropenia occurred together more frequently in non-survivors (39%) than in survivors (14%; $p=0.0007$). In infants greater than 34 weeks gestation, lower ANC, higher immature neutrophil number, and greater immature: total neutrophil ratio at onset of NEC were associated with the need for surgical intervention.¹⁴¹ Although the pathophysiology of neutropenia in NEC is not well-understood, depletion of the circulating neutrophil pool due to emigration into the intestines and peritoneum, and increased margination in the microvasculature are some of the possible causes.^{121,124,125}

Macrophages and Monocytes—The cellular inflammatory response in NEC is characterized by the presence of a macrophage-rich infiltrate.⁵⁴ We have previously shown that gut macrophage populations are normally maintained through continuous recruitment of circulating monocytes and *in situ* differentiation of these cells in the *lamina propria*.^{142,143} Because preterm infants have a limited circulating monocyte pool¹⁴⁴ and lack significant reservoirs of mature monocytes in the bone marrow or elsewhere,¹⁴⁵ we asked whether the migration of circulating monocytes into NEC lesions could result in an acute drop in peripheral blood monocyte counts that may distinguish early NEC from other causes of feeding intolerance.¹⁴⁶ We compared the absolute monocyte counts (AMC) at the onset of feeding intolerance with the last available pre-symptomatic AMC. In patients who developed stage II NEC, the AMC fell from median $1.7 \times 10^9/\text{L}$ (interquartile range, IQR 0.98-2.4) to median 0.8 (IQR 0.62-2.1, $p < 0.05$), whereas in those who developed stage III NEC dropped their AMCs from median $2.1 \times 10^9/\text{L}$ (IQR 0.15-3.2) to median 0.8 (IQR 0.6-1.9, $p < 0.05$). Total white cell counts, ANC, and lymphocyte counts did not change significantly. In the control group (developed feeding intolerance for a reason other than NEC), there was no change in AMC or the white cell, ANC, or lymphocyte counts.

Eosinophils—Eosinophilia, is defined as a blood eosinophil counts exceeding the 95th percentile upper reference limit, ranges from 1180/ μL during the 1st postnatal week to 1560/ μL after 3 weeks.¹⁴⁴ Christensen *et al.*¹⁴⁷ noted eosinophilia in 54 of 275 infants (19.6%) who were evaluated for bloody stools. They hypothesized that premature infants with bloody stools and eosinophilia were likely to have atopic enteropathy and not develop the classical signs of NEC, and were therefore, more likely to have a benign course of disease. However, the rate of *pneumatoxis*, bowel resection, and death in infants with bloody stools and eosinophilia was similar to those with normal blood eosinophil counts. Interestingly, infants with eosinophilia were more likely to have received a RBC transfusion in a 48 hour period prior to passing a bloody stool. These infants with a preceding history of a blood transfusion showed a progressive rise in eosinophil counts. In contrast, infants who developed bloody stools and eosinophilia but did not have a history of a prior transfusion showed reduction in eosinophil counts once the feedings were discontinued and had a relatively benign course of disease. Although the mechanism for transfusion-associated eosinophilia is unclear, exposure to foreign antigens on donor RBCs or to medications administered during the transfusion may play a role.

Basophils—Kim *et al.*¹⁴⁸ showed that low basophil counts ($16.5 \pm 43.0/\mu\text{L}$ in the NEC group vs. $60.6 \pm 131.4/\mu\text{L}$ in controls, $p < 0.001$) at birth may predict the development of NEC. The authors speculated that these differences in basophil hematopoiesis may reflect host interaction with commensal bacteria.¹⁴⁹ However, further study is needed to understand these findings because these differences were identifiable early, likely before the intestinal microbial flora would have been established in these infants.

Lymphocytes—Lambert *et al.*¹⁵⁰ reported that infants who died of fulminant NEC within 48 hours of onset had low lymphocyte counts. They reviewed the medical records of 523 infants with a diagnosis of NEC, of which 35 (6.7%) had a fulminant course. These infants were more likely to have a blood lymphocyte count $< 4000/\mu\text{L}$ ($p = 0.018$), besides having

portal venous air, severe anemia, recent increase in feeding volume or introduction of human milk fortifier, and increased immature to total neutrophil ratio.

Nucleated red blood cells—Infants who develop NEC may have a higher number of nucleated RBCs at birth than their gestation-matched controls. Mandel *et al.*¹⁵¹ compared 23 preterm infants diagnosed with NEC with pair-matched controls who were admitted immediately after a case, had the same gestational age (± 1 week), 1- and 5- minute Apgar scores (± 1), and who did not develop NEC. Exclusion criteria included factors that may influence the ANRBC at birth, such as maternal diabetes, pregnancy-induced hypertension, major congenital malformations, chromosomal abnormalities, hemolysis and perinatal blood loss. Infants who developed NEC had higher absolute nucleated RBC counts than controls, suggesting that these neonates may have experienced intrauterine hypoxemia, which may increase the risk of NEC. These data are consistent with observations by Baschat *et al.*,¹⁵² who showed that nucleated RBC counts $>30/100$ white blood cells beyond 3 days were associated with increased risk of NEC.

Conclusions

In the premature intestine, developmental limitations in both the innate and adaptive arms of the mucosal immune system increase the risk of inflammatory injury and NEC. The systemic inflammatory response during NEC is characterized by elevated circulating cytokine levels and a consistent pattern of hematological abnormalities, which may also play a direct or indirect role in augmenting the mucosal injury. Some of these immunological and hematological abnormalities carry important prognostic information.

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References

1. Neu J, Walker WA. Necrotizing enterocolitis. The New England journal of medicine. 2011; 364:255–264. doi:10.1056/NEJMra1005408. [PubMed: 21247316]
2. Maheshwari A, Corbin LL, Schelonka RL. Neonatal Necrotizing Enterocolitis. Res Rep Neonatol. 2011; 1:39–53.
3. Patel RM, et al. Causes and timing of death in extremely premature infants from 2000 through 2011. The New England journal of medicine. 2015; 372:331–340. doi:10.1056/NEJMoA1403489. [PubMed: 25607427]
4. Maheshwari A, et al. TGF-beta(2) Suppresses Macrophage Cytokine Production and Mucosal Inflammatory Responses in the Developing Intestine. Gastroenterology. 2011; 140:242–253. doi:S0016-5085(10)01423-X [pii]10.1053/j.gastro.2010.09.043. [PubMed: 20875417]
5. Walsh MC, Kliegman RM. Necrotizing enterocolitis: treatment based on staging criteria. Pediatr Clin North Am. 1986; 33:179–201. [PubMed: 3081865]
6. Hsueh W, Caplan MS, Tan X, MacKendrick W, Gonzalez-Crussi F. Necrotizing enterocolitis of the newborn: pathogenetic concepts in perspective. Pediatric and developmental pathology: the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society. 1998; 1:2–16.
7. Bury RG, Tudehope D. Enteral antibiotics for preventing necrotizing enterocolitis in low birthweight or preterm infants. Cochrane Database Syst Rev. 2001:CD000405. doi:CD000405 [pii]. [PubMed: 11279690]

8. de la Cochetiere MF, et al. Early intestinal bacterial colonization and necrotizing enterocolitis in premature infants: the putative role of Clostridium. *Pediatric research*. 2004; 56:366–370. doi: 10.1203/01.PDR.0000134251.45878.D501.PDR.0000134251.45878.D5 [pii]. [PubMed: 15201403]
9. Wang Y, et al. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. *ISME J*. 2009; 3:944–954. doi:ismej200937 [pii]10.1038/ismej.2009.37. [PubMed: 19369970]
10. Maheshwari A, et al. Interleukin-8/CXCL8 forms an autocrine loop in fetal intestinal mucosa. *Pediatric research*. 2004; 56:240–249. doi:10.1203/01.PDR.0000133196.25949.9801PDR.0000133196.25949.98 [pii]. [PubMed: 15181190]
11. Nanthakumar NN, et al. Glucocorticoid responsiveness in developing human intestine: possible role in prevention of necrotizing enterocolitis. *American journal of physiology. Gastrointestinal and liver physiology*. 2005; 288:G85–92. doi:288/1/G85 [pii]10.1152/ajpgi.00169.2004. [PubMed: 15591589]
12. Fusunyan RD, Nanthakumar NN, Baldeon ME, Walker WA. Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes. *Pediatric research*. 2001; 49:589–593. [PubMed: 11264445]
13. Nanthakumar NN, Fusunyan RD, Sanderson I, Walker WA. Inflammation in the developing human intestine: A possible pathophysiologic contribution to necrotizing enterocolitis. *Proceedings of the National Academy of Sciences of the United States of America*. 2000; 97:6043–6048. [PubMed: 10823949]
14. Daig R, et al. Human intestinal epithelial cells secrete interleukin-1 receptor antagonist and interleukin-8 but not interleukin-1 or interleukin-6. *Gut*. 2000; 46:350–358. [PubMed: 10673296]
15. Martin CR, Walker WA. Intestinal immune defences and the inflammatory response in necrotising enterocolitis. *Semin Fetal Neonatal Med*. 2006
16. Nanthakumar N, et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PloS one*. 2011; 6:e17776. doi:10.1371/journal.pone.0017776. [PubMed: 21445298]
17. Claud EC, et al. Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. *Proceedings of the National Academy of Sciences of the United States of America*. 2004; 101:7404–7408. doi:10.1073/pnas.04017101010401710101 [pii]. [PubMed: 15123821]
18. Lotz M, et al. Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells. *The Journal of experimental medicine*. 2006; 203:973–984. doi:jem.20050625 [pii]10.1084/jem.20050625. [PubMed: 16606665]
19. Montgomery RK, Mulberg AE, Grand RJ. Development of the human gastrointestinal tract: twenty years of progress. *Gastroenterology*. 1999; 116:702–731. [PubMed: 10029630]
20. Martin NA, et al. Active transport of bile acids decreases mucin 2 in neonatal ileum: implications for development of necrotizing enterocolitis. *PloS one*. 2011; 6:e27191. doi:10.1371/journal.pone.0027191. [PubMed: 22162748]
21. Mallow EB, et al. Human enteric defensins. Gene structure and developmental expression. *J Biol Chem*. 1996; 271:4038–4045. [PubMed: 8626737]
22. Zhang C, et al. Paneth cell ablation in the presence of Klebsiella pneumoniae induces necrotizing enterocolitis (NEC)-like injury in the small intestine of immature mice. *Disease models & mechanisms*. 2012; 5:522–532. doi:10.1242/dmm.009001. [PubMed: 22328592]
23. MacDonald TT. Accessory cells in the human gastrointestinal tract. *Histopathology*. 1996; 29:89–92. [PubMed: 8818703]
24. Rognum TO, Thrane S, Stoltenberg L, Vege A, Brandtzaeg P. Development of intestinal mucosal immunity in fetal life and the first postnatal months. *Pediatric research*. 1992; 32:145–149. [PubMed: 1508603]
25. Braegger CP, Spencer J, MacDonald TT. Ontogenetic aspects of the intestinal immune system in man. *Int J Clin Lab Res*. 1992; 22:1–4. [PubMed: 1633313]
26. Smythies LE, et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *The Journal of clinical investigation*. 2005; 115:66–75. doi:10.1172/JCI200519229. [PubMed: 15630445]

27. Namachivayam K, et al. Smad7 inhibits autocrine expression of TGF-beta2 in intestinal epithelial cells in baboon necrotizing enterocolitis. *American journal of physiology. Gastrointestinal and liver physiology.* 2013; 304:G167–180. doi:10.1152/ajpgi.00141.2012. [PubMed: 23154975]
28. Takeda K, et al. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity.* 1999; 10:39–49. doi:S1074-7613(00)80005-9 [pii]. [PubMed: 10023769]
29. Spencer J, MacDonald TT, Isaacson PG. Heterogeneity of non-lymphoid cells expressing HLA-D region antigens in human fetal gut. *Clin Exp Immunol.* 1987; 67:415–424. [PubMed: 3301095]
30. Wilders MM, Sminia T, Janse EM. Ontogeny of non-lymphoid and lymphoid cells in the rat gut with special reference to large mononuclear Ia-positive dendritic cells. *Immunology.* 1983; 50:303–314. [PubMed: 6578195]
31. Makori N, et al. Functional and morphological development of lymphoid tissues and immune regulatory and effector function in rhesus monkeys: cytokine-secreting cells, immunoglobulin-secreting cells, and CD5(+) B-1 cells appear early in fetal development. *Clin Diagn Lab Immunol.* 2003; 10:140–153. [PubMed: 12522052]
32. Emami CN, Mittal R, Wang L, Ford HR, Prasadara NV. Recruitment of dendritic cells is responsible for intestinal epithelial damage in the pathogenesis of necrotizing enterocolitis by *Cronobacter sakazakii*. *Journal of immunology.* 2011; 186:7067–7079. doi:10.4049/jimmunol.1100108.
33. Finke D, Acha-Orbea H, Mattis A, Lipp M, Kraehenbuhl J. CD4+CD3- cells induce Peyer's patch development: role of alpha4beta1 integrin activation by CXCR5. *Immunity.* 2002; 17:363–373. [PubMed: 12354388]
34. Spencer J, Finn T, Isaacson PG. Gut associated lymphoid tissue: a morphological and immunocytochemical study of the human appendix. *Gut.* 1985; 26:672–679. [PubMed: 3874811]
35. Husband AJ, Gleeson M. Ontogeny of mucosal immunity--environmental and behavioral influences. *Brain Behav Immun.* 1996; 10:188–204. [PubMed: 8954593]
36. Cornes JS. Peyer's patches in the human gut. *Proc R Soc Med.* 1965; 58:716. [PubMed: 5826210]
37. Bhide SA, Wadekar KV, Koushik SA. Peyer's patches are precocious to the appendix in human development. *Dev Immunol.* 2001; 8:159–166. [PubMed: 11589311]
38. Gebbers JO, Laissue JA. Bacterial translocation in the normal human appendix parallels the development of the local immune system. *Ann N Y Acad Sci.* 2004; 1029:337–343. doi: 1029/1/337 [pii]10.1196/annals.1309.015. [PubMed: 15681775]
39. Spencer J, MacDonald TT, Finn T, Isaacson PG. The development of gut associated lymphoid tissue in the terminal ileum of fetal human intestine. *Clin Exp Immunol.* 1986; 64:536–543. [PubMed: 3491695]
40. Latthe M, Terry L, MacDonald TT. High frequency of CD8 alpha alpha homodimer-bearing T cells in human fetal intestine. *Eur J Immunol.* 1994; 24:1703–1705. [PubMed: 8026531]
41. Cerf-Bensussan N, Guy-Grand D. Intestinal intraepithelial lymphocytes. *Gastroenterol Clin North Am.* 1991; 20:549–576. [PubMed: 1917026]
42. Komano H, et al. Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. *Proc Natl Acad Sci U S A.* 1995; 92:6147–6151. [PubMed: 7597094]
43. Boismenu R, Havran WL. Modulation of epithelial cell growth by intraepithelial gamma delta T cells. *Science.* 1994; 266:1253–1255. [PubMed: 7973709]
44. Kagnoff MF. Current concepts in mucosal immunity. III. Ontogeny and function of gamma delta T cells in the intestine. *Am J Physiol.* 1998; 274:G455–458. [PubMed: 9530144]
45. Howie D, et al. Extrathymic T cell differentiation in the human intestine early in life. *J Immunol.* 1998; 161:5862–5872. [PubMed: 9834065]
46. Gunther U, et al. Phenotypic characterization of CD3-7+ cells in developing human intestine and an analysis of their ability to differentiate into T cells. *J Immunol.* 2005; 174:5414–5422. [PubMed: 15843540]
47. Spencer J, Isaacson PG, Walker-Smith JA, MacDonald TT. Heterogeneity in intraepithelial lymphocyte subpopulations in fetal and postnatal human small intestine. *J Pediatr Gastroenterol Nutr.* 1989; 9:173–177. [PubMed: 2809936]

48. Fichtelius KE. The gut epithelium--a first level lymphoid organ? *Exp Cell Res.* 1968; 49:87–104. [PubMed: 5640703]
49. Koningsberger JC, et al. TCR expression in human fetal intestine and identification of an early T cell receptor beta-chain transcript. *J Immunol.* 1997; 159:1775–1782. [PubMed: 9257840]
50. Williams AM, et al. Intestinal alpha beta T cells differentiate and rearrange antigen receptor genes in situ in the human infant. *J Immunol.* 2004; 173:7190–7199. [PubMed: 15585840]
51. Holtmeier W, Witthoft T, Hennemann A, Winter HS, Kagnoff MF. The TCR-delta repertoire in human intestine undergoes characteristic changes during fetal to adult development. *J Immunol.* 1997; 158:5632–5641. [PubMed: 9190911]
52. Anttila A, et al. T-cell-mediated mucosal immunity is attenuated in experimental necrotizing enterocolitis. *Pediatric surgery international.* 2003; 19:326–330. doi:10.1007/s00383-003-1004-7. [PubMed: 12764628]
53. Pender SL, et al. Matrix metalloproteinases in necrotising enterocolitis. *Pediatric research.* 2003; 54:160–164. [PubMed: 12736398]
54. Mohankumar K, et al. Gut Mucosal Injury in Neonates is marked by Macrophage Infiltration in Contrast to Pleomorphic Infiltrates in Adult: Evidence from an Animal Model. *American journal of physiology. Gastrointestinal and liver physiology.* 2012; 303:G93–G102. doi:ajpgi.00016.2012 [pii]10.1152/ajpgi.00016.2012. [PubMed: 22538401]
55. Maheshwari A, et al. Cytokines associated with necrotizing enterocolitis in extremely-low-birth-weight infants. *Pediatric research.* 2014; 76:100–108. doi:10.1038/pr.2014.48. [PubMed: 24732104]
56. Weitkamp JH, et al. Ontogeny of FOXP3(+) regulatory T cells in the postnatal human small intestinal and large intestinal lamina propria. *Pediatric and developmental pathology: the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society.* 2009; 12:443–449. doi:10.2350/08-09-0533.1.
57. Wildin RS, et al. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nature genetics.* 2001; 27:18–20. doi:10.1038/83707. [PubMed: 11137992]
58. Silverstein AM, Lukes RJ. Fetal response to antigenic stimulus. I. Plasmacellular and lymphoid reactions in the human fetus to intrauterine infection. *Laboratory investigation; a journal of technical methods and pathology.* 1962; 11:918–932.
59. Vignali DA, Collison LW, Workman CJ. How regulatory T cells work. *Nature reviews. Immunology.* 2008; 8:523–532. doi:10.1038/nri2343.
60. Weitkamp JH, et al. Necrotising enterocolitis is characterised by disrupted immune regulation and diminished mucosal regulatory (FOXP3)/effector (CD4, CD8) T cell ratios. *Gut.* 2013; 62:73–82. doi:10.1136/gutjnl-2011-301551. [PubMed: 22267598]
61. Golby S, et al. B cell development and proliferation of mature B cells in human fetal intestine. *J Leukoc Biol.* 2002; 72:279–284. [PubMed: 12149418]
62. Fagarasan S, Kinoshita K, Muramatsu M, Ikuta K, Honjo T. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature.* 2001; 413:639–643. [PubMed: 11675788]
63. Crabbe PA, Nash DR, Bazin H, Eyssen H, Heremans JF. Immunohistochemical observations on lymphoid tissues from conventional and germ-free mice. *Lab Invest.* 1970; 22:448–457. [PubMed: 4911977]
64. Shroff KE, Meslin K, Cebra JJ. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. *Infect Immun.* 1995; 63:3904–3913. [PubMed: 7558298]
65. Haworth JC, Dilling L. Concentration of gamma-A-globulin in serum, saliva, and nasopharyngeal secretions of infants and children. *J Lab Clin Med.* 1966; 67:922–933. [PubMed: 4161936]
66. Brandtzaeg P, Nilssen DE, Rognum TO, Thrane PS. Ontogeny of the mucosal immune system and IgA deficiency. *Gastroenterol Clin North Am.* 1991; 20:397–439. [PubMed: 1917020]
67. Gleeson M, et al. Ontogeny of the secretory immune system in man. *Aust N Z J Med.* 1982; 12:255–258. [PubMed: 6753816]

68. Mellander L, Carlsson B, Hanson LA. Appearance of secretory IgM and IgA antibodies to *Escherichia coli* in saliva during early infancy and childhood. *J Pediatr*. 1984; 104:564–568. [PubMed: 6200589]
69. Hayes JA, Adamson-Macedo EN, Perera S, Anderson J. Detection of secretory immunoglobulin A (SIgA) in saliva of ventilated and non-ventilated preterm neonates. *Neuroendocrinol Lett*. 1999; 20:109–113. [PubMed: 11473239]
70. Shields CJ, et al. Hypertonic saline enhances host response to bacterial challenge by augmenting receptor-independent neutrophil intracellular superoxide formation. *Ann Surg*. 2003; 238:249–257. [PubMed: 12894019]
71. Cripps AW, Gleeson M, Clancy RL. Ontogeny of the mucosal immune response in children. *Adv Exp Med Biol*. 1991; 310:87–92. [PubMed: 1809030]
72. Weemaes C, et al. Development of immunoglobulin A in infancy and childhood. *Scand J Immunol*. 2003; 58:642–648. [PubMed: 14636420]
73. Fitzsimmons SP, et al. Immunoglobulin A subclasses in infants' saliva and in saliva and milk from their mothers. *J Pediatr*. 1994; 124:566–573. [PubMed: 8151471]
74. Bhat NM, et al. The ontogeny and functional characteristics of human B-1 (CD5+ B) cells. *Int Immunol*. 1992; 4:243–252. [PubMed: 1377947]
75. Chen ZJ, et al. Polyreactive antigen-binding B cells are the predominant cell type in the newborn B cell repertoire. *Eur J Immunol*. 1998; 28:989–994. [PubMed: 9541594]
76. Bauer K, et al. Diversification of Ig heavy chain genes in human preterm neonates prematurely exposed to environmental antigens. *J Immunol*. 2002; 169:1349–1356. [PubMed: 12133958]
77. Ogra PL, Losonsky GA, Fishaut M. Colostrum-derived immunity and maternal-neonatal interaction. *Ann N Y Acad Sci*. 1983; 409:82–95. [PubMed: 6347005]
78. Hanson LA, Korotkova M. The role of breastfeeding in prevention of neonatal infection. *Semin Neonatol*. 2002; 7:275–281. [PubMed: 12401297]
79. Hanson LA, et al. The immune response of the mammary gland and its significance for the neonate. *Ann Allergy*. 1984; 53:576–582. [PubMed: 6391286]
80. Takahashi T, et al. Reactivity of secretory IgA antibodies in breast milk from 107 Japanese mothers to 20 environmental antigens. *Biol Neonate*. 2002; 82:238–242. [PubMed: 12381931]
81. Flamand V, et al. CD40 ligation prevents neonatal induction of transplantation tolerance. *J Immunol*. 1998; 160:4666–4669. [PubMed: 9590211]
82. Araujo ED, et al. Evaluation of the secretory immunoglobulin A levels in the colostrum and milk of mothers of term and pre-term newborns. *Braz J Infect Dis*. 2005; 9:357–362. [PubMed: 16410886]
83. Caplan MS, Simon D, Jilling T. The role of PAF, TLR, and the inflammatory response in neonatal necrotizing enterocolitis. *Semin Pediatr Surg*. 2005; 14:145–151. doi:S1055-8586(05)00030-2 [pii]10.1053/j.sempedsurg.2005.05.002. [PubMed: 16084401]
84. Hsueh W, Gonzalez-Crussi F, Arroyave JL. Platelet-activating factor-induced ischemic bowel necrosis. An investigation of secondary mediators in its pathogenesis. *The American journal of pathology*. 1986; 122:231–239. [PubMed: 3080895]
85. Caplan M, Hsueh W, Kelly A, Donovan M. Serum PAF acetylhydrolase increases during neonatal maturation. *Prostaglandins*. 1990; 39:705–714. [PubMed: 2371412]
86. MacKendrick W, Hill N, Hsueh W, Caplan M. Increase in plasma platelet-activating factor levels in enterally fed preterm infants. *Biology of the neonate*. 1993; 64:89–95. [PubMed: 8260551]
87. Furukawa M, Narahara H, Yasuda K, Johnston JM. Presence of platelet-activating factor-acetylhydrolase in milk. *Journal of lipid research*. 1993; 34:1603–1609. [PubMed: 8228643]
88. Caplan MS, Hedlund E, Adler L, Lickerman M, Hsueh W. The platelet-activating factor receptor antagonist WEB 2170 prevents neonatal necrotizing enterocolitis in rats. *J Pediatr Gastroenterol Nutr*. 1997; 24:296–301. [PubMed: 9138176]
89. Ewer AK, et al. The role of platelet activating factor in a neonatal piglet model of necrotising enterocolitis. *Gut*. 2004; 53:207–213. [PubMed: 14724152]

90. Caplan MS, Lickerman M, Adler L, Dietsch GN, Yu A. The role of recombinant platelet-activating factor acetylhydrolase in a neonatal rat model of necrotizing enterocolitis. *Pediatric research*. 1997; 42:779–783. [PubMed: 9396557]
91. Lu J, et al. Dual roles of endogenous platelet-activating factor acetylhydrolase in a murine model of necrotizing enterocolitis. *Pediatric research*. 2010; 68:225–230. doi: 10.1203/00006450-201011001-00439. [PubMed: 20531249]
92. Wang H, et al. Regulation of platelet-activating factor receptor gene expression in vivo by endotoxin, platelet-activating factor and endogenous tumour necrosis factor. *The Biochemical journal*. 1997; 322(Pt 2):603–608. [PubMed: 9065783]
93. Chokshi NK, et al. The role of nitric oxide in intestinal epithelial injury and restitution in neonatal necrotizing enterocolitis. *Seminars in perinatology*. 2008; 32:92–99. doi:10.1053/j.semperi.2008.01.002. [PubMed: 18346532]
94. Potoka DA, et al. NF-kappaB inhibition enhances peroxynitrite-induced enterocyte apoptosis. *The Journal of surgical research*. 2002; 106:7–14. [PubMed: 12127801]
95. Stanford A, et al. Nitric oxide mediates dendritic cell apoptosis by downregulating inhibitors of apoptosis proteins and upregulating effector caspase activity. *Surgery*. 2001; 130:326–332. doi: 10.1067/msy.2001.116411. [PubMed: 11490367]
96. Ford H, Watkins S, Reblock K, Rowe M. The role of inflammatory cytokines and nitric oxide in the pathogenesis of necrotizing enterocolitis. *Journal of pediatric surgery*. 1997; 32:275–282. doi:S0022-3468(97)90194-9 [pii]. [PubMed: 9044137]
97. D'Souza A, et al. Effects of probiotics, prebiotics, and synbiotics on messenger RNA expression of caveolin-1, NOS, and genes regulating oxidative stress in the terminal ileum of formula-fed neonatal rats. *Pediatric research*. 2010; 67:526–531. doi:10.1203/PDR.0b013e3181d4ff2b. [PubMed: 20101198]
98. Nadler EP, et al. Expression of inducible nitric oxide synthase and interleukin-12 in experimental necrotizing enterocolitis. *The Journal of surgical research*. 2000; 92:71–77. doi:10.1006/jsre.2000.5877S0022-4804(00)95877-3 [pii]. [PubMed: 10864485]
99. Zhou Y, Wang Q, Mark Evers B, Chung DH. Oxidative stress-induced intestinal epithelial cell apoptosis is mediated by p38 MAPK. *Biochemical and biophysical research communications*. 2006; 350:860–865. doi:10.1016/j.bbrc.2006.09.103. [PubMed: 17034759]
100. Baregamian N, et al. Intestinal mitochondrial apoptotic signaling is activated during oxidative stress. *Pediatric surgery international*. 2011; 27:871–877. doi:10.1007/s00383-011-2880-x. [PubMed: 21400030]
101. Clark DA, et al. Contribution of oxygen-derived free radicals to experimental necrotizing enterocolitis. *The American journal of pathology*. 1988; 130:537–542. [PubMed: 3348358]
102. Qu XW, Rozenfeld RA, Huang W, Bulkley GB, Hsueh W. The role of xanthine oxidase in platelet activating factor induced intestinal injury in the rat. *Gut*. 1999; 44:203–211. [PubMed: 9895379]
103. Chaaban H, et al. Inter-alpha inhibitor protein level in neonates predicts necrotizing enterocolitis. *The Journal of pediatrics*. 2010; 157:757–761. doi:10.1016/j.jpeds.2010.04.075. [PubMed: 20955849]
104. Ng PC, et al. Host-response biomarkers for diagnosis of late-onset septicemia and necrotizing enterocolitis in preterm infants. *The Journal of clinical investigation*. 2010; 120:2989–3000. doi: 10.1172/JCI40196. [PubMed: 20592468]
105. Edelson MB, et al. Plasma intestinal fatty acid binding protein in neonates with necrotizing enterocolitis: a pilot study. *Journal of pediatric surgery*. 1999; 34:1453–1457. doi:S0022346899000445 [pii]. [PubMed: 10549746]
106. Lobe TE, Richardson CJ, Rassin DK, Mills R, Schwartz M. Hexosaminidase: a biochemical marker for necrotizing enterocolitis in the preterm infant. *Am J Surg*. 1984; 147:49–52. [PubMed: 6691551]
107. Bhandari V, et al. Familial and genetic susceptibility to major neonatal morbidities in preterm twins. *Pediatrics*. 2006; 117:1901–1906. doi:10.1542/peds.2005-1414. [PubMed: 16740829]

108. Viscardi RM, Lyon NH, Sun CC, Hebel JR, Hasday JD. Inflammatory cytokine mRNAs in surgical specimens of necrotizing enterocolitis and normal newborn intestine. *Pediatr Pathol Lab Med*. 1997; 17:547–559. [PubMed: 9211547]
109. Caplan MS, Sun XM, Hseuh W, Hageman JR. Role of platelet activating factor and tumor necrosis factor-alpha in neonatal necrotizing enterocolitis. *The Journal of pediatrics*. 1990; 116:960–964. [PubMed: 2348301]
110. Tan X, Hsueh W, Gonzalez-Crussi F. Cellular localization of tumor necrosis factor (TNF)-alpha transcripts in normal bowel and in necrotizing enterocolitis. TNF gene expression by Paneth cells, intestinal eosinophils, and macrophages. *The American journal of pathology*. 1993; 142:1858–1865. [PubMed: 8506954]
111. Halpern MD, et al. Up-regulation of IL-18 and IL-12 in the ileum of neonatal rats with necrotizing enterocolitis. *Pediatric research*. 2002; 51:733–739. [PubMed: 12032269]
112. Halpern MD, et al. Decreased development of necrotizing enterocolitis in IL-18-deficient mice. *American journal of physiology. Gastrointestinal and liver physiology*. 2008; 294:G20–26. doi: 10.1152/ajpgi.00168.2007. [PubMed: 17947451]
113. Maheshwari A, Christensen RD, Calhoun DA, Dimmitt RA, Lacson A. Circulating CXC-chemokine concentrations in a murine intestinal ischemia-reperfusion model. *Fetal Pediatr Pathol*. 2004; 23:145–157. [PubMed: 15768860]
114. Benkoe T, et al. Interleukin-8 predicts 60-day mortality in premature infants with necrotizing enterocolitis. *Journal of pediatric surgery*. 2014; 49:385–389. doi:10.1016/j.jpedsurg.2013.05.068. [PubMed: 24650462]
115. Bhatia AM, Stoll BJ, Cismowski MJ, Hamrick SE. Cytokine levels in the preterm infant with neonatal intestinal injury. *American journal of perinatology*. 2014; 31:489–496. doi:10.1055/s-0033-1353437. [PubMed: 23966125]
116. Edelson MB, Bagwell CE, Rozycki HJ. Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. *Pediatrics*. 1999; 103:766–771. [PubMed: 10103300]
117. Harris MC, et al. Cytokine elaboration in critically ill infants with bacterial sepsis, necrotizing enterocolitis, or sepsis syndrome: correlation with clinical parameters of inflammation and mortality. *The Journal of pediatrics*. 2005; 147:462–468. doi:10.1016/j.jpeds.2005.04.037. [PubMed: 16227031]
118. Lodha A, Asztalos E, Moore AM. Cytokine levels in neonatal necrotizing enterocolitis and long-term growth and neurodevelopment. *Acta Paediatr*. 2010; 99:338–343. doi:APA1600 [pii]10.1111/j.1651-2227.2009.01600.x. [PubMed: 19922507]
119. Kling PJ, Hutter JJ. Hematologic abnormalities in severe neonatal necrotizing enterocolitis: 25 years later. *Journal of perinatology*. 2003; 23:523–530. doi:10.1038/sj.jp.72109837210983 [pii]. [PubMed: 14566346]
120. O'Neill JA Jr. Neonatal necrotizing enterocolitis. *Surg Clin North Am*. 1981; 61:1013–1022. [PubMed: 7031925]
121. Hutter JJ Jr. Hathaway WE, Wayne ER. Hematologic abnormalities in severe neonatal necrotizing enterocolitis. *The Journal of pediatrics*. 1976; 88:1026–1031. [PubMed: 1271173]
122. Patel CC. Hematologic abnormalities in acute necrotizing enterocolitis. *Pediatr Clin North Am*. 1977; 24:579–584. [PubMed: 896319]
123. Kenton AB, et al. Severe thrombocytopenia predicts outcome in neonates with necrotizing enterocolitis. *Journal of perinatology: official journal of the California Perinatal Association*. 2005; 25:14–20. doi:7211180 [pii]10.1038/sj.jp.7211180. [PubMed: 15526014]
124. Ververidis M, et al. The clinical significance of thrombocytopenia in neonates with necrotizing enterocolitis. *Journal of pediatric surgery*. 2001; 36:799–803. doi:S0022-3468(01)04730-3 [pii]10.1053/jpsu.2001.22964. [PubMed: 11329593]
125. Ragazzi S, Pierro A, Peters M, Fasoli L, Eaton S. Early full blood count and severity of disease in neonates with necrotizing enterocolitis. *Pediatric surgery international*. 2003; 19:376–379. doi: 10.1007/s00383-003-1014-5. [PubMed: 12756597]
126. Sola MC, Del Vecchio A, Rimsza LM. Evaluation and treatment of thrombocytopenia in the neonatal intensive care unit. *Clin Perinatol*. 2000; 27:655–679. [PubMed: 10986634]

127. Hsueh W, et al. Neonatal necrotizing enterocolitis: clinical considerations and pathogenetic concepts. *Pediatric and developmental pathology: the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society*. 2003; 6:6–23. doi:10.1007/s10024-002-0602-z.
128. Colarizi P, et al. Circulating thrombopoietin levels in neonates with infection. *Acta Paediatr*. 1999; 88:332–337. [PubMed: 10229048]
129. Oygur N, et al. Thrombopoietin levels of thrombocytopenic term and preterm newborns with infection. *American journal of perinatology*. 2001; 18:279–286. doi:10.1055/s-2001-16990. [PubMed: 11552180]
130. Brown RE, et al. Effects of sepsis on neonatal thrombopoiesis. *Pediatric research*. 2008; 64:399–404. doi:10.1203/PDR.0b013e318181ad49. [PubMed: 18552713]
131. Sonntag J, Wagner MH, Waldschmidt J, Wit J, Obladen M. Multisystem organ failure and capillary leak syndrome in severe necrotizing enterocolitis of very low birth weight infants. *Journal of pediatric surgery*. 1998; 33:481–484. doi:S0022-3468(98)90092-6 [pii]. [PubMed: 9537561]
132. Osborn DA, et al. T and Tk antigen activation in necrotizing enterocolitis: manifestations, severity of illness, and effectiveness of testing. *Archives of disease in childhood. Fetal and neonatal edition*. 1999; 80:F192–197. [PubMed: 10212080]
133. Hall N, et al. T cryptantigen activation is associated with advanced necrotizing enterocolitis. *Journal of pediatric surgery*. 37:791–793. doi:S0022346802646147 [pii] (2002). [PubMed: 11987103]
134. Boralessa H, et al. RBC T activation and hemolysis in a neonatal intensive care population: implications for transfusion practice. *Transfusion*. 2002; 42:1428–1434. doi:trf237 [pii]. [PubMed: 12421215]
135. Schutzman DL, Porat R. Glucose-6-phosphate dehydrogenase deficiency: another risk factor for necrotizing enterocolitis? *The Journal of pediatrics*. 2007; 151:435–437. doi:S0022-3476(07)00648-8 [pii]10.1016/j.jpeds.2007.06.041. [PubMed: 17889084]
136. Detlefsen B, Boemers TM, Schimke C. Necrotizing enterocolitis in premature twins with twin-to-twin transfusion syndrome. *Eur J Pediatr Surg*. 2008; 18:50–52. doi:10.1055/s-2007-965788. [PubMed: 18302071]
137. Singh R, et al. Association of necrotizing enterocolitis with anemia and packed red blood cell transfusions in preterm infants. *Journal of perinatology: official journal of the California Perinatal Association*. 2011; 31:176–182. doi:10.1038/jp.2010.145. [PubMed: 21273983]
138. Szabo JS, Mayfield SR, Oh W, Stonestreet BS. Postprandial gastrointestinal blood flow and oxygen consumption: effects of hypoxemia in neonatal piglets. *Pediatric research*. 1987; 21:93–98. [PubMed: 3797137]
139. Caplan MS, Jilling T. New concepts in necrotizing enterocolitis. *Curr Opin Pediatr*. 2001; 13:111–115. [PubMed: 11317050]
140. Reber KM, Nankervis CA, Nowicki PT. Newborn intestinal circulation. *Physiology and pathophysiology. Clin Perinatol*. 2002; 29:23–39. [PubMed: 11917738]
141. Schober PH, Nassiri J. Risk factors and severity indices in necrotizing enterocolitis. *Acta Paediatr Suppl*. 1994; 396:49–52. [PubMed: 8086683]
142. Maheshwari A, et al. Epithelial Cells in Fetal Intestine Produce Chemerin to Recruit Macrophages. *American journal of physiology. Gastrointestinal and liver physiology*. 2009; 297:G1–G10. doi:90730.2008 [pii]10.1152/ajpgi.90730.2008. [PubMed: 19443732]
143. Smythies LE, et al. Mucosal IL-8 and TGF-beta recruit blood monocytes: evidence for cross-talk between the lamina propria stroma and myeloid cells. *Journal of leukocyte biology*. 2006; 80:492–499. doi:jlbg.1005566 [pii]10.1189/jlb.1005566. [PubMed: 16793909]
144. Christensen RD, Jensen J, Maheshwari A, Henry E. Reference ranges for blood concentrations of eosinophils and monocytes during the neonatal period defined from over 63 000 records in a multihospital health-care system. *Journal of perinatology*. 2010; 30:540–545. doi:jp2009196 [pii]10.1038/jp.2009.196. [PubMed: 20054336]
145. van Furth R, Sluiter W. Distribution of blood monocytes between a marginating and a circulating pool. *J Exp Med*. 1986; 163:474–479. [PubMed: 3944542]

146. Remon J, et al. Acute drop in blood monocyte count differentiates NEC from other causes of feeding intolerance. *Journal of perinatology: official journal of the California Perinatal Association*. 2014; 34:549–554. doi:10.1038/jp.2014.52. [PubMed: 24674979]
147. Christensen RD, et al. Neonates presenting with bloody stools and eosinophilia can progress to two different types of necrotizing enterocolitis. *Journal of perinatology: official journal of the California Perinatal Association*. 2011 doi:10.1038/jp.2011.163jp2011163 [pii].
148. Kim D-H, S-P B, Hahn W-H, Song J-H, M-H O. Low Basophil Count and Red Cell Distribution Width at Birth May Predict the Development of Neonatal Necrotizing Enterocolitis: A Matched Control Study. *Soonchunhyang Med Sci*. 2013; 19:61–64.
149. Hill DA, et al. Commensal bacteria-derived signals regulate basophil hematopoiesis and allergic inflammation. *Nature medicine*. 2012; 18:538–546. doi:10.1038/nm.2657.
150. Lambert DK, et al. Fulminant necrotizing enterocolitis in a multihospital healthcare system. *Journal of perinatology*. 2012; 32:194–198. doi:jp201161 [pii]10.1038/jp.2011.61. [PubMed: 21566569]
151. Mandel D, et al. Nucleated red blood cells in preterm infants who have necrotizing enterocolitis. *The Journal of pediatrics*. 2004; 144:653–655. doi:10.1016/j.jpeds.2004.01.055S0022347604001003 [pii]. [PubMed: 15127005]
152. Baschat AA, et al. Nucleated red blood cell counts in the first week of life: a critical appraisal of relationships with perinatal outcome in preterm growth-restricted neonates. *Am J Obstet Gynecol*. 2007; 197:286 e281–288. doi:S0002-9378(07)00749-1 [pii]10.1016/j.ajog.2007.06.020. [PubMed: 17826423]

Key points

- Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in preterm infants born prior to 32 weeks gestation or with a birth weight less than 1500 grams
- Bacterial flora plays a central pathophysiological role in NEC
- Premature intestine is at risk of NEC because of mucosal sensitivity to bacterial products and paucity of mechanisms that normally limit the interaction of luminal bacteria with mucosal cells
- The onset of NEC is associated with elevated plasma concentrations of several inflammatory cytokines. Increased circulating interleukin-8 concentrations may provide prognostic information
- Low circulating TGF- β concentrations on the first postnatal day may predict later occurrence of NEC
- Hematological abnormalities such as thrombocytopenia, disseminated intravascular coagulation, increased or decreased neutrophil counts, eosinophilia, and anemia occur frequently in infants with NEC
- In a premature infant with feeding intolerance, an acute drop in peripheral blood monocyte concentrations compared to the last pre-symptomatic blood counts may be an early indicator of NEC

Best Practices

What is the current practice?

Infants who develop NEC frequently show immunological and hematological abnormalities such as elevated circulating cytokine levels, thrombocytopenia, increased or decreased neutrophil counts, low monocyte counts, and anemia. These findings show high sensitivity and in some infants, might convey important diagnostic and prognostic information. In others, the clinical utility of these tests may be limited due to low specificity and the overlap with co-morbidities such as bacterial or fungal sepsis, or cholestasis.

What changes in current practice are likely to improve outcomes?

Many hematological abnormalities associated with NEC have been identified in recent years and still need confirmation in larger studies and dissemination of the information to the clinical community. Cytokine measurements, although straightforward and relatively inexpensive, are not routinely available yet in clinical laboratories. Further study is needed to refine these measurements and determine whether the diagnostic accuracy of these tests could be improved if used in combination.

Is there a Clinical Algorithm?

No. Due to low specificity, immunological and hematological abnormalities are likely to be less useful as diagnostic tests in infants with NEC. Instead, these findings are more likely to find utility as 'early-warning' systems that indicate a need for clinical monitoring and/or imaging. Elevated plasma concentrations of interleukin (IL)-8, transforming growth factor- β , and IL-6, and the detection of worsening thrombocytopenia or an acute drop in monocyte counts are some of the more promising findings. In infants at risk of transfusion-associated NEC, a rise in blood eosinophil counts may be important.

Summary Statement

NEC is characterized by elevated circulating cytokine levels and a consistent pattern of hematological abnormalities. Some of these findings may provide important diagnostic and prognostic information.

Table 1
Development of Peyer's Patches in the Human Fetus

11 wks gestation	PP anlagen with HLA-DR ⁺ CD4 ⁺ lymphoid cells
16 wks gestation	Appearance of T and B cells
	First appearance of CD8 ⁺ cells *
	B cell maturation with appearance of surface IgM and IgD
16-18 wks gestation	Appearance of CD5 ⁺ B-1 cells
	Surface IgA on B cells
18-20 wks gestation	Appearance of PP zonation into B and T cell areas
24 wks gestation	PP are macroscopically identifiable
0-4 wks postnatal	Germinal center formation

* Fetal Peyer's patch T cells are predominantly of the CD4⁺ phenotype