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Cytokine levels in the preterm infant with neonatal intestinal injury

and relationship with outcome

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Cytokine levels in the preterm infant with neonatal intestinal injury and relationship with outcome

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Abstract

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Background: Necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (SIP) are common newborn surgical diseases primarily affecting preterm infants. Increased levels of proinflammatory mediators may play a role in short and long-term outcomes in the preterm infant. Purpose: To characterize the cytokine response in preterm newborns with surgical NEC or SIP before and after surgical treatment as compared to non-septic, preterm surgical newborns and to relate this to intestinal disease (NEC vs. SIP) and short-term outcome. Methods: The study was a 14-month prospective, cohort study of neonates undergoing surgery or drainage for NEC or SIP or surgical ligation of patent ductus arteriosus (PDA, the non-septic control group). Bioplex xMAP technology was used to analyse 6 inflammatory markers (IL-2, IL-6, IL-8, IL-1β, IFN-Ɣ, and TNF-α). Prospectively collected clinical data included: preoperative risk factors, indications for surgery or drainage, extent of intestinal disease, and early postoperative morbidity and mortality. Results: NEC had much higher median preoperative levels than SIP and PDA subjects of the cytokines IL-6 (NEC: 8381 pg/ml; SIP: 36 pg/ml; PDA: 25 pg/ml), IL-8 (NEC: 18438 pg/ml; SIP: 2473 pg/ml; PDA: 1110 pg/ml), and TNF-α (NEC: 161 pg/ml; SIP: 77 pg/ml; PDA: 61 pg/ml), and IL-1β (NEC: 85 pg/ml; SIP: 35 pg/ml; PDA: 24 pg/ml). NEC-totalis had the highest levels of IL-6, IL-8, and IL-1β, but only IL-8 was significantly different from limited NEC (28141 pg/ml vs. 11429 pg/ml). Salvage laparotomy (reoperation) after primary peritoneal drainage was associated with lower early postoperative IL-6 and IL-1β (postoperative days 1 and 2) as well as TNF-α levels (postoperative days 2 and 5). Early mortality (<30 days) was associated with elevated preoperative IL-6, IL-8, and TNF-α, but 5 of 7 deaths were in subjects with NEC-totalis. Conclusions: Surgical NEC is a profoundly more proinflammatory disease than SIP, which is closer to a non-septic neonate. A strong proinflammatory response is associated with early postoperative death, likely due to its association with NEC-totalis.

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INTRODUCTION

Necrotizing enterocolitis (NEC) is an inflammatory disease of the intestine that affects primarily preterm neonates. Despite major advances in neonatal care in the past decades, intestinal perforation from NEC or spontaneous intestinal perforation (SIP) remains one of the most common and devastating complications of prematurity. Over 50,000 babies are born with birth weights <1500 grams annually (1). The risk of intestinal perforation is highest for the very low birth weight newborns, which leads to increased morbidity and mortality among preterm infants. The risk of NEC increases by 3% for each 250 gram decrease in birth weight, and SIP affects 2% of newborns with birth weights < 1500 grams (2).

The etiology of NEC is still unclear but appears to be a proinflammatory disease. Multiple factors, such as feeding, intestinal immaturity, inflammatory mediators, ischemia and infectious organisms, are thought to contribute to the pathogenesis of NEC (3). Hematopoietic cytokines have direct effects on the developing intestine and may initiate and propel the progression of NEC. Activation of the toll-like receptors by bacterial endotoxin leads to an inflammatory cascade, culminating in intestinal inflammation (4). The clinical manifestation of the postoperative inflammatory response is characterized by capillary leak, tissue edema, hypotension, oliguria and acidosis. The cytokine mediators of the systemic inflammatory response system include interleukin-1, tumor necrosis factor-alpha, interleukin-6 and interleukin-8, though immune system function (pattern of production and response to cytokines) varies with age (5,6). Plasma levels of interleukin-6 are elevated five- to ten-fold in septic babies with neonatal NEC (7). NEC-totalis, or total intestinal gangrene, is a particular aggressive form of NEC and is a fatal disease. The etiology and inflammatory profile of this subset of NEC has not been studied.

Spontaneous intestinal perforation (SIP) is a distinct clinical entity from neonatal NEC but is often treated in a similar manner. Studies of treatment strategies for neonatal intestinal perforation in the preterm infant usually include both the diagnoses of NEC and SIP together.

Newborns with SIP typically develop the disease earlier, usually have not been fed, and do not exhibit radiographic pneumatosis intestinalis (8). Distinguishing between NEC and SIP can be difficult in infants without obvious pneumatosis intestinalis. Although newborns with SIP are likely to have lower birth weight and gestational age and to present earlier in the hospital course before the initiation of enteral feeds, overlap exists between the clinical presentation of SIP and NEC (9). Thus, clinical characteristics alone may not always distinguish between these entities. Biomarkers could aid in classifying intestinal perforations as NEC or SIP. Currently, however, there are no published studies describing the inflammatory cytokine profile of SIP.

Peritoneal drainage is more often utilized for this group of subjects, but some surgeons prefer laparotomy as a first line of treatment. Although outcomes are worse with NEC than SIP, a prospective observational study showed a neurodevelopmental advantage with laparotomy over peritoneal drainage in neonates with either NEC or SIP (10). Currently, the NICHD-sponsored Neonatal Network NEC surgical trial is investigating the neurodevelopmental outcomes in very low birth weight babies undergoing laparotomy and peritoneal drainage for both NEC and SIP. However, because the indications for these treatment strategies vary so much between individual surgeons and institutions, identification of clinical parameters which may favor one treatment strategy over the other will likely remain unsettled after this trial.

Recently, levels of proinflammatory mediators have been correlated with long-term developmental outcomes. In small studies, neonatal cytokine levels tended to be greater in the infants later found to have abnormal cognitive and psychomotor outcomes (11). This suggests that increased serum levels of proinflammatory cytokines may contribute to the poor neurodevelopment observed in the very low birth weight infant with intestinal disease. Increased neonatal levels of interleukin-8 have recently been linked to the development of cerebral palsy (12). Certain proinflammatory cytokines are known to be toxic to the developing white matter. The pre-myelinating oligodendrocytes of the developing brain are extremely vulnerable to injury from cytokines such as interleukin-6, tumor necrosis factor-alpha, and interferon-gamma (13). It

is this injury that is felt to be a major factor in the diffuse white matter injury frequently seen after sepsis and NEC.

Intestinal perforation caused by NEC, as compared to SIP, is associated with worse neurodevelopmental and survival outcomes at 1 year (9, 14). The differences in the cytokine profile of these two disease processes, however, have not been described. Given the association between proinflammatory mediators, the septic response, and neurodevelopmental outcomes, it is conceivable that disease processes with unfavorable cytokine profiles could be associated with worse short- and long- term outcomes (Figure 1a). Altering this cytokine profile by either surgical treatment strategies that alleviate this proinflammatory response or medical treatments that target the cytokine response could influence the approach to NEC and SIP in the preterm infant. The aims of this study are to: 1) characterize the perioperative systemic inflammatory response profile in preterm newborns requiring surgery or drainage for necrotizing enterocolitis (NEC) or spontaneous intestinal perforation (SIP), or patent ductus arteriosus (PDA) ligation (Figure 1b) and 2) to evaluate the systemic inflammatory response profile in preterm newborns receiving drainage for necrotizing enterocolitis (NEC) or spontaneous intestinal perforation (SIP) with treatment failure as defined by need for reoperation (Figure 1c).

Hypothesis 1:

Newborns with NEC or SIP have an increased in the proinflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-χ) as compared to preoperative levels in the non-septic comparison newborns undergoing PDA ligation, regardless of gestational age. Newborns with NEC-totalis have the greatest increase in the preoperative proinflammatory cytokines (IL-6, IL-8, IL-1β, TNF-α, and IFN-χ) as compared to those in newborns with limited NEC.

H**ypothesis 2**:

Newborns who require reoperation by salvage laparotomy after undergoing primary *drainage* for NEC or SIP have persistent postoperative elevations (>24 hours) of IL-6, IL-8, IL-1β, TNF-α, IFN-𝛘 as compared to those without reoperation.

Proinflammatory mediators are clearly part of the pathogenesis of NEC (15). Moreover, these same cytokines have been implicated in adversely affecting the long-term neurodevelopmental outcomes in preterm infants (12, 16-18). The role of the systematic inflammatory response in SIP has not previously been defined. This study is the first of its kind to evaluate disease-type (NEC vs. SIP), extent of disease (totalis versus limited NEC), and treatment outcomes with cytokine profiles.

BACKGROUND

I. *Newborn Cytokine Response and Outcomes*

Perinatal inflammation has been implicated in the development of periventricular leukomalacia, the central nervous system change associated with very low birth weight infants with neurodevelopmental impairment (19). Certain proinflammatory cytokines are known to be toxic to the developing white matter. In cell culture, TNF-α and IFN-Ɣ have been shown to be toxic to developing oligodendroglia (20). Periventricular leukomalacia (PVL) has been shown to be associated with increased staining for the proinflammatory cytokines TNF-α IL-1β, and IL-6 in autopsy specimens (21). Clinically, preterm infants with neonatal sepsis and infection have been shown to be at increased risk of developing PVL. Maternal chorioamnionitis is associated with a six fold risk of a preterm infant developing PVL (22, 23). The proinflammatory cytokines IL-8, IL-12, IL-17, TNF-β, IL rα, macrophage inflammatory protein 1β were found to be altered on days 0-4 of life in infants who later developed cerebral palsy (12).

NEC has been found to be associated with white matter injury, which is likely the result of the proinflammatory mechanisms seen with sepsis and NEC. Infants with NEC who have undergone surgery for intestinal perforation have a higher incidence of PVL (24). Higher levels of the cytokine IL-6 has been associated with neurodevelopmental impairment in infants with advanced or surgical NEC (11). This suggests that increased serum levels of proinflammatory cytokines may contribute to the poor growth and neurodevelopment observed in the very low birth weight infant with intestinal disease. A better understanding of the interaction between inflammatory cytokines and neurodevelopment may help to develop strategies to minimize secondary neural injury in preterm infants.

The morbidity and mortality from SIP, although better than NEC, is significantly worse than that for infants without intestinal perforation. SIP is associated with a two-fold risk of neurodevelopmental impairment and death compared to infants of comparable gestational age (14). It is assumed that SIP is less likely to be associated with a proinflammatory response than

NEC, but no studies of the cytokine response in SIP have been published. Given the overlap clinically between SIP and NEC, understanding the proinflammatory profile in these diseases may help to define better strategies for minimizing secondary neural injury and predicting outcome.

II. ***NEC, SIP and Treatment Strategies***

Preterm intestinal injury is major cause of morbidity and mortality, and the optimum surgical treatment is uncertain. The traditional approach to preterm infants with intestinal necrosis or perforation has been a formal laparotomy, which involves a large incision across the abdomen with resection of necrotic and/or perforated intestine. In critically ill infants or low birth weight infants (<1500 grams), primary peritoneal drainage through a small abdominal incision has been used as an alternative to laparotomy (25). There is controversy over which treatment is preferable. Some have used preoperative diagnosis of spontaneous intestinal perforation as opposed to neonatal necrotizing enterocolitis (NEC) as the indication for primary peritoneal drainage as opposed to laparotomy. Necrotizing enterocolitis is a severe inflammatory condition of the bowel leading to sepsis, intestinal pneumatosis and necrosis, and/or perforation. Spontaneous ileal perforation (SIP) is thought to be a distinct clinical entity that occurs in very low birth weight, preterm newborns in which intestinal perforation occurs in the absence of pneumatosis or necrosis. These two disease entities occur in similar risk-profile infants and are often treated similarly. However, previous observational, multi-centered studies did not find survival advantage of primary peritoneal drainage over laparotomy for suspected SIP (26). In a large, multi-centered, randomized trial no differences in outcomes were seen between neonates randomized to laparotomy and primary peritoneal drainage (27). However, this trial focused only on mortality and prolonged need for parenteral nutrition. Subgroup analysis found no differences by diagnosis or gestational age. Yet, strong biases still exist between treatment strategies. These biases are based on individual surgeons and neonatologists clinical experience regarding appropriate treatment choices for individual subjects based on their full clinical picture.

Among extremely low birth weight babies, neonatal NEC is associated with adverse neurodevelopmental outcomes (15). In a multi-centered, observational study looking at both neurodevelopmental and mortality outcomes, outcomes were worse with NEC as well as with peritoneal drainage (10). These findings have led to a large, multi-centered, randomized trial assessing the role of initial surgical treatment in maximizing optimal neurodevelopmental survival. However, obstacles to this trial include heterogeneity of the subject population and selection bias due to surgeon preferences. This reluctance by surgeons and neonatologists to randomize to either a laparotomy or peritoneal drainage treatment strategy stems from a strong belief that some subgroups have better outcomes with one treatment strategy, while others do better with another. From previous observational studies mentioned above, preoperative diagnosis, birth weight, or gestational age alone, however, do not seem to be adequate criteria for a treatment algorithm (10).

III. *The Effects of Gestational Age*

Gestational age is clearly associated with both mortality and neurodevelopmental impairment. Neurological and developmental disability in very low birth weight and preterm babies is present in nearly 50% of survivors (28). Adult medical disabilities increase with decreasing gestational age. Outcomes begin to improve dramatically after 27 weeks gestational age. A frequent criticism of the observational studies comparing drainage versus laparotomy for intestinal perforation is the consistent lower average gestational age of infants who receive drains compared to laparotomy. Given the relationship between gestational age and outcomes, gestational age remains an important confounder in these studies.

The relationship between gestational age and cytokine response to intestinal insult is less well-defined. Very low birth weight (<1500 gram) infants are able to mount an inflammatory response to infectious insults. IL-10, Il-6, and regulated upon activation, normal T-cell expressed, and secreted (RANTES) were shown to accurately predict the onset of disseminated intravascular coagulation in severely septic preterm weight infants (29). In a study of 25 inflammation-

associated proteins in very low birth weight infants before 28 weeks gestation, a broad, wide-spread inflammatory response was sustained for up to 2 weeks (30). The concentrations of some proteins increased with postnatal age. The persistence of postnatal inflammatory proteins in newborns <28 weeks born after intrauterine inflammation demonstrates that these infants can mount a postnatal inflammatory response. However, whether the cascade of systematic inflammation varies by intestinal maturity and gestational age is uncertain.

METHODS

I. *Study Design*

A prospective cohort study was conducted with time zero defined as the time when the participant was determined to require surgical intervention. The population in the cohort was neonates admitted to Egleston NICU with: 1) the diagnosis of NEC, SIP, or PDA and 2) plan for surgical intervention for this diagnosis. Participants were followed for 30 days from enrollment. If participants were back-transferred to the referring hospital, the participants were followed until discharge from the neonatal intensive care unit at Egleston.

II. *Inclusion Criteria*

Eligible subjects included infants admitted to the Egleston neonatal intensive care unit for surgical treatment of NEC, SIP, or PDA. Surgical treatment of NEC and SIP could either be by laparotomy or by bedside placement of a surgical drain.

III. *Exclusion Criteria*

Newborns with evidence of documented congenital infection, major congenital anomaly, prior laparotomy or drain placement, or prior episodes of NEC or SIP were excluded from participation in the study.

IV. *Justification for PDA Group*

To define normal, perioperative concentrations of each cytokine, samples were obtained from stable neonates undergoing elective ligation of a patent ductus arteriosus (PDA). PDA is a common condition in preterm infants in which failure of the connection between the aorta and pulmonary artery to close results in persistence of the fetal circulation pattern. Newborns that undergo this procedure are of similar age and birth weights as those who undergo treatment for NEC and SIP. In addition, operative interventions for PDA ligation and for NEC or SIP typically occur in the neonatal intensive care unit in our hospital system, avoiding the stress of transport and changes in ventilatory management. Although the perioperative proinflammatory response has not been clearly defined in preterm infants, increased levels of proinflammatory mediators

have been measured postoperatively. Interleukin-6 and the acute phase reactants C-reactive protein and prealbumin have been found to be elevated after both laparotomy and thoracotomy (13). Moreover, surgery in the neonate results in an increase in oxygen consumption and resting energy expenditure immediately after surgery with a return to normal levels by 12-24 hours. The increase in resting energy expenditure is significantly greater in infants undergoing a major operation than a minor procedure (14).

In this study, the non-septic comparison group is an important non-septic control which can provide gestational age appropriate comparisons. The preoperative levels provide a non-septic baseline normal level for age. The perioperative levels allows us to factor the cytokine response to surgical intervention into our analysis of the proinflammatory response and defines the time it takes for these cytokine levels to return to preoperative levels. Thus, measuring the perioperative cytokine response in newborns undergoing PDA ligation provides a non-septic, perioperative cytokine profile in neonates of similar gestational age and birth weight and in a similar operative environment.

V. *Response Variables*

A. Hypothesis 1: Association between preoperative cytokine levels and disease

Response Variable: Preoperative cytokines levels (IL-2, IL-6, IL-8, IL-1β, TNF-α, IF-δ)

B. Hypothesis 2: Association of outcome with perioperative cytokine levels

Response Variable: Treatment failure defined by need for reoperation

VI. *Predictor Variables*

A. Hypothesis 1: Association between preoperative cytokine levels and disease

Predictor variable: Disease diagnosis – NEC (NEC totalis and limited NEC), SIP, or non-septic PDA (comparison group)

Confounder: SIP and PDA subjects may be overall lower in gestational age. Cytokine response to disease could vary with gestational age. Unknown confounders associated with gestational age my also influence disease-type and cytokine response (Figure 1b).

B. Hypothesis 2: Association of outcome with perioperative cytokine levels

Predictor variable: cytokine levels from preoperative to postoperative day 6

Confounder: SIP and PDA subjects may be overall lower in gestational age. Other confounders associated with this decreased gestational age or low birth weight include increased perinatal anoxia, risk of respiratory failure, intraventricular hemorrhage, perinatal steroids, blood transfusions, all which may influence outcome. Cytokine response to disease could vary with gestational age or with unknown confounders associated with low gestational age (Figure 1c).

VII. ***Inflammatory Marker Sample Collection and Processing***

Blood was collected for measurements of proinflammatory cytokines at the following time periods: prior to surgery, immediately postoperatively, and daily while the subject remained in the intensive care unit with indwelling lines (maximumof 6 days). Blood was be transferred onto filter paper, allowed to dry for ten minutes, and tubed to the laboratory at Egleston where the dried blood spot samples (DBSS) were stored on campus at – 20 degrees C, batched, and sent to Dr. Mary Cismowski at The Research Institute at Nationwide Children's Hospital at the end of recruitment. Specimen was be extracted from DBSS in extraction buffer on a micro plate shaker as described by Skogstrand *et al* (33-35). Bioplex xMAP technology will be used to analyse 6 inflammatory markers (IL-2, IL-6, IL-8, IL-1β, TNF-α, IF-δ).

VIII. Sample Size Calculations

A. Hypothesis 1: Association between preoperative cytokine levels and disease

Because the large effect size expected between NEC and SIP and PDA subjects, a large sample was not expected to be needed. There are no reported levels for SIP, so no reference value could be used. For IL-6, for example, a value of 50 pg/ml was expected for the non-

septic comparison (PDA group) with a standard deviation of 25 pg/ml and an effect size of 100% increase (100 pg/ml) for experimental groups. This was based on prior comparisons of suspected and surgical NEC (15). Using a power of 0.80 and an α=0.017 (corrected for multiple comparisons), a sample size of 6 subjects per group was necessary.

B. Hypothesis 2: Association of outcome with perioperative cytokine levels

The expected reoperation rate for drained subjects is 20%. Thus, an inherent imbalance exists between the treatment failure (salvage laparotomy) and treatment success groups (1:4). For IL-8, for example, a difference of 1000 pg/ml was expected with a standard deviation of 700 pg/ml (large variance) for each group. Using a power of 0.8 and α=0.05 (choosing only one postoperative day, postoperative day 2, to analyze), a total sample size of 30 subjects receiving drain would be required. However, no published values of postoperative cytokine levels in preterm infants are available for precise calculations.

IX. Statistical Analysis

A. Hypothesis 1: Association between preoperative cytokine levels and disease

The primary analyses were performed in subject groups with NEC, SIP, and PDA by preoperative diagnosis. Subject characteristics were compared using ANOVA test for continuous variables and Fisher's exact test for categorical comparisons. For preoperative cytokine analyses, median preoperative levels with interquartile ranges were determined for each group. Due to the skewness of the cytokine data, the nonparametric Kruskal-Wallis test was used to analyze the association between disease and preoperative cytokine levels. A Bonferroni correction for multiple analysis was used, resulting in $\alpha = 0.017$ due to three comparison groups (PDA, SIP, NEC).

In order to account for gestational age, a linear regression model was utilized for the cytokines found to be significant by the previous nonparametric analysis. The distribution of age and diagnosis was examined, and a gestational age cut point made based on the best balance of subjects with NEC and SIP between age categories. Median levels of preoperative cytokines

were reported for both gestational age categories by diagnosis, but overall median levels were not compared among groups due to sample size.

The influence of gestational age on cytokine levels was evaluated using linear regression modeling with *preoperative cytokine levels* as the outcome (y-axis) and the predictor variables of interest *diagnosis* (SIP = 0 and NEC = 1; x-axis). The potential predictors *gestational age* (dichotomous with gestational age ≤ 26.5 weeks = 0 and gestational age > 26.5 weeks = 1) and *the gestational age*diagnosis* interaction were evaluated for inclusion in the model as follows. A two-way ANOVA test was performed to look for interaction between diagnosis and gestational age by age category. In addition, the full model with the confounder of gestational age (as a dichotomous categorical variable) as well as with the interaction term of age category*diagnosis were compared to the model without these terms. The F-statistic was used to determine the utility of these additional predictors in the linear regression model. Expected mean levels for SIP and NEC with 95% confidence intervals were recorded for IL-6, IL-8, IL-1β and TNF-α.

Median cytokine levels for infants with NEC (excluding totalis) and NEC-totalis were compared using the non-parametric Mann-Whitney test using $\alpha = 0.05$ as significant.

B. Hypothesis 2: Association of outcome with perioperative cytokine levels

Log transformed, median cytokine levels (preoperative day 0 – postoperative day 6) for infants who required a salvage laparotomy after initial drain placement for treatment of SIP or NEC was compared to those who were treated alone using Mann-Whitney test for each day ($\alpha = 0.05$). Because of small sample sizes, no adjustments for multiple comparisons were made.

Median preoperative cytokine levels for infants with NEC or SIP who died were compared using the non-parametric Mann-Whitney test ($\alpha = 0.05$).

RESULTS

I. Characteristics of enrolled subjects

Between October 1, 2010 and December 1, 2011, data were collected on 36 subjects with the preoperative diagnoses of NEC, SIP, and PDA admitted to Egleston NICU for surgical intervention of their disease. Two subjects with NEC were term infants, which were not specifically excluded in the exclusion criteria, but were not part of analysis due to the original aim of the study. Thirteen of these thirty-four subjects were diagnosed with NEC (5 NEC-totalis, 8 limited NEC), 13 with SIP, and 8 with PDA for ligation (Figure 2). Maternal and infant characteristics of participants by diagnosis are listed in Table 1. All subjects with SIP received a primary peritoneal drain as initial treatment. Of these 13 SIP subjects, 10 survived without reoperation, 2 required reoperation and survived, and 1 died within a 30 day period. Of the 8 subjects with limited NEC, 2 received a primary peritoneal drain, and 6 received a laparotomy. Of the 2 NEC subjects who received an initial drain, 1 required reoperation and subsequently died. Of the 6 limited NEC subjects who received a laparotomy, 1 required reoperation and all survived. All 5 subjects with NEC-totalis underwent a primary laparotomy and died from their fatal intestinal disease.

II. Diagnosis and preoperative cytokine levels

Preoperative median cytokine levels by diagnosis of NEC, SIP, and PDA are shown in Table 2. One subject with NEC-totalis did not have a preoperative blood spot, leaving 33 subjects for preoperative cytokine analysis. Gestational age was categorized into $\leq$ 26.5 weeks gestation and >26.5 weeks gestation. This cut point led to the most balance between the SIP and NEC groups. Using this cut point, the lower categorical gestational age included 7 PDA subjects, 8 SIP subjects, and 5 NEC subjects. The higher categorical gestational age included 1 PDA subject, 5 SIP subjects, and 8 NEC subjects.

When not stratified by age, median levels of the proinflammatory mediators IL-6, IL-8, IL-1β, and TNF-α were highest in the NEC diagnosis (Table 2). The median level of IL-6 in NEC

was more than 200-fold higher and IL-8 more than 7-fold higher than either SIP or PDA median levels (IL-6: NEC 8381 pg/ml vs. PDA 25 pg/ml, $p<0.001$; NEC vs. SIP 36 pg/ml, $p < 0.005$; IL-8: IL-8: NEC 18438 pg/ml vs. SIP 2473 pg/ml, $p<0.005$; NEC vs. SIP 1110 pg/ml, $p < 0.05$). Median level of IL-1β and TNF-α were also significantly higher in the NEC group (TNF-α: NEC 161 pg/ml vs. SIP 77 pg/ml, $p<0.005$; NEC vs. PDA 71 pg/ml, $p < 0.005$; IL-1β: NEC 85 pg/ml vs. SIP 31 pg/ml, $p = 0.016$; NEC vs. PDA 24 pg/ml, $p < 0.005$).

Linear regression was used to examine the role of gestational age and cytokine levels in those which were found to be important in the overall analysis by non-parametric comparisons of preoperative cytokine levels and diagnosis (IL-6, IL-8, IL-1β, and TNF-α). Only the diagnosis NEC and SIP were compared in the model; PDA was not included. Gestational age was categorized into $\leq$ 26.5 weeks gestation and >26.5 weeks gestation. Results of two-way ANOVA tests for each preoperative cytokine with gestational age category and gestational age category interaction with diagnosis as variables did not show a significant age category or interaction as a predictor for IL-6, IL-8, IL-1β, and TNF-α. In addition, comparison of linear regression models with these additional predictor variables (age category and interaction of age category and diagnosis) did not yield any significant age category or interaction predictors. Therefore, for each of the cytokines IL-6, IL-8, IL-1β, and TNF-α, a linear regression model was performed with the model: preoperative cytokine level (pg/ml) = intercept (pg/ml) + slope (pg/ml) *diagnosis with the intercept representing preoperative cytokine levels for SIP and the slope representing the difference in preoperative cytokines for NEC versus SIP. Results for intercepts and slopes for each model with standard error are listed in Table 3. Using this model, the slope for diagnosis was significantly different from zero for IL-6, IL-8, and TNF-α. The R^2 for these models were IL-6: 0.18, IL-8: 0.29; and TNF-α: 0.18.

Preoperative median cytokine levels by comparing groups of NEC (not totalis) with NEC-totalis are listed in Table 4. Median levels of IL-8 were significantly higher in the group with NEC-totalis compared to NEC (not totalis) (28141 pg/ml versus 11429 pg/ml; $p=0.03$).

III. Outcomes and cytokine levels

Characteristics of infants who required reoperation with salvage laparotomy after initial primary peritoneal drainage are shown in Table 5. Although no significant differences in subject characteristics were found, 2/3 (67%) of subjects who required salvage laparotomy had received postnatal steroids preoperatively, while only 1/12 (8%) who did not require salvage laparotomy had received postnatal steroids. Perioperative cytokine levels for infants who required a salvage laparotomy after primary peritoneal drainage as compared to those who were treated with a drain alone were log transformed and analyzed. Mean values for log-transformed cytokines IL-6, IL-1β, and TNF-α were lower in subjects who required salvage laparotomy after drain placement on early postoperative days: IL-6 (postoperative days 1: 5.04 vs. 4.06, p=0.02; and postoperative day 2: 3.61 vs. 3.01, p= 0.016), IL-1β (postoperative days 1: 3.64 vs. 3.16, p = 0.03; and postoperative day 2: 3.45 vs. 2.74, p= 0.016), and TNF-α (postoperative day 2: 4.24 vs. 3.85, p = 0.01) (Figure 3). In addition, mean values for log-transformed cytokines TNF-α were lower in subjects who required salvage laparotomy after drain placement on the later postoperative day 5 (4.31 vs. 3.99, p=0.04).

Characteristics of subjects with NEC or SIP who died within 30 days of presentation are listed in Table 6. Five of 7 subjects had care withdrawn immediately postoperatively due to NEC-totalis, which is not survivable. Preoperative median cytokine levels of those who survived and died within 30 days are compared in Table 7. Survivors of NEC and SIP had significantly lower preoperative levels of IL-6 (37 pg/ml versus 15327 pg/ml, $p<0.01$), IL-8 (2752 pg/ml versus 28141 pg/ml, $p<0.005$) and TNF-α (83 versus 301 pg/ml, $p<0.05$).

DISCUSSION

This study is the first to demonstrate different cytokine profiles for subjects with surgical NEC as compared to SIP. It was found in this study that surgical NEC had significantly higher preoperative levels of IL-6, IL-8, and TNF-α, and IL-1β than either SIP or PDA. NEC-totalis had the highest levels of IL-6, IL-8, and IL-1β, but only IL-8 was significantly different from NEC (not totalis) at $\alpha = 0.05$. These data suggest that proinflammatory signaling is a prominent feature of surgical NEC but not of SIP. These results have potential application to the classification, pathophysiology, treatment strategies, and outcome prediction for preterm infants with neonatal intestinal perforation and inflammation.

The significant proinflammatory cytokine levels seen in NEC and NEC-totalis as opposed to SIP have implications for the pathophysiology of these diseases. The pathophysiology of NEC and the role of the inflammatory cascade has been the subject of decades of investigation. The initiation of toll-like receptors and ultimate release of proinflammatory cytokines is thought to be the final common pathway towards neonatal NEC (36-38). High levels of IL-6, IL-8, TNF-α, and IL-1β have identified in surgical NEC compared to non-surgical NEC (39). However, although NEC and SIP are felt to have a different pathophysiology, no prior studies have been published on the cytokine profile of SIP. This study suggests that proinflammatory signaling, even in the very ill newborn requiring significant hemodynamic and respiratory support, is a lesser feature of SIP.

Elevated proinflammatory cytokine levels were associated with surgical NEC and NEC-totalis, and therefore, may be a novel predictor variable of disease classification. A high degree of agreement between preoperative clinical diagnosis and intraoperative diagnosis (95%) has been previously reported (10). However, in those newborns with intestinal perforation who only receive a peritoneal drain without subsequent laparotomy, the true classification cannot be confirmed. In addition, very short segment NEC and SIP can look similar at operation, and ultimate postoperative classification may be biased by preoperative information. In this study,

preoperative IL-6, IL-8, TNF-α, and IL-1β were highly positively correlated with disease-type (NEC versus SIP; Table 2). The outlier for the SIP group was one subject with very high preoperative cytokine levels who received only a drain and who therefore did not have the diagnosis confirmed. Although no pneumatosis was seen on X-ray, the subject had been receiving trophic feeds, so potentially may have had NEC rather than SIP. For the NEC group, there were three outliers with lower cytokine levels: 1 who had < 5cm short segment NEC, which some define as SIP; one with true gangrenous long-segment NEC; and one with NEC but no resection required. The overlap in the clinical presentation and even operative findings of these diseases make misclassification errors possible (9). A larger, multicenter study would allow for the sensitivity and specificity of preoperative IL-6, IL-8, TNF-α, and IL-1β as a predictor of surgical NEC, NEC-totalis, and SIP to be better defined.

This study is also the first to attempt to correlate the perioperative proinflammatory response to surgical outcomes. Reoperation by salvage laparotomy after primary peritoneal drainage was associated with lower early postoperative IL-6 (postoperative days 1 and 2), IL-1β (postoperative days 1 and 2), and TNF-α (postoperative days 2 and 5) levels. However, only 3 subjects (20%) required salvage laparotomy, and the drainage subjects consisted of both NEC (2) and SIP subjects (13), so random error is a consideration in interpreting these results. Biological explanations for these attenuated post-operative levels include depressed wound healing due to postnatal administration of steroids or abnormal inflammatory levels resulting in poor wound healing and subsequent need for reoperation. A strong proinflammatory response was associated with early postoperative death, but this was likely due to the association of cytokine response and the diagnosis of NEC-totalis.

Treatment of intestinal perforation in very low birth weight babies includes both laparotomy with intestinal resection and intraperitoneal drain placement. The optimal treatment is controversial and the subject of an ongoing nationwide randomized clinical trial. Since the indications for a salvage laparotomy for newborns that were treated initially with drainage are not

standardized, biological markers of intestinal injury and healing could assist in surgical choices, including early reoperation with salvage laparotomy in order to prevent secondary brain injury. Given the association between inflammation and neurodevelopment, identifying treatment strategies that minimize the level of systemic inflammatory response may have implications for improving long-term outcomes. In this study, the large effect size of NEC on the preoperative proinflammatory cytokines IL-6, IL-8, TNF-α, and IL-1β allowed for conclusions about disease class and cytokine profile to be made despite moderate sample sizes. However, for outcome differences, sample sizes were not adequate to make reliable conclusions, although some differences were noted in the early postoperative days.

The strengths of this study included 1) the novelty of examining cytokines in SIP; 2) the ability to measure multiple cytokines using minimal blood sampling, and 3) access to detailed clinical parameters and outcome assessments. Proinflammatory cytokines have been implicated in adversely affecting the long-term neurodevelopmental outcomes in preterm infants (18, 19, 40-42) This study is the first of its kind to try to link disease-type (NEC vs. SIP), extent of disease, and treatment outcomes with cytokine profiles. The small quantities of blood required for these and future analyses were minimal risk to the newborns, and led to high enrollment among families approached for study inclusion.

Weaknesses of this study included 1) confounding by neonatal risk factors for disease, 2) skewness of the data with outliers; 3) small sample size; 4) potential for misclassification; and 5) missing data. In this study, gestational age was chosen as the only confounder to control by using the linear regression modeling with a categorical age cut point. However, many potential confounders including postnatal steroids, age at diagnosis, perinatal hypoxia-ischemia, and antibiotic usage exist. Other, potential unrecognized confounders may exist in this complex pathway from disease to outcome (Figure 1a). If cytokine levels could be collected in subjects randomized to laparotomy or drainage, confounding may be eliminated, provided random error did not lead to group imbalances. Because of outliers, the data tended to be skewed (to the right

with SIP and PDA, to the left with NEC). Although log transformation helped somewhat, the data remained skewed, so that nonparametric examination was required.

Another inherent issue in the study of inflammation and disease lies in the interpretation of the results. A large variety of cytokines have been associated with NEC (36-39). However, levels of the counter inflammatory cytokines are also elevated in NEC (15). Some cytokines, such as IL-6, have both pro- and anti-inflammatory properties. The balance between the inflammatory and anti-inflammatory signaling may be more important than single cytokine levels. Some studies have attempted to look at ratios of proinflammatory: anti-inflammatory responses (15). More information about the relationship of these pro- and anti-inflammatory cytokines in preterm infants and the interaction between them is needed to help understand the newborn response to intestinal inflammatory disease.

In conclusion, surgical NEC is a profoundly more proinflammatory disease than SIP, which is closer to a non-septic neonate. A strong proinflammatory response is associated with early postoperative death, likely due to the association with NEC-totalis. Lower postoperative inflammatory response is associated with drain failure, as defined by need for reoperation with salvage laparotomy, but numbers were small and postnatal steroid administration was imbalanced between the groups. Further studies, possibly with increased single-institution sample sizes or as part of the multi-institutional study may help further define the association of the neonatal inflammatory response to NEC and SIP with neonatal outcomes.

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TABLES & FIGURES

Figure 1a. Causal diagram illustrating the possible complex relationships between diagnosis, treatment, cytokines, and outcomes

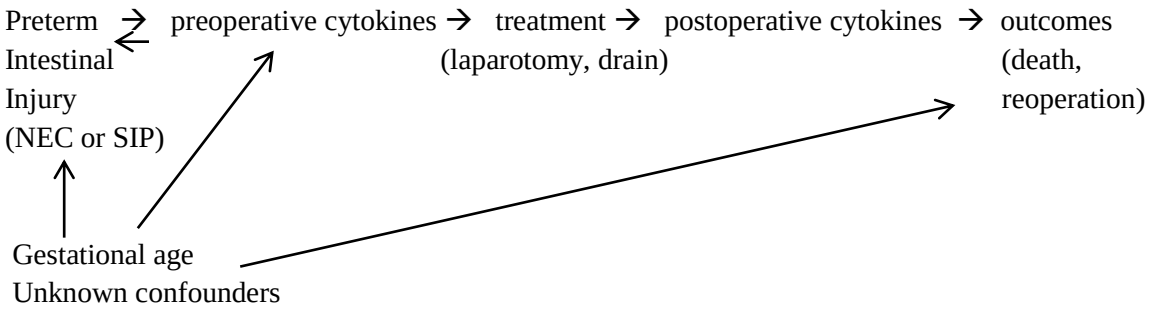


Figure 1b. Causal diagram illustrating the portion of the causal pathway addressed in aim 1, which examines the association between diagnosis and preoperative cytokine levels

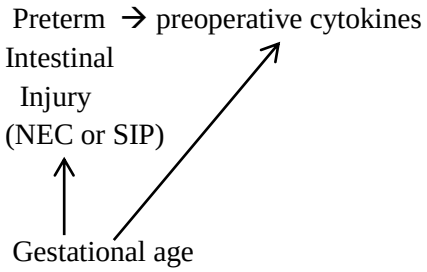
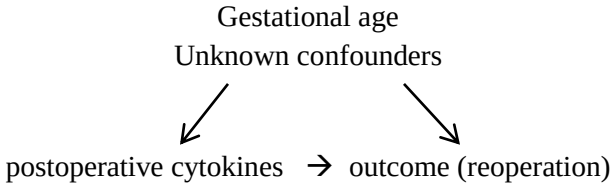
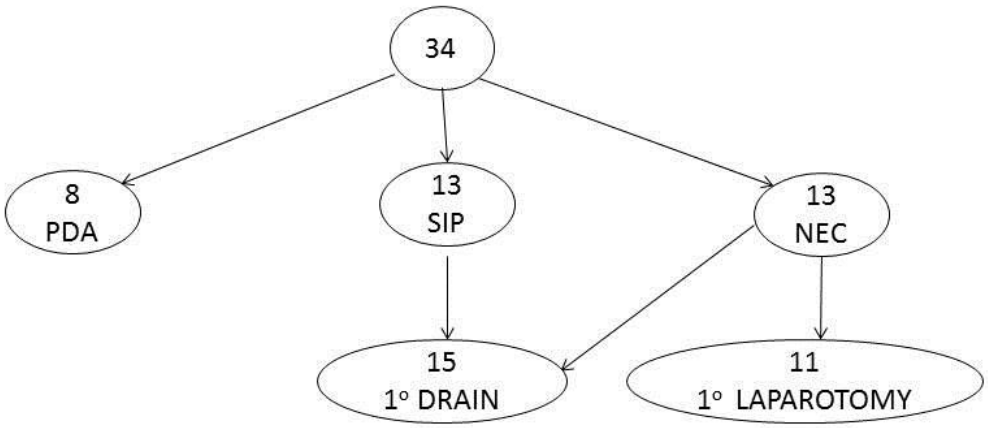


Figure 1c. Causal diagram illustrating the portion of the causal pathway addressed in aim 2, which examines the association between postoperative cytokine levels and outcome (reoperation)



PDA: PDA ligation; SIP: Spontaneous intestinal perforation; NEC: Necrotizing enterocolitis

Figure 2. Diagnoses, treatments and outcomes of 34 newborns included in the study



13 SIP 1° DRAIN	2 NEC 1° DRAIN	11 NEC 1° LAPAROTOMY
10 survived no reoperation	1 died with reoperation	6 survived no reoperation
1 died	1 survived	5 died of NEC-totalis
2 survived with reoperation		

PDA: PDA ligation; SIP: Spontaneous intestinal perforation; NEC: Necrotizing enterocolitis

Table 1. Maternal and infant characteristics of subjects by diagnosis

	NEC (n=12)	SIP (n=13)	PDA (n=8)	p-value
Maternal characteristics				
Mean age in years (SD)	29.8(6.2)	28.5(6.1)	23.4(4.5)	0.06
Multiple birth	2 (15%)	7 (54%)	3 (38%)	0.12
Hypertension	1 (8%)	4 (31%)	1 (13%)	0.39
Preterm Rupture of Membranes	0 (0%)	2 (15%)	1 (13%)	0.44
Antenatal steroids	7 (54%)	9 (69%)	5 (63%)	0.9
Cesarean section	10 (77%)	10 (77%)	3 (38%)	0.16
Infant characteristics				
Mean gestational age (wks, SD)	27.5 (2.6)	25.5 (1.0)	24.9 (2.1)	0.011*
Mean birth weight (grams, SD)	1022 (419)	816 (162)	857 (438)	0.31
Males	2 (15%)	9 (69%)	5 (63%)	0.016**
Apgar at 1 minute (SD) Apgar at 5 minutes (SD)	5.1 (2.2) 7.3 (1.3)	3.2 (2.2) 5.5 (2.7)	4.4 (2.4) 7.4 (0.9)	0.12 0.05
Indomethacin	7 (54%)	8 (61%)	8 (100%)	0.11
Postnatal steroids	0	3 (23%)	0	0.09
Preoperative IVH	4 (31%)	7 (54%)	4 (50%)	0.56
Grade 3 or 4 IVH	0	5 (38%)	3 (38%)	0.03
Admission WBC (SD)	7163 (3457)	18937 (14513)	12073(7169)	0.017*
Admission pressors	6 (46%)	6 (46%)	2 (25%)	0.62
Preoperative HFV	1 (8%)	2 (15%)	1 (13%)	1
Admission PDA	5 (38%)	6 (44%)	8 (100%)	------
Initial Laparotomy	11 (85%)	0	-------	-------

PDA: PDA ligation; SIP: Spontaneous intestinal perforation; NEC: Necrotizing enterocolitis SD: standard deviation; wks: weeks; IVH: intraventricular hemorrhage; WBC: white blood cell count; HFV: high frequency ventilation; *ANOVA with $\alpha = 0.017$ (Bonferroni correction): Admission WBC: NEC vs. SIP p=0.014; gestational age: NEC vs. SIP p=0.015; **Fishers exact test: Males: SIP vs. NEC p=0.015

Table 2. Preoperative cytokine levels (median pg/ml with IQR) by diagnoses of PDA ligation (n=8), Spontaneous intestinal perforation (SIP, n=13), and Necrotizing enterocolitis (NEC, n=12). Results are further stratified by age categories (≤ 26.5 weeks and >26.5 weeks)

		PDA** (n=8)	SIP (n=13)	NEC (n=12)	p-value
IL-6	≤ 26.5 wks	27(13-36)	41(24-356)	7940 (206-14113)	
	>26.5 wks	10	23 (23-37)	8821 (119-27456)	
	Total	25 (12-35)	36 (23-46)	8381 (163-18330)	<0.001‡
IL-8					
	≤ 26.5 wks	1433 (599-1982)	2613 (1435-5419)	18415 (7627-30000)	
	>26.5 wks	254	1338 (705-4721)	18461 (1941-26283)	
	Total	1110 (471-1805)	2473 (1059-4721)	18438 (5610-28141)	0.001‡
IL-1β					
	≤ 26.5 wks	25(21-28)	44 (25-58)	161(90-181)	
	>26.5 wks	18	31 (15-31)	75 (42-92)	
	Total	24 (20-27)	31 (23-46)	85 (43-171)	<0.001‡
TNF-α					
	≤ 26.5 wks	72(64-94)	80 (73-110)	169 (153-388)	
	>26.5 wks	62	66 (54-79)	132(86-290)	
	Total	71(63-86)	77 (66-83)	161(124-302)	0.001‡
IFN-Ɣ					
	≤ 26.5 wks	88 (79-123)	93 (69-99)	141 (103-202)	
	>26.5 wks	87	79 (69-80)	130 (71-163)	
	Total	88(83-108)	92(79-102)	135 (84-170)	0.24
IL-2					
	≤ 26.5 wks	21 (18-24)	28 (26-28)	34 (29-37)	
	>26.5 wks	23	23 (21-25)	21 (21-26)	
	Total	22(19-24)	26(23-29)	26(21-32)	0.14

PDA: PDA ligation; SIP: Spontaneous intestinal perforation; NEC: Necrotizing enterocolitis

IL-6: interleukin-6; IL-8 : interleukin-8; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor alpha; IFN-Ɣ: interferon gamma; IL-2: interlekin-2

*α = 0.017 (Bonferroni correction) ‡Kruskal-Wallis test

** Only one subject was in the >26.5 weeks age category for PDA, so no range is reported

IL-6: PDA vs. NEC p=0.0008; IP vs. NEC p = 0.002; IL-8: IL-8: PDA vs. NEC p=0.002; IP vs. NEC p = 0.02

TNF-α: PDA vs. NEC p=0.001; IP vs. NEC p < 0.005; IL-1β: PDA vs. NEC p<0.005; IP vs. NEC p = 0.016

Table 3. Expected mean cytokine values for SIP (intercept) and NEC (intercept + slope (pg/ml)) using linear regression model

Cytokine	Expected value for SIP (pg/ml) (95% CI)	Expected value for NEC (pg/ml) (95% CI)	p-value for slope estimate*
IL-6	2384 (0-7380)	10826 (5625-16027)	<0.05*
IL-8	5059 (45-10072)	16852 (11634-22070)	<0.01*
IL-1β	84 (0-168)	129 (41-217)	0.49
TNF-α	104 (32-177)	229 (154-305)	<0.05*

IL-6: interleukin-6; IL-8 : interleukin-8; IL-1β: interleukin-1β;

TNF-α: tumor necrosis factor alpha

Model: preoperative cytokine level (pg/ml) = intercept(pg/ml) + slope(pg/ml) *diagnosis where diagnosis SIP = 0 and diagnosis NEC = 1.

Intercept represents expected value for preoperative cytokine (pg/ml) for diagnosis of spontaneous intestinal perforation (SIP). Slope represents the expected difference in preoperative cytokine (pg/ml) for diagnosis of necrotizing enterocolitis (NEC).

CI: confidence interval

*$\alpha = 0.05$; p-value for test of slope = 0.

Table 4. Preoperative cytokine levels (median pg/ml with interquartile ranges) for infants with NEC (excluding totalis) and NEC-totalis

Cytokine	NEC (not totalis) n=8	NEC-totalis (n=4)	p-value**
IL-6	4073 (98-11467)	21999 (10510-28728)	0.07
IL-8	11429 (2768-18438)	28141 (23045-30000)	0.03**
IL-1β	82 (38-192)	86 (61-136)	0.93
TNF-α	143 (103-201)	301 (208-350)	0.20
IFN-Ɣ	135 (79-173)	143 (97-170)	0.93
IL-2	27 (22-36)	23 (21-28)	0.35

NEC: Necrotizing enterocolitis

NEC-totalis: Necrotizing enterocolitis-totalis

IL-6: interleukin-6; IL-8 : interleukin-8; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor alpha; IFN-Ɣ: interferon gamma; IL-2: interlekin-2

**Mann-Whitney test; $\alpha = 0.05$

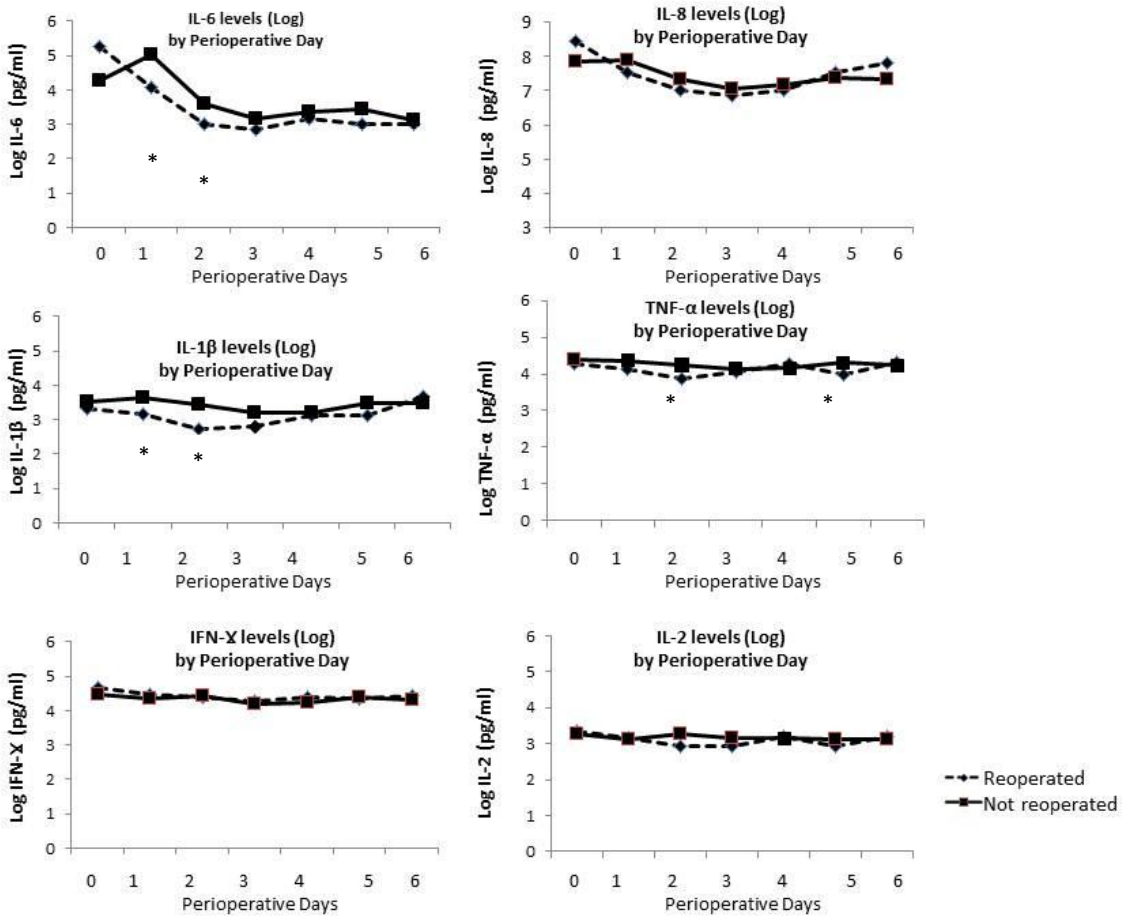
Table 5. Characteristics of subjects who required reoperation (salvage laparotomy) after initial drain placement for treatment of SIP or NEC as compared to those who were treated with a drain alone

	Reoperation (n=3)	No Reoperation (n=12)	p-value
NEC	1 (33%)	1 (8%)	0.37
SIP	2 (67%)	11 (92%)	0.37
Mean gestational age (wks, SD)	25. 7 (0.6)	25.3 (1.1)	0.62
Mean birth weight (grams, SD)	874 (47)	804 (168)	0.50
Mean initial WBC (SD)	15986 (16503)	18037 (13967)	0.83
Preoperative HFV	1 (33%)	2 (17%)	0.52
Preoperative pressors	2 (67%)	5 (42%)	0.57
Postnatal steroids	2 (67%)	1 (8%)	0.15
Postnatal indocin	1 (33%)	9 (75%)	0.24

SIP: Spontaneous intestinal perforation; NEC: Necrotizing enterocolitis

Wks: weeks; SD: standard deviation; WBC: white blood cell count; HFV: high frequency ventilation

Figure 3. Log of mean daily cytokine levels (preoperative day 0 – postoperative day 6) for subjects who required a salvage laparotomy after initial drain placement for treatment of SIP or NEC as compared to those who were treated with a drain alone



Dashed lines with diamonds represent mean log cytokine levels (pg/ml) of those subjects who required reoperation (Reoperated) while the solid lines with squares represent mean log cytokine levels (pg/ml) of those subjects who did not require further surgical intervention (Not reoperated). Day 0 represents the preoperative level and Days 1-6 are the subsequent postoperative days.

IL-6: interleukin-6; IL-8 : interleukin-8; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor alpha; IFN-Ɣ: interferon gamma; IL-2: interlekin-2; *Mann-Whitney test, $\alpha = 0.05$

IL-6 : postoperative day 1, $p=0.02$; postoperative day 2, $p<0.05$; IL-1β: postoperative day 1, $p<0.05$, postoperative day 2, $p<0.05$; TNF-α: postoperative day 2, $p=0.01$; postoperative day 5, $p<0.05$

Table 6. Characteristics of individual subjects with NEC or SIP who died

Diagnosis	Gestational Age	Initial treatment	Reoperation	Cause of death
NEC-totalis	27	Laparotomy	No	NEC-totalis
NEC-totalis	29	Laparotomy	Yes	NEC-totalis
NEC-totalis	28	Laparotomy	No	NEC-totalis
NEC-totalis	26	Laparotomy	No	NEC-totalis
NEC-totalis	29	Laparotomy	No	NEC-totalis
SIP	25	Drain	No	Intracerebral bleed
NEC	26	Drain	Yes	Respiratory failure

Each row represents an individual subject.

NEC-totalis: Necrotizing enterocolitis totalis

NEC: Necrotizing enterocolitis

SIP: Spontaneous intestinal perforation

Table 7. Preoperative cytokine levels (median in pg/ml with interquartile ranges) for subjects with necrotizing enterocolitis or spontaneous intestinal perforation who died and survived

Cytokine	Died (n=6)	Survived (n=19)	p-value*
IL-6	15327(4480-27456)	37 (23-455)	<0.01*
IL-8	28141 (19808-30000)	2752 (1059-9134)	<0.005*
IL-1β	86 (42-161)	34 (22-75)	0.10
TNF-α	301 (127-387)	83 (71-134)	<0.05*
IFN-Ɣ	143 (102-178)	93 (69-130)	0.10
IL-2	24 (21-29)	26 (23-30)	0.70

IL-6: interleukin-6; IL-8 : interleukin-8; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor alpha; IFN-Ɣ: interferon gamma; IL-2: interlekin-2

*Mann-Whitney test, $\alpha = 0.05$