Predicting Disease Severity of Necrotizing Enterocolitis: How to Identify Infants for Future Novel Therapies

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──────────── ABSTRACT ────────────

Necrotizing enterocolitis (NEC) remains a very devastating problem within the very low birth weight neonatal population. Several experimental therapies are being tested in animal models and soon may be ready for human trials. Despite this progress, we currently have no way to identify infants who would be optimal targets for therapy. Specifically, we are unable to predict which infants will progress to the more severe Bell's stage of disease that may necessitate surgery. Ideally, an algorithm could be constructed that would encompass multiple neonatal and maternal risk factors as well as potential biologic markers of disease so that these infants could be identified in a more timely fashion. This review summarizes the known risk factors and biomarkers of disease in hopes of stimulating clinical research to identify such an "early warning" NEC algorithm.

Key words:
Biomarkers, necrotizing enterocolitis, neonate, predictors, risk

INTRODUCTION

Necrotizing enterocolitis (NEC) remains one of the most devastating intra-abdominal emergencies in the newborn infant, particularly those of low birth weight.[1] Medical management of the more severe cases of NEC is often inadequate, thereby warranting surgical resection of necrotic bowel. In many cases, however, surgical resection leaves the infant with a suboptimal length of intestine that inhibits appropriate nutritional absorption. Therefore, these infants usually require long-term parenteral nutrition and are exposed to the risks and side effects of this therapy. Active research is underway to understand the mechanisms associated with the intestinal ischemia, bacterial translocation, sepsis and organ failure often associated with NEC.[2,3]

In order to optimize the utility of novel future therapies, it becomes necessary to be able to identify at risk infants before the progression of disease so that therapeutic intervention can be delivered before surgical resection of the bowel is required. Repeated attempts to identify clinical parameters that would reliably identify infants with NEC most likely to progress to severe disease have thus far been unsuccessful.[4,5] Several previous studies utilizing the Score of Neonatal Acute Physiology and the Metabolic Derangement Acuity score have failed to be able to accurately predict surgical NEC.[6] Whereas some studies have claimed to be able to predict surgical NEC based on several laboratory parameters, these have been most notable in small studies and have not been reproducible on a larger scale.[7]

In view of the high morbidity and mortality associated with surgical NEC, early detection of ischemic or necrotic bowel before surgical intervention could potentially improve outcomes. Identifying specific maternal and neonatal risk factors as well as biomarkers for surgical NEC may aid in creating a predictive algorithm for at-risk infants. Therefore, the purpose of this review is to identify predictive characteristics, including neonatal, maternal, radiographic and biologic factors that may be able facilitate early prediction of surgical NEC so that novel treatment modalities can be effectively implemented.

NEONATAL RISK FACTORS

There are several neonatal risk factors for developing NEC that have been very consistent throughout the literature. These include prematurity, enteral formula feeding and incomplete gastrointestinal colonization by bacteria. Additional factors that may contribute to NEC

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onset include red blood cell transfusion and increased or prolonged respiratory and ventilator support. Each of these risk factors has an individually established incidence of NEC. However, when multiple risk factors are present, the incidence of NEC increases dramatically.

NEC is most prevalent in premature infants of very low birth weight.[8] With an inverse relationship between postmenstrual age and incidence of NEC, only 7-15% of all NEC cases occur in term or late preterm infants.[9,10] More than 85% of cases occur in infants <1500 g or <32 weeks gestational age.[9] This translates to an incidence of approximately 11-15% in the very low birth weight population as determined by the National Institute of Child Health and Human Development Neonatal Research Network.[11,12] Several contributing factors of NEC may be related to intestinal prematurity, including immature intestinal peristalsis, which may allow bacteria to adhere and translocate more easily.[13,14] Additional factors include decreased or different intestinal mucous production,[15] increased intestinal permeability,[16] decreased gastric acid production, immature proteolytic enzymes,[17] and immature gut hormones and enzymes for digesting enteral nutrition.[18]

Formula feedings are well-known to be an independent risk factor for the development of NEC. Formula feeds may contribute to disease incidence in that they have higher levels of free fatty acids, which are toxic to cells.[19] Formulas also lack a number of beneficial factors including secretory IgA, lysozyme and platelet activating factor acetylhydrolase, which may serve to protect the intestine during times of stress.[20] However, delaying the introduction of progressive enteral feeds does not appear to affect the risk of developing NEC.[21,22] There are no data from randomized trials of formula milk versus maternal breast milk for feeding preterm and/or low birth weight neonates.[23] However, there are several randomized controlled trials comparing human donor breast milk to formula. A meta-analysis of these trials showed that preterm infants fed with formula had twice the incidence of NEC as compared to those receiving donor milk,[24] although only one study utilized human milk fortifier in the donor milk group, a practice that is commonly used today. Additional data would suggest that there is a dose-related benefit of human milk intake. In a retrospective analysis of the National Institute of Child Health and Human Development Neonatal Network Glutamine Trial, researchers noted that for each 10% increase in the proportion of human milk consumption, the incidence of NEC was lower by a factor of 0.83.[25] In another study, only 3% of infants who received more than 50% as human milk developed NEC, whereas 10% of infants who received <50% of their total enteral intake as human milk developed NEC.[26]

Other recent studies have targeted blood transfusions and have proposed that red blood cell transfusion may be an independent risk factor for developing NEC in the premature infant population.[21,27-29] This phenomenon, a variant of Transfusion Associated Gut Injury, has been called Transfusion Associated Necrotizing Enterocolitis (TANEC). The etiology is felt to be immunologic, possibly through alterations of mesenteric arterial reactivity and nitric oxide pathways.[30] Approximately 25-40% of infants who developed NEC were found to have received a blood transfusion within 48 h of disease onset.[31] In addition, infants who were being fed in the 48 h period prior to transfusion were eight times more likely to develop NEC than infants who were neither fed nor transfused.[27] A 2012 meta-analysis of 12 studies clearly demonstrated an association between NEC and transfusion, however a cause and effect relationship has yet to be established. Neonates who developed TANEC were younger by 1.5 weeks, were 500 g lower in birth weight, were more likely to have a patent ductus arteriosis and were more likely to be receiving ventilator support.[32] Some institutions have now developed protocols to limit transfusions in this population and to ensure that enteral feedings do not occur at the time of blood transfusions.

Other potential inherent risk factors such as race and gender are not readily discussed in the literature. Gender may play a role in disease pathology, but there are no conclusive studies to suggest that the incidence of NEC occurs more frequently in one gender versus the other. There is some data noting that males may have a higher risk of NEC associated death as compared to females.[33] Several separate studies have examined race as a potential risk factor. One study noted that black neonates had a higher incidence of NEC, but this did not reach significance.[34] A subsequent study noted that race only played a role in males, as black males had a higher prevalence of NEC compared with non-black males and females of other races.[35]

Racial and gender disparities are likely related to genetic variations in these populations. Genetic risk factors associated with NEC have previously not been well-characterized. Nonetheless, it certainly seems intuitive that variations in the genetic code may predispose certain individuals to NEC. Many of the genetic variations noted are single nucleotide polymorphisms (SNPs), which involve the transition of one nucleotide to another nucleotide. Most of the variations occur in non-coding sequences with only about 2-3% being found in the promoter regions or coding exons of the gene.[36] Genetic variations in toll-like receptor (TLR) signaling have been explored since this receptor has been shown to play an important role in disease onset. SNPs in *TLR2, TLR4, TLR5, TLR9, IRAK1* and *TIRAP* genes did not appear to be associated with NEC.

However, variations in *NfKB1*, and *NfKBIA* were associated with NEC.[37] Further studies have examined genetic variations in other growth factors and cytokines that may predispose to NEC. These include but are not limited to vascular endothelial growth factor,[38] interleukin (IL)-12,[39] IL-18 and IL-4 receptor alpha.[36,40]

Certain physiologic parameters have also been implicated as potential risk factors for NEC. A U.S. study examining a database of over 15,000 neonates demonstrated a positive correlation between the number of days of mechanical ventilation and the development of NEC. A possible etiology could be colonization of the respiratory track and bacterial overgrowth associated with intubation. They also saw a positive relationship between the incidence of NEC and use of antenatal glucocorticoids, absence of an umbilical artery catheter, postnatal use of glucocorticoids or indomethacin, low Apgar scores at 5 min and vaginal delivery.[41] Another study also independently appreciated that premature infants who required increased respiratory support to maintain oxygenation during the early neonatal period were 13 times more likely to develop NEC. When combined with lack of breast milk feeds, the likelihood of NEC increased 28.6 times compared with the controls.[42]

The onset of NEC is undoubtedly multifactorial and a discussion of infant risk factors would not be complete without mentioning the intestinal microbiome. Although there is little doubt that microbes play a vital role in the development of NEC, the identity of specific causative pathogens has not been identified.[43,44] Additional suggestions that intestinal microbes play a significant role in disease pathogenesis come from data demonstrating the benefit and decreased incidence of NEC with the use of supplemental probiotics.[45-47] However, at this time, there is no predictive assay or factor to determine if an individual's intestinal microbiome puts them at risk for the development of NEC.

One potential correlation between incidence of NEC and bacterial infections was seen between the number of nosocomial infections that infants incurred in the neonatal period and the development of NEC.[34] Frequent infections may decrease the native immune response of the infant, making them more susceptible to pathogens. Vulnerability to infection may also be a result of overuse of antibiotics and the creation of drug resistant bacterial strains,[48] or to the breakdown of native defense mechanisms such as may be seen by increasing gastric pH through the use of histamine-blocker therapy.[49,50]

MATERNAL RISK FACTORS

The maternal variables that contribute to fetal development and correlate with NEC are not well-defined. As we are currently unable to accurately predict the incidence of NEC in neonates, many investigators have turned to studying these maternal risk factors. A recent study examined plausible maternal risk factors and correlated them to the development of NEC. Maternal smoking, hypertension, diabetes, body mass index, type of delivery and conduct of labor were among the variables inspected. Maternal cigarette smoking was the only maternal risk factor that significantly correlated with the development of NEC.[51]

Maternal exposures to antibiotics and steroids in the prenatal period have also been postulated as potential antecedents to the development of NEC. The neonatal microbiome is greatly dependent on the maternal vaginal and gastrointestinal flora.[52,53] Maternal broad spectrum antibiotic exposure may alter this native flora which may then place the infant at increased risk for the development of NEC.[54-57] Administration of antibiotics for the antenatal management of pregnancy complications such as premature rupture of membranes, preterm labor, or chorioamnionitis is common in clinical practice. A few studies have suggested a correlation between maternal exposure of amoxicillin/clavulanate and the development of NEC,[58-60] while other studies have not found such an association.[61,62] The data for antenatal steroid use is also controversial, as several studies noted no significant increase in the incidence of NEC associated with prenatal use of steroids,[63-65] while a much larger study did appreciate a correlation.[41]

Placental pathology has also been felt to contribute to the onset of NEC. One study examined over 5000 placentas from high risk pregnancies and discovered that placentas from infants with surgically treated NEC had significant evidence of vascular pathology such as placental infarcts.[66] In addition, evidence of placental infection (chorioamnionitis or villitis) plus evidence of a fetal inflammatory response were more readily present in placentas from infants with surgical NEC when compared with unaffected infants.[67] Another study found no correlation with placental pathology and NEC, but the population of this study was small and was confined to a single institution.[51]

The belief that abnormal placental vascular resistance can contribute to the predisposition of NEC is quite legitimate.[68-71] The theory is that placental resistance causes a centralized diversion of blood flow from the splanchnic mesentery. In a multi-institutional U.S. study examining 404 neonates, infants with NEC had statistically higher umbilical artery Doppler indices prenatally, 5 min Apgars <7 and higher umbilical cord artery base deficit. NEC was also more likely with a combined variable consisting of umbilical artery absent or reversed end diastolic velocity, absent or reversed ductus venosus a-wave, or umbilical vein pulsations.[72] Additional studies examined uterine

artery Doppler waveforms. In 83% of infants with NEC in a single study, there were noted bilateral uterine artery protodiastolic notches and a mean resistance index greater than the 95th percentile. In addition, within the fetus, there was noted absent or retrograde diastolic blood flow through the aortic isthmus.[73] All these parameters would suggest increased placental vascular resistance and associated central splanchnic shunting away from the intestinal mesentery.

RADIOGRAPHIC PREDICTORS

The modified Bell's stage is currently the most noted classification scheme in existence for NEC. The scale is based entirely on broad clinical and radiographic findings and is not specific for NEC, nor is it predictive of disease severity. In fact, some investigators feel that these staging criteria should be abandoned and other alternative methods to identify NEC embraced.[74] The seminal paper by Bell *et al.*, used a combination of radiographic and bedside clinical criteria to gauge the severity of NEC. Over time, it has gone well beyond just staging criteria and has become essential to thecapture and reporting of infants with NEC. It was later modified to distinguish between perforated and non-perforated NEC.[75]

The main imaging modality that has been used to identify progression of disease has been the abdominal plain film. Despite its frequent use, individual radiographic signs of NEC do not readily correlate with disease severity. The presence of multiple signs, however, may increase radiographic predictability.[76,77] In one study, investigators described the sensitivity and specificity for pneumatosis intestinalis (44% and 100%, respectively), portal venous gas (13% and 100%), free air (52% and 92%) and a gasless abdomen (32% and 92%, respectively).[78]

A recent study of radiographic findings crafted a numeric score designed to indicate relative certainty that a patient was progressing in severity of NEC. This scale was termed the Duke abdominal assessment scale (DAAS). It is a 10 point scale that progresses from 0, or "normal gas pattern", through "fixed or persistent dilation of bowel loops", to a score of 10 which is indicative of "pneumoperitoneum".[79] This study noted increasing DAAS scores with increasing disease severity and noted that patients were more likely to undergo operative intervention with every one point increase in DAAS score.[80]

Ultrasonography has become an added adjunct to the radiographic evaluation of neonates suspected of having NEC. Echogenic dots and dense granular echogenicities can often be found in infants with early stage NEC.[54] A recent study examining 44 neonates who had 55 sonograms correlated ultrasound findings to radiographic imaging and clinical outcomes. Focal fluid collections, echogenic free fluid (likely debris), increased bowel wall echogenicity and bowel wall thickness were statistically significant for predicting unfavorable outcomes. Conversely, anechoic free peritoneal fluid predicted a good outcome.[81]

In some instances where perforation was present in the absence of free intraperitoneal air, ultrasound was able to detect echoic free fluid and bowel wall thickening.[82] The sensitivity of free air at abdominal radiography as a positive sign for surgical NEC with perforation is about 40%, whereas there can be 100% sensitivity by appreciating the absence of color flow during ultrasound.[83] Despite its added utility, the quality and accuracy of ultrasound imaging is extremely operator dependent. In addition, many centers do not have ultrasound capabilities within the facility 24 h a day, which may also limit its diagnostic utility.

BIOLOGICAL MARKERS

As previously mentioned, clinical parameters alone have been inadequate in predicting progression to surgical NEC. Many investigators are therefore suggesting that a variety of biologic markers be investigated. Identifying a specific biomarker or panel of biomarkers may allow for physicians to detect severe cases of NEC before operative intervention is required. Ideal biomarkers should be able to differentiate NEC from sepsis and should also increase with severity of NEC.

A recent study from Hong Kong examined three intestinal biomarkers, liver-fatty acid binding protein, which is expressed by enterocytes and hepatocytes, intestinal-fatty acid binding protein (I-FABP), which is expressed solely by enterocytes and trefoil factor 3, which is expressed in the mucin-producing epithelial cells and goblet cells. In this particular study, all three factors, as well as the combined value, known as the LIT score, were significantly higher in the NEC groups when compared with septicemia or control groups. These biomarkers and the LIT score were also higher in NEC non-survivors compared with survivors. A LIT score >4.5 identified surgical NEC with a sensitivity of 83% and specificity of 100%.[84]

Studies looking at infants with Bell's stage II/III NEC were observed to have higher levels of I-FABP to creatinine ratios with increasing severity of disease. In addition, I-FABP: Cr ratios decreased after successful non-operative management as well as after surgery.[85] Other studies confirmed the utility of the use of I-FABP and saw that I-FABP levels were elevated in those necessitating surgery or who succumbed to NEC when compared to conservatively treated patients. Urinary I-FABP levels were only useful

once a clinical suspicion was made, however and may not be an adequate screening tool for NEC.[86]

A 2012 prospective multicenter study assessed another potential biomarker, S100 myeloid related protein (S100 A8/A9), a key player in the innate immune response. The authors concluded that this marker was significantly elevated in infants with surgical NEC when compared to those with sepsis or controls. They defined the optimal cutoff value at 3.0 mg/ml by a receiver operating characteristic curve analysis. With this assay they maintained a sensitivity of 100%, specificity of 96.4%, positive predictive value of 88.9% and negative predictive value of 100%.[87] Due to its accuracy, the S100 A8/A9 levels may be a promising tool to detect subclinical NEC.

Fecal concentrations of S100A12, a marker of intestinal inflammation, were also assessed. In a prospective study of 145 preterm infants, SA100A12 was significantly higher in infants with severe NEC at the onset of disease and at 4-10 days prior to onset. Unfortunately, the sensitivity and specificity were fairly low at 76% and 56% respectively.[88] Other fecal markers, including lactoferrin and calprotectin have also been studied with mixed enthusiasm. Both are inflammatory markers of gastrointestinal disease. In a small study of 14 newborns with NEC and 63 healthy infants, there were no noted differences in fecal concentrations of lactoferrin or calprotectin.[89] However, others found that calprotectin could actually serve as a biomarker if corrected for gestational age. Sensitivity and specificity for distinguishing moderate NEC from healthy infants and those with intestinal distress was fairly high with this correction.[90]

Other serum markers, such as C-reactive protein (CRP), serum amyloid A (SAA) and procalcitonin have been studied as potential biomarkers for the diagnosis and follow up of NEC. These markers may serve to distinguish advanced disease as opposed to screening, as infants with NEC stages II and III were significantly higher than those with only sepsis or stage I disease.[91] Negative levels of CRP might indicate another process, such as ileus.[92] A recent multi-institution, multiyear retrospective study of 220 neonates with Bell's stage two or greater found that CRP was significantly elevated 3 days prior to diagnosis in infants with stage three disease. In addition, these infants were more likely to have a higher immature to total neutrophil ratio, as well as lower platelet count and pH.[93] In another study, SAA was higher in NEC infants than controls, but significantly lower in NEC infants when compared to those with sepsis. When inspected separately, the SAA levels of stage II NEC were higher than controls and stage I NEC, but similar to those with sepsis.[94] This may indicate that SAA is not a suitable biomarker to distinguish NEC from sepsis, but may be used to show progression of disease once diagnosed.

Inflammatory and anti-inflammatory cytokines have been extensively studied in the pathogenesis of NEC.[1] Many cytokines are also elevated in neonatal sepsis which makes utilizing them as biomarkers for NEC somewhat difficult. Nonetheless, a few cytokines, including IL-8 and IL-6 may be useful in detecting severity of disease. Studies assaying IL-8 have been able to differentiate the degree of bowel involvement in NEC. A cutoff of 449 pg/ml provided specificity of 82% and sensitivity of 83% in discriminating focal from multifocal and panintestinal disease. Likewise, a cutoff of 1388 pg/ml had specificity and sensitivity of 78% and 77% respectively for discriminating panintestinal disease from multifocal and focal disease.[95] IL-6 may also be a useful cytokine biomarker. A study done by Harris *et al*., found that IL-6 levels were elevated 5-10 fold in infants with bacterial sepsis plus NEC, as compared to infants with sepsis alone or controls.[96] This study also noted that IL-6 levels were higher in non-survivors of NEC, indicating that IL-6 elevations are related to increased mortality.

Examination of the umbilical cord has also been considered to identify infants at risk for NEC. The theory is that infants have inefficient antioxidant systems and are unable to counteract the harmful effects of free radicals. An Italian study recruited 332 patients at three European neonatal intensive care units. They saw that cord blood levels of several markers of oxidative stress, non-protein bound iron, advanced oxidation protein products and total hydroperoxides were significantly increased in babies with NEC compared to healthy babies.[97]

Vital sign and bioimpedence monitoring may be effective alternatives to traditional serum or fecal biologic markers. Abnormal heart rate characteristics have previously been implemented as a biomarker for sepsis and neonatal mortality.[98] In a randomized clinical trial involving over 3000 patients, heart rate characteristic monitoring significantly reduced mortality in very low birth weight infants.[99] Reduced heart rate variability and transient decelerations often occur in preclinical sepsis and may be applicable in NEC. In a 2013 U.S. study, investigators found that patients who developed surgical NEC had significantly higher baseline heart rate characteristic indices 1-3 days before diagnosis. Likewise, at the time of diagnosis, infants with surgical NEC had higher indices.[100]

Ideally, it would be beneficial to screen the infant's mother in the antenatal period for discrepancies in various biomarkers or immune modulators so as to be able to predict and prepare for the problem in the infant. Some have inspected the same biomarkers in the mother that they

have inspected in the infants. A U.S. study assayed maternal levels of IL-6, CRP and matrix metalloproteinase-9 between 24 and 32 weeks gestation in order to determine if elevations in these biomarkers could predict preterm labor or neonatal morbidity. Elevations in these markers above the 90th percentile were associated with preterm birth at gestational ages <32 weeks. There was no correlation however, between these levels and the development of NEC.[101]

A very recent study published in *Gut* combined clinical parameters with analysis of several urinary biomarkers, namely a family of fibrinogen peptides and found that the combination of parameters and biomarkers into an "ensemble algorithm" greatly increased the clinician's ability to predict the severity of NEC. Authors noted that prior to utilizing the ensemble that 40% of patients were not able to be accurately predicted. With the combination of parameters, all patients with surgical NEC were able to be appropriately categorized. This study utilized a small cohort from a larger population of individuals with NEC and certainly needs to be verified on a larger scale.[102]

Based on the available literature, there are clearly a number of serum, fecal and physiologic biomarkers exist that may be able to assist in readily identifying infants with NEC. Identifying the appropriate cut-off values between normal, septic and NEC infants will be paramount in identifying appropriate screening assays for NEC. In addition, identifying a panel of biomarkers will likely increase the sensitivity above any single test and may allow for a timelier implementation of novel therapies.

CONCLUSION

Surgically treated NEC can be very devastating. Surviving infants require attentive healthcare and often need invasive catheters for parenteral nutrition. Our current ability to medically treat NEC is limited. There are currently new therapies in development,[103-107] which have the potential to limit the extent of disease if administered before its deleterious effects. A scoring system to predict surgical NEC and mortality needs to be established in order to achieve this goal. To that end, a reliable and validated scoring algorithm composed of many of the neonatal and maternal risk factors mentioned within, as well as predictable radiographic and biologic markers, may facilitate earlier diagnosis and treatment patterns for NEC.

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