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Depth of Bacterial Invasion in Resected Intestinal Tissue Predicts Mortality in Surgical Necrotizing Enterocolitis

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Abstract

Objective—Up to a third of all infants who develop necrotizing enterocolitis (NEC) require surgical resection of necrotic bowel. We hypothesized that the histopathological findings in surgically-resected bowel can predict the clinical outcome of these infants.

Study design—We reviewed the medical records and archived pathology specimens from all patients who underwent bowel resection/autopsy for NEC at a regional referral center over a 10-year period. Pathology specimens were graded for the depth and severity of necrosis, inflammation, bacteria invasion, and pneumatosis, and histopathological findings were correlated with clinical outcomes.

Results—We performed clinico-pathological analysis on 33 infants with confirmed NEC, of which 18 (54.5%) died. Depth of bacterial invasion in resected intestinal tissue predicted death from NEC (odds ratio 5.39 per unit change in the depth of bacterial invasion, 95% confidence interval 1.33-21.73). The presence of transmural necrosis and bacteria in the surgical margins of resected bowel was also associated with increased mortality.

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A.M., J.I.R., and S.C.A. designed the study, J.I.R., S.C.A., S.M., S.A.G., and A.M. collected data, A.A.L. and R.R. provided critical tools and expertise for data analysis, A.M. wrote the manuscript. All the authors reviewed and approved the final manuscript

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Conclusions—Depth of bacterial invasion in resected intestinal tissue predicts mortality in surgical NEC.

Keywords

feeding intolerance; clinico-pathological; necrosis; bacterial overgrowth; neonate; outcome

INTRODUCTION

Necrotizing enterocolitis (NEC) continues to be a leading cause of morbidity and mortality in premature infants.[1, 2] Although approximately one-half of all infants with NEC respond to bowel rest, antibiotics, and supportive medical measures, many develop progressive bowel disease requiring surgical intervention.[3] In the sub-group of infants with NEC who require surgery, up to half may undergo laparotomy and require resection of necrotic bowel. In this study, we investigated the hypothesis that the histopathological findings in intestinal tissue resected for NEC could be useful to the clinician in predicting the clinical outcome of these infants.

Histopathologically, the most prominent findings of NEC include coagulative necrosis, bacterial overgrowth/invasion, *pneumatosis intestinalis* (gaseous cysts in the bowel wall), and inflammatory changes.[4, 5] Although the severity and extent of these findings may vary from one patient to another, the literature is scant on clinico-pathological correlation in NEC and histopathology reports are often of limited utility to the clinician beyond confirmation of the diagnosis. To investigate whether the histopathological findings in surgically-resected NEC tissue carries predictive information, we reviewed the medical records and archived pathology specimens from all patients who underwent bowel resection/autopsy for NEC at a regional referral Center over a 10-year period.

PATIENTS AND METHODS

Demographic and clinical information

A retrospective chart review was performed after approval by the Institutional Review Board on infants with a diagnosis of NEC (Bell stages II or III)[6] treated at the University of Illinois Hospital, Chicago during the period Jan 2001- Jun 2012. Demographic characteristics including birth weight, gestational age, gender, ethnicity (African-American, Caucasian, Latino, or other), and mode of delivery were noted. We also recorded clinical information including Apgar scores, age at initiation of feedings, blood culture-proven sepsis prior to onset of NEC, assisted ventilation and pressor support during the first 24 hours after the onset of NEC, central line days, patent *ductus arteriosus*, indomethacin therapy, severity of intraventricular hemorrhage, age of onset of NEC, and the clinical presentation of NEC (abdominal distension, pre-feeding residuals ≥30% of the feeding volume, bloody stools), length and region of resected bowel, and disposition (death *vs*. discharge to home).

Histopathological evaluation

Specimens of bowel tissue from above patients with NEC, either resected during surgery or obtained at autopsy, were identified in pathology archives. Autopsy specimens were carefully screened for signs of post-mortem autolysis such as epithelial denudation in areas not affected by NEC.[7] To minimize sampling errors, sections were obtained from all the available tissue blocks. In more recent cases, tissue samples were obtained from different parts of the resected bowel specimen.

Hematoxylin & eosin-stained tissue sections were digitized using the Hamamatsu Nanozoomer 2 slide scanner (Hamamatsu Photonics KK, Hamamatsu City, Japan) and evaluated by two independent, blinded observers using a digital slide viewing software program (NDP-view, Hamamatsu). The following information was recorded for each section: area of necrosis on the slide (per cent), scored as 0, 1 (0-25%), 2 (26-50%), 3 (51-75%), and 4 (76-100%); maximum depth of necrosis, scored as 0, 1 (mucosa), 2 (submucosa), 3 (*muscularis propria*), and 4 (transmural); severity of inflammation, scored as 0 (no inflammatory cells), 1 [up to 50 inflammatory cells/high power field (hpf)], 2 (51-200 inflammatory cells/hpf, 3 (>201 cells/hpf); depth of inflammatory infiltrates, scored as 0, 1 (mucosa), 2 (submucosa), 3 (muscularis propria), and 4 (transmural); depth of bacterial invasion, scored as 0, 1 (mucosa), 2 (submucosa), 3 (muscularis propria), and 4 (transmural), and area in each section covered by pneumatosis intestinalis (per cent).

Statistical Analysis

Statistical analysis was performed using the IBM SPSS software (IBM Corporation, Armonk, NY). Inter-observer agreement was quantified using the kappa (κ) coefficient.[8] Data were defined as parametric if the following conditions were satisfied: (a) measurement was on a continuous scale; (b) consecutive data points were equidistant; (c) normal distribution, as defined by the Kolmogorov-Smirnov[9] and Shapiro-Wilk[10] tests; and (d) equality of variance, measured by Levene's test.[11] Descriptive statistics were computed, and univariate comparisons were made by the Mann-Whitney U[12] or the Student's t[13] test. Frequencies were compared by the Fisher's exact test.[14]

Binomial logistic regression was performed in SPSS using the 'enter' method with bootstrapping. R^2 was defined following Nagelkerke.[15] Highly-correlated variables were identified by Spearman's correlation[16] and multicollinearity diagnostics (tolerance <0.2 and variance inflation factors >10).[17] Confidence intervals were based on profile likelihoods and p-values were based on Wald's test.[18] Data were further analyzed by binary recursive partitioning to develop classification and regression trees (CARTs; software program XL-STAT, Addinsoft, New York, NY).[19] Nodes with ≥3 subjects were represented by pie charts depicting the number of infants who died (black) *vs*. those discharged home (grey), placed inside an outer ring that reflected outcomes in the parent node. For each node, the size (number of infants), percentage, and purity (% of infants with the dominant outcome) were depicted.

The predictive accuracy of the CART was measured by computing receiver-operating characteristics and estimating the area under the curve.[20] All statistical tests were two-sided and the results were considered significant at $p<0.05$.

RESULTS

We identified 84 patients who received a diagnosis of NEC (Bell's stages II-III) and were treated at the regional, referral neonatal intensive care unit at the University of Illinois, Chicago, Illinois during the period Jan 2001- Jun 2012. The medical records of these infants were reviewed to confirm that their clinical presentation, course, and radiological/laboratory data were consistent with a diagnosis of NEC. Three infants who developed bowel perforation within the 1st postnatal week were excluded as their clinical course was consistent with a diagnosis of spontaneous intestinal perforation. Twenty-three (63.8%) infants underwent laparotomy and surgical resection of affected bowel. Eleven (30.5%) received a peritoneal drain, and 4 of these infants subsequently underwent a secondary laparotomy and bowel resection. We also included 6 infants who died from acute complications of NEC and underwent autopsy soon after death. Thus, the final clinical-pathological analysis included 33 infants.

Demographic and clinical characteristics

The clinical characteristics of the 33 patients included in the clinical-pathological analysis were representative of the larger group of all infants who received a diagnosis of confirmed NEC at our Center during the study period (Table 1). Similarly, there was no difference between infants who underwent surgical bowel resection *vs*. patients from whom pathology specimens were obtained at autopsy (data not depicted). All further analysis was limited to the 33 infants with available histopathological data.

The median age of onset of NEC was 19 days [inter-quartile range (IQR) 9-29.5 days], at a median post-menstrual age (PMA) of 31.3 weeks (IQR 27.8-32.6 weeks). Males developed NEC at a later chronological age than females (median onset 26 days, IQR 17-32 days in males *vs*. 10 days, IQR 6-25 days in females, $p=0.02$), and also at a later PMA (median 32 weeks, IQR 30-32 weeks in males *vs*. 28 weeks, 26.3-32 weeks in females, $p=0.01$).

All 33 infants had abdominal distension at presentation. Fourteen of these 33 (42.4%) infants presented with increased pre-feed residuals, whereas bloody stools were recorded in 18/33 (54.5%). The 27 infants who underwent laparotomy and bowel resection were operated upon at median 2 days after the onset of NEC (IQR 0-4.5 days). Sixteen of these 33 (48.5%) infants were intubated and received assisted ventilation, and 18/33 (54.5%) received pressors during the first 24h after onset of NEC. The time from onset of NEC to surgery correlated with gestational age ($r=0.405$, $p=0.019$) and birth weight ($r=0.558$, $p=0.001$). Blood cultures were positive in 2/33 (6%; grew *Escherichia coli* and *Staphyloccus aureus*, respectively). Both these infants died. At surgical resection, parts of the jejunum were removed in 12/27 (44.4%), ileum in 27/27 (100%), and colon in 7/27 (25.9%). Twenty-two of these 27 (81.5%) patients underwent resection of more than one bowel regions.

Eighteen of our 33 (54.5%) patients died from NEC. Infants who died had lower birth weights (971±744 g vs. 1204±328 g in survivors, p=0.003). The gestational age of the two groups was 27.6±3.9 weeks and 28±1.9 weeks, respectively (p=0.18). There were no differences in the age at initiation of feedings, chronological age or PMA at onset of NEC, gender, or clinical presentation. There was a trend for the non-survivors to have lost more bowel to resection that did not reach significance (median 12 cm, IQR 7.7-75.5 cm in non-survivors *vs*. 7.5 cm, IQR 4.4-11.9 cm among survivors, p=0.089).

The length of hospital stay did not correlate with the length of resected bowel. There was a trend towards shorter length of hospital stay among infants who died from NEC than the survivors (median 35 days, IQR 26-178 *vs*. 110 days, IQR 70-149 for survivors, p=0.2).

Histopathological findings

The median number of tissue sections evaluated per patient was 3 (range 1-6). All tissue sections were evaluated by two independent observers. A high degree of inter-observer consistency was observed; the κ-values for necrosis, inflammation, bacterial overgrowth, and pneumatosis were 0.97, 0.94, 0.91, and 0.94, respectively.

Coagulative necrosis was the dominant finding, defined as loss of nuclei and diminished cytoplasmic staining, but with relatively-preserved, ‘ghost-like’ crypt-villus histoarchitecture (Fig. 1). Although necrotic changes were seen in all 33 (100%) tissue samples, there was considerable variability in the depth of necrosis (measured from luminal to serosal surface; median grade 3 or extending to the muscular layer, IQR 1-4) and in the total area affected in each tissue section (median grade 2 or 26-50% of the section, IQR grades 1-4). Most cases showed a strong correlation between the extent and depth of necrosis (r=0.807, p<0.001), although some sections were marked by narrow, lingular bands of necrosis extending towards the serosa. Necrosis was severe (grades 3 and 4) in 21/33 (63.6%), and transmural in 17/33 (51.5%) cases.

Inflammatory infiltrates were seen in 27/33 (81.8%) cases, comprised mainly of macrophages (62±8% cells/hpf) and neutrophils (18±6% cells/hpf; (Fig. 1, *inset*). The median severity of inflammatory changes was grade 1 (0-50 inflammatory cells/hpf; IQR grades 1-2). Severe inflammation (grade 3, or ≥201 inflammatory cells/hpf) was seen in 14/33 (42.4%) cases. A few eosinophils were noted in the involved areas in 4 (12%) infants. Inflammatory pseudomembranes and a crypt abscesses were seen in 4 (12%) infants.

The median depth of inflammatory infiltrates was grade 2 (extending to the submucosa; IQR 1-4). Leukocyte infiltrates were frequently deeper in infants with severe inflammatory changes (grade 3 or >201 inflammatory cell/hpf; r=0.623, p<0.001). Leukocyte infiltrates extended to the muscularis and serosa in 13/33 (39.4%) cases, and the depth of inflammatory changes correlated with the depth of bacterial invasion (r=0.372, p=0.03).

Bacteria were identified within bowel tissue affected by NEC from 20/33 (60.6%) infants, but not in the intact ‘skip’ areas. Specific stains (Brown and Brenn method) allowed for enhanced identification of bacteria (Fig. 2).[21] Most sections showed mixed populations of cocci and bacilli. Bacterial colonies could be identified in both the lumen and in tissues in

6/33 (18%) infants. In the bowel wall, bacteria were identified in the mucosa, submucosa, *muscularis*, and serosa in 3 (9.1%), 6 (18.2%), 10 (30.3%), and 1 (3%) cases, respectively. The presence of bacteria correlated with the extent (r=0.693, $p<0.001$) and depth of necrosis (r=0.799, $p<0.001$), depth of leukocyte infiltrates (r=0.454, $p=0.008$), and *pneumatosis* (as described below). Fungal hyphae were seen in 2/33 (6%) patients. Both these patients had severe necrosis and the hyphae were seen in the submucosa.

Pneumatosis was seen in 14/33 (42.4%) cases, located most frequently in the submucosa. In positive sections, pneumatosis involved a median 20% (IQR 5-31.25%) of the tissue on the slide. Pneumatosis was more likely to be seen in infants with severe bowel necrosis [12/21 (57%) cases with severe, grade 3-4 necrosis *vs*. 2/12 (16.7%) with grade 1-2 necrosis, $p=0.03$], and in those with identifiable bacteria on tissue sections [12/20 (60%) cases with bacteria *vs*. 2/13 (15.4%) cases with no bacteria, $p=0.015$].

Reparative/regenerative changes such as epithelial regeneration (replacement of columnar epithelium with cuboidal/flattened cells, granulation tissue formation, fibrosis, and villus atrophy[4] were seen in 22/33 (66.7%) cases. Vascular thrombi in capillaries and small vessels, and focal hemorrhages were also common and seen in 25/33 (75.7%) cases. The presence of regenerative changes did not correlate with the time elapsed between the onset of NEC and surgery ($p=0.2$).

Clinico-pathological correlation

Gestational age correlated with the severity of inflammation (r=0.426, $p=0.034$). Males were more likely to have severe necrosis (12/19 males vs. 6/14 females, $p=0.036$). Compared to females, males showed a tendency to have greater depth of necrosis ($p=0.078$) and affected area ($p=0.084$) that did not reach significance. Male infants were also more likely to show greater depth of bacterial invasion (median grade 2, IQR 0-3 in males vs. median 0, IQR 0-2.25 in females, $p=0.05$) and show pneumatosis (11/19 males vs. 3/14 females, $p=0.04$). Males were also more likely to have more extensive *pneumatosis* in a given section (median 5%, IQR 0-25% in males vs. median 0, IQR 0-1.25% in females, $p=0.017$).

Clinical presentation with increased pre-feed residuals was not associated with specific histopathological findings or outcome. Infants who presented with bloody stools were more likely to lose greater lengths of bowel to resection (median 12.6 cm, IQR 8.7-36.5 cm in infants presenting with hematochezia *vs*. 7 cm, IQR 4.1-8 cm, $p=0.021$), and show severe bowel necrosis [hematochezia noted in 16/21 (76.2%) infants with severe necrosis *vs*. 2/12 (16.7%) of those who did not, $p=0.001$] and identifiable bacteria on tissue sections [hematochezia noted in 14/20 (76.2%) infants with identifiable bacteria *vs*. 4/13 (30.8%) of those who did not, $p=0.03$]. There was a trend towards finding pneumatosis that did not reach significance ($p=0.09$). Bloody stools were not associated with mortality ($p=0.2$). The need for assisted ventilation or pressors in the first 24h after onset of NEC also did not correlate with specific histopathological findings.

Age at onset of NEC correlated with the depth of necrosis (r=0.343, $p=0.05$), identification of bacteria on tissue sections (r=0.463, $p=0.007$), depth of bacterial invasion (r=0.339, $p=0.05$), presence of pneumatosis (r=0.367, $p<0.03$), and the extent of pneumatosis

(r=0.425, *p*=0.014). The time elapsed between onset of NEC and surgery showed a negative correlation with the length of resected bowel (r=-0.69, *p*=0.004). We looked for, but did not find an association between inflammation and time between the onset of NEC and surgery.

To determine whether the combination of the length of resected bowel with specific histopathological findings may improve our ability of predict clinical presentation or mortality, we multiplied length with the severity grades for depth of necrosis, severity of inflammation, and the depth of bacterial invasion). Assuming that the severity of these histopathological findings was distributed uniformly across the entire length of the resected bowel segment, these computed variables would depict the total 'burden' of necrotic or inflamed bowel, or bacterial translocation. Length*depth necrosis correlated with a presentation with bloody stools and time elapsed between onset of NEC and surgery, length* severity of inflammation correlated with gestational age, and length*depth of bacterial invasion with age at NEC. However, these computed variables did not show any novel associations that had not already been identified with the component variables (length of resected bowel, necrosis, inflammation, or depth of bacterial invasion).

Outcome

In univariate analysis, infants with bacteria identifiable in the bowel wall were more likely to have died than those who did not have identifiable bacteria on tissue sections [14 deaths among the 20 (70%) infants with identifiable bacteria *vs*. 4/13 (30.8%) with no bacteria, p = 0.03]. There was a trend towards increased mortality in infants with severe bowel necrosis that did not reach statistical significance (p=0.08). To identify predictors of mortality, we next performed logistical regression using the following independent variables: gestational age, birth weight, chronological age at NEC, PMA, time elapsed between the onset of NEC and bowel resection, depth of necrosis, severity and depth of inflammatory changes, depth of bacterial invasion, and the extent of pneumatosis. The best-fitting model included the depth of necrosis, severity of inflammation, depth of bacterial invasion, and the extent of pneumatosis (χ^2 = 10.1, p=0.039; Nagelkerke R^2=0.353) and predicted 15/18 (83.3%) deaths. Each unit change in the depth of bacterial invasion increased the odds of death by a factor of 5.39 [95% confidence interval (CI) 1.33-21.73, *p*=0.018; bootstrap CI 0.56-22.11, *p*=0.008].

When the model was re-computed after exclusion of the one full-term infant in our study group, the accuracy improved (χ^2 = 13.69, p=0.008; Nagelkerke R^2=0.47). In this analysis, depth of bacterial invasion increased the odds of death by a factor of 11.21 (CI 1.68-74.92, *p*=0.013). Depth of necrosis also showed a small but statistically-significant impact (odds ratio 0.17, CI 0.03-0.97, *p*=0.046). Depth of bacterial invasion remained a significant predictor of death even after statistically controlling for the effect of gestational age, birth weight, age at NEC, PMA, involved bowel region, or the time between the onset of NEC and surgery/autopsy.

We next used binary recursive partitioning to develop CARTs using the covariates listed above. Mortality increased as a function of the depth of bacterial invasion, particularly when bacteria were detected in the submucosa and deeper layers (grades 1.5-2.5; Fig. 3). Mortality

was also influenced by gestational age, with infants born at <=27.5 weeks at greater risk of death at a given depth of bacterial invasion. The predictive accuracy of this model was 62%.

Transmural necrosis and bacterial overgrowth in surgical margins

We further asked if the detection of transmural necrosis with bacterial overgrowth in the surgical margins may indicate incomplete removal of necrotic tissue and, therefore, predict mortality. We were able to obtain the surgical margins from 22 of the 33 infants. Transmural necrosis and bacterial overgrowth was noted in the surgical margins in 8 (36.4%) cases, and 6 of these 8 (75%) infants died. A representative tissue section from one of these infants is shown in Fig. 4. Only 4/14 (28.6%) of the infants who did not have transmural necrosis in surgical margins died (p=0.048).

DISCUSSION

Our clinico-pathological analysis shows that the depth of bacterial invasion in resected bowel tissue can predict mortality in infants with NEC, particularly in those born at a gestational age ≤27 weeks. Our findings also indicate a need for careful evaluation of the resected bowel segment for transmural necrosis and bacterial overgrowth in the surgical margins, which can provide clinically-important information. Although the histopathology of NEC has been described previously,[4, 5] our study is, to our knowledge, the first to investigate whether histopathological findings in bowel resected for NEC can predict mortality in surgical NEC.

In our study, the depth of bacterial invasion in the resected bowel emerged not only as the leading predictor of mortality in NEC but also showed a strong correlation with all other findings that comprise the pathoanatomy of NEC, including the extent and depth of coagulative necrosis, leukocyte infiltrates, and *pneumatosis*. These findings support current pathophysiological models where gut luminal bacteria play a central role in NEC.[22] Even though some bacterial proliferation may be expected in devitalized/ischemic bowel tissue, increasing evidence now indicates that bacterial overgrowth, low species diversity and early colonization with gammaproteobacteria (*Enterobacteriaceae, Pseudomonadaceae*) and *Clostridia* likely precede NEC development.[23, 24, 25, 26] The role of bacteria in the pathogenesis of NEC is illustrated by the exclusive occurrence of NEC after postnatal bacterial colonization; intestinal injury in the sterile *in utero* microenvironment may cause strictures or atresia, but not NEC.[27] Similarly, the postnatal ‘latency’ before the typical onset of NEC in the late 2nd-3rd postnatal weeks may also be related to the time needed for bacterial flora to be established in the intestine.[28] *Pneumatosis*, the radiological hallmark of NEC, indicates the accumulation of gaseous products of bacterial fermentation in the bowel wall. Finally, the pathogenic role of bacteria in NEC is supported by evidence that enteral antibiotics can reduce the incidence of NEC and related mortality.[29]

In premature infants, depth of bowel necrosis was a minor, although significant, predictor of mortality in NEC. Historically, coagulative necrosis in NEC has been viewed as evidence of splanchnic vascular immaturity and ischemic injury to the bowel.[4, 30, 31] In this pathophysiological model, ischemic injury disrupted the gut epithelial barrier and promoted bacterial translocation, triggering an exaggerated and destructive mucosal inflammatory

response. However, if ischemic injury was the initiating event in NEC, coagulative necrosis should have emerged as a key determinant of the severity of NEC and related mortality. In this context, the observed contribution of necrosis to NEC-related mortality was smaller than anticipated. Although the reasons for this discrepancy are unclear, a few possibilities merit consideration. Premature infants in the post-menstrual age range of 28-40 weeks have the capacity to grow more bowel than at any other time in life,[32] which, within certain limitations, could impart some tolerance to the loss of bowel to necrosis, resection, or scarring. The timing of surgical resection and removal of devitalized bowel needs consideration but in our study, there was no difference in the time elapsed between the onset of NEC and surgery in infants with minor *vs*. severe necrosis. Finally, the role of bacterial flora as a driver of necrosis (as opposed to bacterial translocation in necrosed tissue) also needs consideration. In rodent and swine models, bacteria and bacterial products can trigger NEC-like injury by inducing secondary mediators such as platelet-activating factor.[33, 34, 35] Bacterial toxins could also produce necrotic lesions; in avian necrotic enteritis, Clostridial toxins such as the α-toxin, the β_2-toxin, and NetB can induce bowel necrosis through direct cytotoxicity.[36] Although such toxins have not been identified in NEC so far, the preponderance of specific bacterial genera in patients who go on to develop NEC raises interesting possibilities for the role of pathogen-derived factors.

In our cohort, males developed NEC at a later chronological age and PMA, and were also more likely to have severe necrosis, greater depth of bacterial invasion, and severe *pneumatosis* on histopathology. Age at onset of NEC was independently associated with these histopathological findings and therefore, was a likely confounder in this equation. However, gender differences in the risk and severity of NEC have been described previously.[37, 38, 39] Patients with severe necrosis were more likely to have presented with hematochezia, which could be explained by the involvement of mucosal and submucosal vessels. However, presentation with bloody stools was not predictive of adverse outcome. Bloody stools have been considered an infrequent sign of NEC in premature infants, except perhaps in the setting of a viral infection.[40, 41] In our study, there were no demographic differences between the infants who presented with bloody stools *vs*. others who did not.

Detection of transmural necrosis and bacteria in the surgical margins of the resected bowel segment also predicted mortality. Although the presence of transmural necrosis in surgical margins may indicate ‘incomplete excision’ of a necrotic bowel segment, clear demarcation of viable from necrotic bowel tissue can be a daunting, if not impossible, task in the setting of acute NEC.[42, 43] Cognizant of the limitations in visual identification of necrotic bowel and also of the long-term morbidity of short-bowel syndrome, most surgeons take a conservative approach and remove only the most obviously devitalized bowel.[42, 44, 45] Further study is needed to determine whether there may be a role for rapid frozen-section evaluation of the surgical margins of the resected bowel within the operating room.

Our study has generated new, clinically-relevant data on histopathological findings in NEC, and has also provided novel, although indirect, information on the relative contribution of bacterial factors *vs*. ischemia in the pathogenesis of NEC. We acknowledge the need for cautious interpretation of our findings in view of the small sample size, and the limitations of retrospective design and single-center format, which increase the risk of bias.[46] There is a

need to validate these findings in a larger, multi-centric cohort, which may also allow the inclusion of additional clinical/laboratory predictors in the statistical models.[39, 47, 48, 49] Further study is also needed to investigate whether there is a role for rapid frozen-section evaluation of the surgical margins of the resected bowel in the operating room. Finally, our study does not provide information on the depth of bacterial invasion in non-resected bowel that is affected but not completely devitalized, indicating a need for focused investigation of bacterial translocation in preclinical models of NEC-like injury.

CONCLUSIONS

We show that the depth of bacterial invasion in surgically-resected bowel tissue can predict mortality in infants with surgical NEC, particularly in those born at gestational age ≤27 weeks. The presence of transmural necrosis and bacteria in surgical margins of the resected bowel was also associated with increased mortality.

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Abbreviations

NICU	neonatal intensive care unit
NEC	necrotizing enterocolitis
PDA	patent *ductus arteriosus*
IVH	intraventricular hemorrhage
VLBW	very low birth weight

FI	feeding intolerance
IQR	inter-quartile range
CI	confidence interval

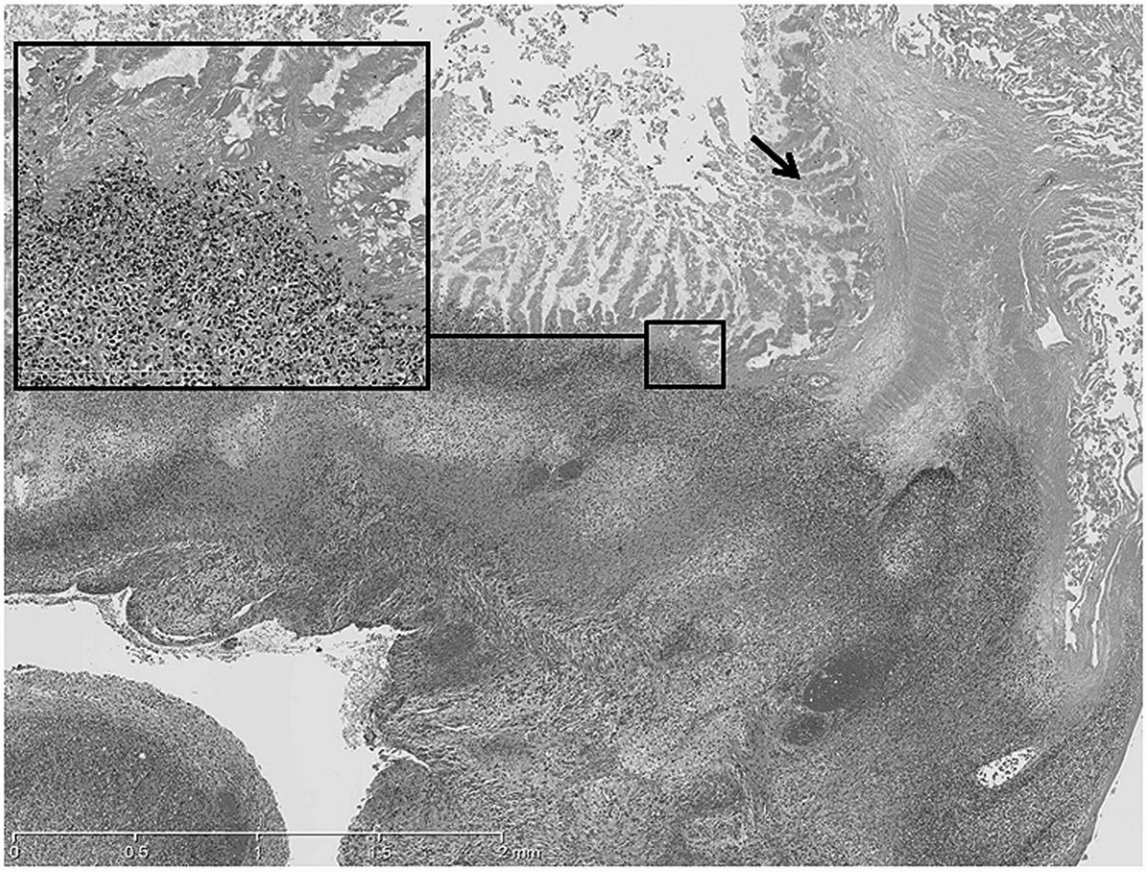

Fig. 1. Coagulative necrosis and inflammation in bowel tissue resected for NEC

H&E-stained tissue section (magnification 25x) shows coagulative necrosis and inflammatory changes. Coagulative necrosis is characterized by the loss of nuclei and cytoplasmic staining but with relatively-preserved 'ghost-like' crypt-villus histoarchitecture (arrow). *Inset:* Higher magnification image (magnification 100x) highlights inflammatory infiltrates, comprised mainly of macrophages and neutrophils.

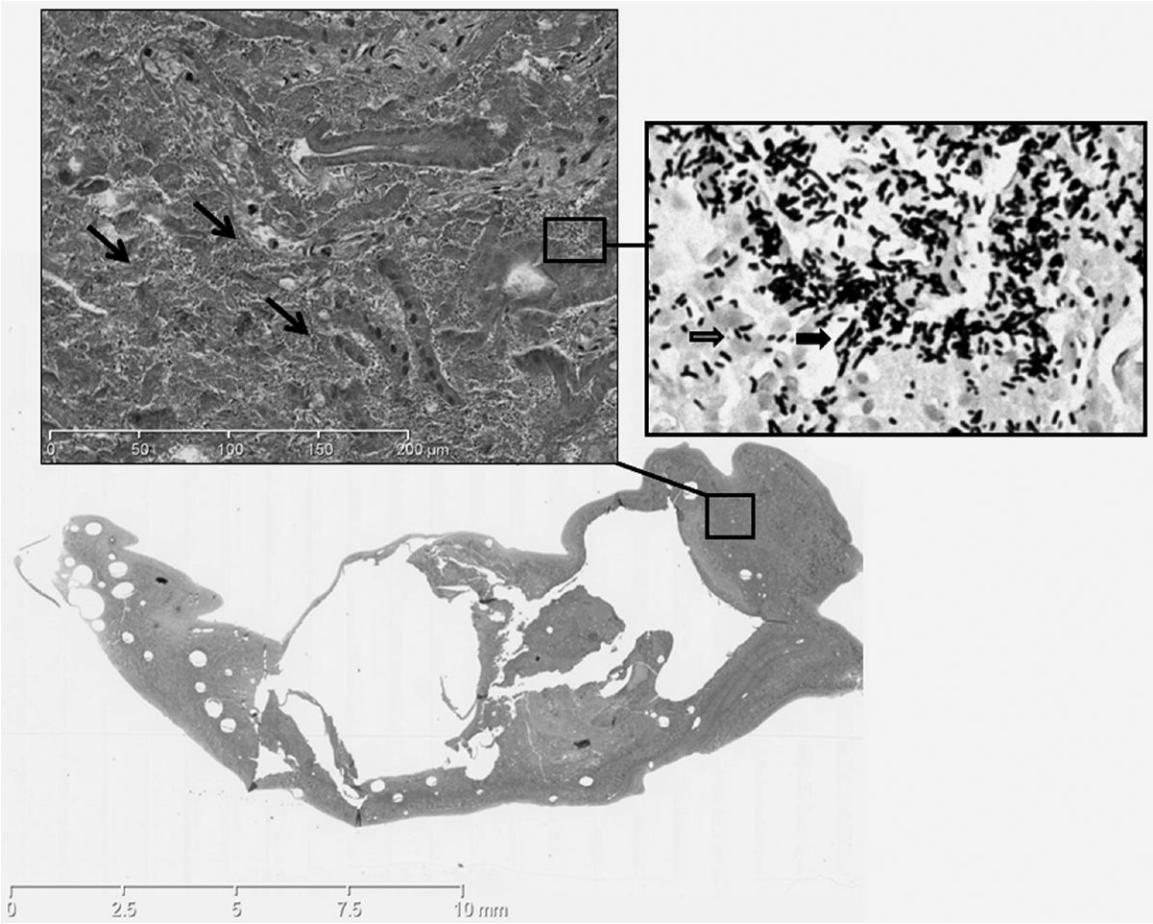

Fig. 2. Bacterial overgrowth in bowel tissue resected for NEC

H&E-stained section (magnification 6.3x) shows bowel tissue resected for NEC. *Insets:* higher magnification (magnification 100x) shows bacteria with discrete hematoxylin staining (arrows) on a background of necrotic tissue. Image on the right from a serial section (Brown and Brenn stain, 1000x) shows Gram-positive rods (blue; solid arrow) and Gram-negative cocci (open arrow) in the tissue.

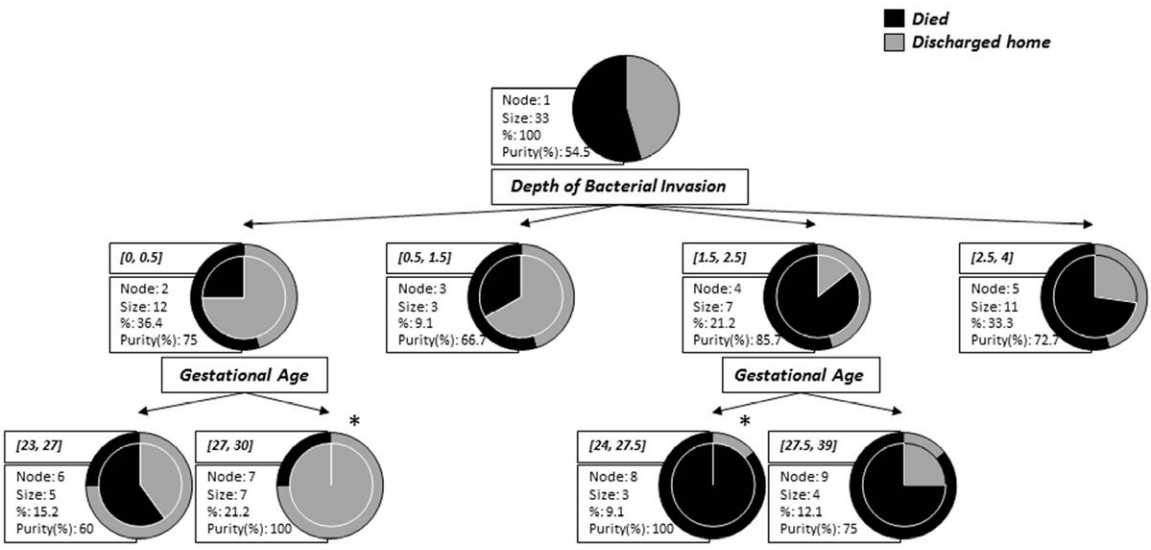

Fig. 3. Depth of bacterial invasion in resected bowel predicts mortality in NEC

Classification and regression tree showing higher mortality in NEC when the depth of bacterial invasion in resected bowel was ≥ grade 1.5 (bacteria detected in the submucosa and deeper layers). Mortality was also influenced by gestational age, with younger infants at greater risk of death at a given depth of bacterial invasion. * indicates $p<0.05$

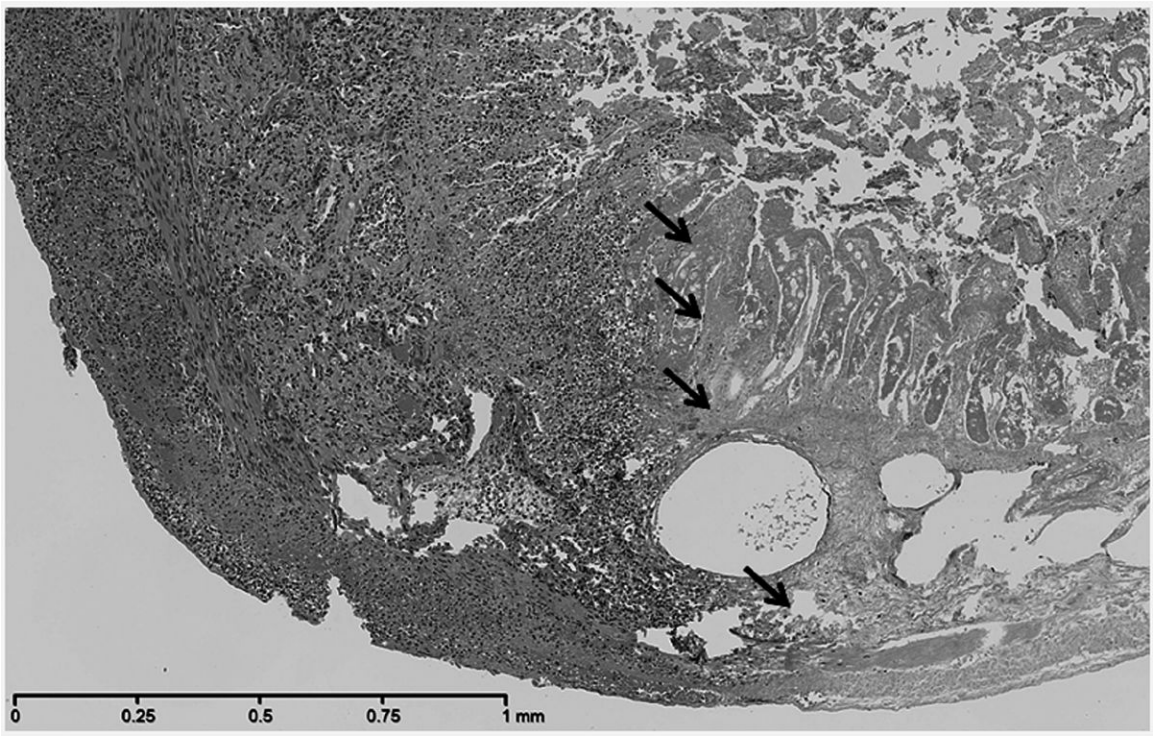

Fig. 4. Transmural necrosis and bacterial overgrowth in surgical margins of resected bowel

H&E-stained tissue section (magnification 50x) obtained from the surgical margins of resected bowel shows transmural necrosis (indicated by arrows), along with other pathognomonic features of NEC such as inflammation, bacterial overgrowth, and pneumatosis.

Table 1

Demographic characteristics

Characteristic	All NEC (n=81)	Surgical NEC + NEC autopsies (n=33)
Birth weight (g); median (interquartile range)	1098 (771-1286)	1077 (690-1307)
Gestational age (weeks); median (interquartile range)	28 (24.5-31.5)	28 (25.5-29.5)
Male sex – n (%)	42 (51.8)	21 (58.3)
Ethnicity – n (%)		
African-American	61 (75.3)	25 (69.4)
Caucasian	8 (9.8)	5 (13.8)
Latino	12 (14.8)	6 (16.7)
Mode of delivery		
Cesarean section	36 (44.4)	14 (38.9)
Vaginal	24 (29.6)	11 (30.6)
Outborn (%)	15 (18.5)	9 (25)
5-min Apgar <6 – n (%)	18 (12.3)	5 (13.9)
Age at feeding (days; median (interquartile range)	4 (2-9)	3 (2-8)
Percentage receiving some mother's milk	32	28
Patent ductus arteriosus – n (%)	42 (51.9)	18 (36)
Indomethacin – n (%)	26 (32.1)	15 (41.7)
Duration of antibiotics after birth (days; median (interquartile range)	3 (0-7)	3 (0-10)
Intraventricular hemorrhage ≥Grade 2 – n (%)	7 (8.6)	4 (11.1)
Central line† – n (%)	61 (75.3)	30 (83.3)
Positive blood culture‡ – n (%)	31 (38.3)	15 (41.7)
Onset of NEC (postnatal age in days); median (interquartile range)	20 (7-62)	19 (9-29.5)
RBC transfusion within 24-48h of diagnosis - n (%)	18 (22.2)	11 (30.6)