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Systemic Inflammation Associated with Severe Intestinal Injury in Extremely Low Gestational Age Newborns

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Abstract

To define the role of systemic inflammation in infants with intestinal perforation (IP) and necrotizing enterocolitis (NEC), we measured 25 blood protein concentrations on days 1, 7, and 14 in 939 infants born before 28 weeks' gestation. On days 7 and 14, infants with NEC had elevated levels of CRP, serum amyloid A (SAA), IL-6, and IL-8. Infants with IP had elevated levels of CRP and insulin growth factor binding protein-1 on day 7 and elevated CRP, SAA, TNF-receptor-2, and matrix metalloproteinase-9 levels on day 14. A better understanding of systemic inflammation might help prevent and treat these disorders.

Keywords

Infant, premature; necrotizing enterocolitis; intestinal perforation; cytokines

INTRODUCTION

Necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (IP) are the leading causes of severe intestinal injury in the extremely low gestational age newborn (ELGAN). The etiology of NEC remains unknown with no clinically reliable predictive factors except for receipt of enteral feedings prior to diagnosis and an inverse correlation with gestational age and birth weight.¹ A role for inflammation in the pathogenesis of NEC is suggested by histologic characteristics of resected bowel that include neutrophil and leukocyte infiltration.²

The etiology of IP is also unknown. Similar to NEC, the incidence of IP varies inversely with gestational age and birth weight; however, when compared to infants with NEC, infants with IP tend to be less mature and of a lower birth weight.³ In addition, receipt of postnatal steroids and indomethacin has frequently been identified as risk factors.⁴ In contrast to NEC, the bowel tends to have considerably less inflammation.^{3,5}

Although, NEC and IP appear to be distinct entities, these two disease processes might be along the same spectrum of disease.⁶ Among the inflammatory biomarkers whose concentrations have tended to be elevated in the blood of preterm infants with NEC are platelet activating factor (PAF), tumor necrosis factor- α (TNF- α), serum amyloid-A (SAA), C-reactive protein (CRP), and interleukins-6, 8 and 10.⁷⁻¹³ However, little is known about the role of the inflammatory response and systemic biomarkers in IP and how these proteins differ from those whose concentrations are elevated in NEC.

The objective of this study was to evaluate and compare blood concentrations of inflammation-related proteins in extremely low gestational age newborns with NEC and IP; and to determine to what extent these profiles differ from those of premature infants who did not develop NEC or IP.

METHODS

Sample

The sample for this analysis is a subset of 1506 infants enrolled in the ELGAN Study, a prospective cohort study designed to identify characteristics and exposures that increase the risk of structural and functional neurologic disorders in preterm infants.¹⁴ During the years 2002-2004, women delivering between 23 and 27 6/7 weeks of gestation at one of 14 participating institutions were asked to enroll in the study. Informed consent was obtained and each of the individual institutional review boards approved the enrollment and consent processes including the primary institution Children's Hospital, Boston.

Of the 1200 children who survived until age 2 years adjusted age, 1102 had a developmental assessment near that time, and of those 939 had blood spots available for protein measurements. A full description of the methods of the ELGAN Study is provided elsewhere.¹⁴

Data Collection

Infant characteristics—Gestational age estimates were based on a hierarchy of the quality of available information. Most desirable were estimates based on the dates of embryo retrieval or intrauterine insemination or fetal ultrasound before the 14th week of gestation (62%). When these were not available, reliance was placed sequentially on a fetal ultrasound at 14 or more weeks of gestation (29%), last menstrual period without fetal ultrasound (7%), and GA recorded in the log of the NICU (1%). The birth weight birth weight Z score for each infant was calculated using a standard data set.¹⁵

Placenta histology and bacteriology—Different parts of the placenta were sampled for histological examination, while a sterile biopsy of the chorionic plate was cultured. Details are presented elsewhere.¹⁶

Disease classification of intestinal injury—We apply the term NEC to stage IIIb of the Modified Bell's staging criteria, which required surgical intervention, an exploratory laparotomy or placement of a Penrose drain.¹⁷ Only 9 infants had medical NEC (Bell Stage IIa – IIIa), which required pneumatosis but not surgical intervention. In light of reports citing an increased risk of morbidity and mortality only in infants with surgical NEC (NEC), we included these 9 infants with medical NEC in the referent group.^{18,19}

A spontaneous intestinal perforation (IP) was defined as a gastrointestinal perforation documented on radiograph in the absence of other clinical features of NEC.

Blood collection and protein measurements—Blood was collected on filter paper (Schleicher & Schuell 903) during the first two postnatal weeks on protocol days 1 (range 1-3), 7 (range 5-8) and 14 (range 12-15). Dried blood spots were stored at –70°C in sealed bags with desiccant until processed.

Protein analyses were performed in the Laboratory of Genital Tract Biology, Brigham & Women's Hospital. Preparation and analyses of the dried blood spot samples for analyses has been previously described in detail.²⁰⁻²² Proteins were measured in duplicate using the Meso Scale Discovery multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD). This electrochemiluminescence system has been validated by comparisons with traditional ELISA²³ and produces measurements that have high content validity.^{21,22}

The multiplex assays measuring up to 10 proteins simultaneously were optimized to allow detection of each biomarker within the linearity range of the eluted samples. The total protein concentration in each eluted sample was determined by BCA assay (Thermo Scientific, Rockford, IL) using a multilabel Victor 2 counter (Perkin Elmer, Boston, MA), and the measurement of each analyte was normalized to milligrams of total protein.

We quantified the concentrations of the following 25 proteins: Interleukin (IL)-1 β , IL-6, IL-6R (R = receptor), tumor necrosis factor (TNF)- α , TNF-R1, TNF-R2, IL-8, MCP-1 (chemokine (C-C motif) ligand (CCL)2; monocyte chemotactic protein-1), MCP-4 (CCL13; monocyte chemoattractant protein-4), MIP-1 β (CCL4; macrophage inflammatory protein-1 β), RANTES (CCL5; regulated upon activation, normal T cell expressed, and secreted), I-TAC (chemokine (C-X-C motif) ligand (CXCL)11; interferon-inducible T cell alpha-chemoattractant), ICAM-1 (cluster of differentiation (CD)54; intercellular adhesion molecule-1), ICAM-3 (CD50), VCAM-1 (CD106; vascular cell adhesion molecule-1), E-selectin (CD62E; E-SEL), MMP-1 (matrix metalloproteinase-1), MMP-9 (matrix metalloproteinase-9), CRP (C-reactive protein), SAA (serum amyloid A), MPO (myeloperoxidase), vascular endothelial growth factor (VEGF), VEGF-R1, VEGF-R2, and IGFBP-1 (insulin growth factor binding protein-1).

Statistical Analyses—We evaluated the generalized null hypothesis that the risks of NEC and IP are not associated with blood protein concentrations in the highest quartile for gestational age interval (23-24, 25-26, 27 gestational weeks) on the day the blood was collected. Because NEC and IP are mutually exclusive and both are best compared to the same referent group of infants who have neither entity, we created multinomial logistic regression models. Each of these models was adjusted for gestational age. The risk of NEC and IP associated with a protein concentration in the highest quartile was estimated with an odds ratio (OR) and its 95% confidence interval (CI).

Because assisted ventilation is associated with systemic inflammation in animals²⁴, adult humans²⁵, late preterm and term infants²⁶, and preterm infants²⁷⁻³¹ and because infants in our sample who developed NEC or IP were more likely than others to be on a ventilator, we wanted to tease out the contribution of assisted ventilation to associations between systemic inflammation and both NEC and IP. Rather than control for ventilation, we chose instead to carry out separate multivariable analyses in a sample restricted to children (including referent/controls) who were ventilated on day 7.

RESULTS

35 of the 939 infants (4%) were diagnosed with NEC, and 3% (27/939) were diagnosed with IP (Table 1). While approximately half the cases of NEC were identified during the first two postnatal weeks, 70% of IP occurred in the first two postnatal weeks. Regardless of when they were identified, all infants with either of these two diagnoses are included in the analyses presented here and compared to the 877 other infants.

Infants with NEC or IP were more likely than infants with neither diagnosis to be male, of lower gestational age, lower birth weight, and severely growth restricted (Table 2). Babies who developed NEC or IP were more likely than others to be ventilated on day 7. While only about half the babies who had neither NEC nor IP were ventilated on day 14, three quarters of babies who developed NEC, and almost all babies who developed IP were on a ventilator then. The recovery of an organism from the placenta was more likely among infants who developed NEC than among all other infants.

The concentrations of the proteins measured on day 1 in blood from infants with either NEC or IP did not differ significantly from those of infants without either of these diagnoses (Table 3). A systemic inflammatory signal is clearly present on both days 7 and 14 among children who developed NEC. This is most evident for CRP, SAA, IL-6, and IL-8.

Although, a lower risk of NEC was observed among newborns who had elevated concentrations of CCL5 on day 7, this was not seen on day 14.

In contrast, among children who developed IP, blood concentrations of only CRP and IGFBP-1 were elevated on day 7, whereas on day 14 the concentrations of CRP, SAA, TNF-R2, and MMP-9 were prominently elevated. As with NEC, IL-6 and IL-8 hover close to statistical significance on day 14.

Infants with elevated concentrations of CRP and IL-8 for even one day were approximately three times more likely than others to have NEC (Table 4). Elevated concentrations of four

proteins on two days a week apart, CRP, SAA, IL-6, and IL-8, identified infants who had prominently increased odds of NEC. Increased levels of CCL5 did not remain significant when viewing the data in this manner.

An elevated concentration of a protein on one day only was not associated with an increased risk of IP. However, similar to NEC, increased levels of CRP, SAA, and IL-8 for two or more days a week apart were associated with increased odds of IP. The doubling of IP risk associated with elevated concentrations of IL-6, TNF-R2, and MMP-9 on two or more days did not achieve statistical significance.

Even among a subsample restricted to infants who were ventilated on postnatal day 7, those who developed NEC were considerably more likely than infants who did not develop NEC or IP to have elevated concentrations of inflammation-related proteins on both days 7 and 14 (Table 5). In contrast, the systemic inflammation among infants who developed IP was less pronounced, achieving statistical significance in this relatively small sample to CRP on days 7 and 14, and SAA on day 14 only.

DISCUSSION

Elevated blood levels of classic markers of systemic inflammation (CRP, SAA, IL-6, and IL-8) were consistently associated with an increased risk of *both* NEC and IP during the first two postnatal weeks. In addition, infants who developed IP tended to have elevated blood concentrations of IGFBP on day 7, and TNF-R2 and MMP-9 on day 14. Unique to NEC was the association of a reduced risk of disease with an elevated level of CCL5 on day 7.

Our finding that both NEC and IP have similar systemic signatures raises several issues. First, severe intestinal injury might lead to a systemic inflammatory response. This systemic response might explain the increased risk of other co-morbidities seen with severe intestinal injury during the NICU course³² and poor long term neurodevelopmental outcome.^{18,19} Second, these two disorders might represent diagnoses across the same disease spectrum⁶. If this is indeed the case, then the characteristics of this spectrum should be identified. Our findings raise the possibility that some components of bowel disease in the preterm newborn represent sets of responses to inflammatory stimuli. Endogenous characteristics that lead an infant to have one rather than the other disorder should be sought.

Third, some of the inflammation-related proteins associated with NEC and IP have also been found significantly elevated in other neonatal disease processes such as chronic lung disease and sepsis.^{20,33,34} This raises the possibility that inflammation contributes to the pathogenesis of other neonatal diseases that have occurred more frequently than expected in newborns who develop NEC.³² Better understanding of the mechanisms that mediate the hyper-inflammatory response of the premature infant might help reduce the occurrence and severity of these inflammation-attributable neonatal disorders.

It is possible that the similar systemic signatures in inflammatory proteins observed in NEC and IP might be due to shared exposures unrelated to intestinal pathology including mechanical ventilation and major surgery. In a subsample restricted to children mechanically ventilated on postnatal day 7, we found an increase in a greater number of

inflammatory proteins in NEC compared to IP. This finding is supported in the current literature regarding different pathogenic mechanisms in NEC versus IP. Because of differences in clinical and epidemiologic characteristics, NEC has been viewed as having a pathogenesis that differs from that of IP.^{35,36} Compared to NEC, IP tends to be identified clinically earlier after birth, in less mature infants, is less likely to be associated with enteral feeding, and instead is more likely to be associated with postnatal exposure to indomethacin and glucocorticoids.³⁷ In addition, IP is characterized by only minimal histologic pneumatosis and inflammation. Our finding of less systemic inflammation in newborns with IP than in newborns with NEC, especially evident in our restricted cohort of mechanically ventilated infants, is in keeping with the histology. This might reflect the association of NEC, but not IP, with intestinal organisms.^{38,39}

It is also possible that some/much of the inflammation we see in infants who developed NEC or IP is a consequence of the body's inflammatory response to "major" surgery⁴⁰. Because NEC and IP are so closely related to "major" surgery in our sample, we cannot separate the contributions of surgery from the contributions of the disorders that required surgery.⁴¹

We found that elevated blood concentrations of CCL5 (also known as RANTES) in the first postnatal week were associated with a *reduced* risk of NEC, but not of IP. CCL5 is a highly pleiotropic chemokine⁴², which, in addition to pro-inflammatory effects, also appears to reduce inflammation and reduce the risk of organ damage in animal models of inflammation-mediated diseases.^{43,44} In the ELGAN Study sample, low CCL5 concentrations in the blood were associated with increased risk of early pulmonary dysfunction⁴⁵ and bronchopulmonary dysplasia²⁰, as well as structural indicators of brain damage.⁴⁶ Others have found that low CCL5 concentrations in the blood are associated with increased risk of proliferative retinopathy of prematurity.⁴⁷ Thus, our finding that infants who developed NEC tended to have low CCL5 concentrations in the blood is in keeping with the view that high concentrations of CCL5 reduce the risk of inflammation-related disorders in the very preterm newborn.

Because intestinal tissue was not available we cannot comment on the importance of protein concentrations at the tissue level. Factors not found to be significantly elevated in blood can still be important mediators of intestinal inflammation at the local tissue level. For example, infants in our study who had IP tended to have non-significant elevated systemic levels of IGFBP, MMP-9 and TNF-R2, biomarkers shown to be important in animal models of NEC.⁴⁸⁻⁵⁰

A major strength of our study is the measurement of 25 inflammation-related proteins in blood collected on three separate occasions a week apart from each other. It is reasonable to view measurements of each protein on each of the three sampling days as snapshots of potentially short-lived, inflammatory processes. In adult humans and animals, interleukins have short half-lives.⁵¹ We do not know the duration in preterm newborns of the half-life of IL-6 and IL-8, the two interleukins whose elevated concentrations were associated with NEC. However, a group of our subjects show tendencies to have inflammatory processes that last at least a week.^{22,52-55} The half-life of IL-6 and of IL-8 might be prolonged in very

preterm newborns, or their concentrations in blood might be elevated for days if a positive reinforcement loop contributes to sustained inflammation.⁵³ Consequently, we cautiously offer the possibility that the three snapshots provide more information than just about the hours close to the time the blood was collected.

Our study also has other strengths, including a large number of infants, selection of infants on the basis of gestational age and not birth weight, prospectively collected data, and protein data of high quality, and high content validity.

This study also has several limitations. First, we relied on clinical judgment for the diagnosis of IP, perhaps resulting in some misclassification. Second, we were not able to identify the exact day of IP or NEC onset. As a result, we do not know if the protein elevations we identified preceded, accompanied, or followed the clinical onset of disease. The date of the diagnosis of NEC or IP would not have provided information about when the processes leading to intestine damage first began. Without that information we cannot offer with certainty any pronouncements about how much of what we found in the blood indicates antecedents and how much reflects consequences of bowel damage. Third, because all subjects in this study had to survive until age 2 years, we might have missed the most severe instances of systemic inflammation, including those that resulted in or accompanied processes leading to death. However, despite these limitations our systemic evaluation of biomarkers in the first two postnatal weeks provides an understanding of the systemic inflammatory state of the premature infant with definite bowel injury and the first to evaluate and compare systemic biomarker profiles in extremely low gestational age newborns with NEC and IP.

CONCLUSION

The patterns of elevated blood concentrations of inflammation-related proteins associated with NEC and IP are more similar than different. Because other neonatal disease processes such as chronic lung disease and sepsis share some of these protein elevation profiles, the similarities we see between NEC and IP might reflect common responses to tissue injury rather than common antecedents. Understanding the mechanisms that regulate the neonatal inflammatory response might have broad preventative and therapeutic potential in diseases related to prematurity.

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Table 1

Number of infants categorized by their bowel disease classification and by the postnatal week when the diagnosis was made.

Disease classification	Postnatal week when the diagnosis was made					Total
	1	2	3	4	> 4	
NEC (Stage IIIb)	7	11	6	2	9	38
Isolated perforation	9	10	1	0	7	27
Neither*						877
Total	16	21	7	2	16	939

* These children were discharged without ever receiving a diagnosis of NEC Stage IIIb or isolated perforation.

Table 2

Percent of children with diagnosis classifications who also had the characteristics listed on the left. Values are column percents.

		Diagnosis			
Characteristic		NEC* (N=35)	IP** (N=27)	Referent Infants N=877)	Row N
Infant					
Sex	Male	71	63	51	493
Gestational age (wks)	23-34	40	33	19	189
	25-26	51	59	46	435
Birth weight (g)	750	54	59	35	343
Birth weight Z-score	< -2	9	11	5	50
Ventilated on	Postnatal day 7	83	85	59	573
	Postnatal day 14	74	96	58	555
Placenta					
Bacteriology	Any organism	61	40	48	414
Histology	Any inflammation	33	44	38	343

* Bell stage IIIb

** Intestinal perforation

Table 3

Odds ratio (and 95% confidence interval) of the bowel disorder listed at the top of each column among children who had a concentration in the top quartile (for gestational age and day specimen was obtained) of the protein(s) listed on the left relative to that of children who did not have an elevated concentration of the same protein(s) collected at the specified postnatal ages and adjusted for gestational age (23-24, 25-26, 27 weeks). **Bold** items are statistically significantly greater than 1.0 and ***bold italic*** items are statistically significantly less than 1.0.

Protein	NEC*			Isolated Perforation		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Systemic						
CRP	1.0 (0.5, 2.4)	2.7 (1.3, 5.5)	5.0 (2.4, 11)	1.8 (0.8, 3.9)	2.7 (1.2, 6.1)	7.7 (3.1, 19)
SAA	0.8 (0.3, 1.9)	2.0 (0.99, 4.2)	4.1 (2.0, 8.6)	0.6 (0.2, 1.7)	2.0 (0.9, 4.4)	7.3 (2.9, 18)
MPO	1.3 (0.6, 2.7)	1.0 (0.4, 2.1)	0.8 (0.3, 1.7)	1.1 (0.5, 2.6)	1.1 (0.5, 2.6)	1.5 (0.6, 3.6)
Cytokines						
IL-1 β	0.7 (0.3, 1.6)	1.5 (0.7, 3.0)	1.3 (0.6, 2.7)	1.2 (0.5, 2.6)	1.1 (0.5, 2.4)	2.0 (0.9, 4.8)
IL-6	0.8 (0.4, 2.0)	2.5 (1.2, 5.1)	2.0 (0.98, 4.3)	1.4 (0.6, 3.3)	1.7 (0.7, 3.7)	2.3 (0.99, 5.6)
IL-6R	0.9 (0.4, 2.1)	0.9 (0.4, 2.0)	0.7 (0.3, 1.7)	1.1 (0.5, 2.6)	0.6 (0.2, 1.6)	0.7 (0.3, 2.0)
TNF- α	1.3 (0.6, 2.8)	1.7 (0.8, 3.5)	1.4 (0.6, 2.9)	0.4 (0.1, 1.2)	2.0 (0.9, 4.4)	2.1 (0.9, 4.9)
TNF-R1	1.0 (0.4, 2.2)	1.1 (0.5, 2.3)	1.8 (0.9, 3.9)	1.3 (0.5, 2.9)	1.1 (0.4, 2.7)	1.4 (0.5, 3.4)
TNF-R2	1.0 (0.4, 2.3)	1.2 (0.6, 2.5)	1.9 (0.9, 4.0)	1.2 (0.5, 2.8)	0.8 (0.3, 2.1)	3.6 (1.5, 8.6)
Chemokines						
IL-8	1.3 (0.6, 2.9)	2.9 (1.4, 5.8)	2.1 (1.01, 4.5)	0.8 (0.3, 2.0)	1.6 (0.7, 3.7)	2.4 (1.03, 5.8)
MCP-1	0.7 (0.3, 1.8)	1.8 (0.9, 3.8)	1.6 (0.7, 3.4)	2.2 (0.97, 4.8)	1.1 (0.4, 2.7)	1.1 (0.4, 2.8)
MCP-4	1.3 (0.6, 2.8)	1.1 (0.5, 2.3)	1.0 (0.4, 2.3)	1.4 (0.6, 3.1)	0.6 (0.2, 1.6)	2.4 (1.00, 5.6)
MIP-1B	0.9 (0.4, 2.1)	1.0 (0.5, 2.2)	1.3 (0.6, 2.8)	0.9 (0.4, 2.2)	0.8 (0.3, 2.0)	1.5 (0.6, 3.7)
RANTES	1.3 (0.6, 2.9)	0.3 (0.1, 0.8)	0.5 (0.2, 1.3)	0.8 (0.3, 2.0)	0.6 (0.2, 1.6)	0.6 (0.2, 1.8)
I-TAC	0.7 (0.3, 1.7)	0.9 (0.4, 2.0)	0.9 (0.4, 2.2)	0.3 (0, 1.1)	1.0 (0.4, 2.5)	1.3 (0.5, 3.2)
Adhesion						
ICAM-1	1.1 (0.5, 2.5)	1.0 (0.5, 2.2)	2.1 (1.01, 4.4)	0.8 (0.3, 2.0)	0.6 (0.2, 1.7)	1.4 (0.5, 3.4)
ICAM-3	0.8 (0.3, 1.9)	0.9 (0.4, 2.0)	0.6 (0.2, 1.5)	1.0 (0.4, 2.4)	0.9 (0.3, 2.2)	2.1 (0.9, 5.0)
VCAM-1	0.9 (0.4, 2.0)	0.5 (0.2, 1.3)	0.3 (0.1, 1.00)	0.9 (0.4, 2.3)	0.5 (0.2, 1.3)	0.7 (0.2, 1.9)
E-SEL	0.7 (0.3, 1.7)	1.3 (0.6, 2.7)	1.1 (0.5, 2.4)	0.6 (0.2, 1.6)	1.0 (0.4, 2.4)	1.0 (0.4, 2.5)
MMPs						
MMP-1	0.8 (0.3, 1.8)	0.7 (0.3, 1.7)	0.8 (0.3, 1.8)	0.8 (0.3, 2.0)	0.9 (0.3, 2.2)	0.8 (0.3, 2.2)
MMP-9	0.5 (0.2, 1.4)	0.7 (0.3, 1.7)	1.2 (0.5, 2.6)	1.0 (0.4, 2.4)	1.0 (0.4, 2.4)	2.9 (1.2, 6.9)
Growth factor						
VEGF	1.4 (0.6, 3.0)	0.7 (0.3, 1.7)	1.0 (0.4, 2.2)	1.1 (0.5, 2.5)	0.6 (0.2, 1.5)	1.1 (0.4, 2.8)
VEGF-R1	1.5 (0.7, 3.1)	1.2 (0.6, 2.5)	1.4 (0.7, 2.9)	0.5 (0.2, 1.4)	0.8 (0.3, 2.0)	1.1 (0.5, 2.7)
VEGF-R2	1.3 (0.6, 2.8)	1.1 (0.5, 2.4)	1.4 (0.6, 2.9)	1.0 (0.4, 2.4)	0.7 (0.3, 1.7)	2.1 (0.9, 4.9)
IGFBP-1	1.1 (0.5, 2.4)	1.7 (0.8, 3.6)	1.1 (0.5, 2.5)	0.5 (0.2, 1.4)	2.4 (1.05, 5.3)	1.0 (0.4, 2.7)

Protein	NEC*			Isolated Perforation		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
N	31	33	31	27	26	22
Total N	861	667	786	861	667	786

* Bell stage IIIb

Table 4

Odds ratio (and 95% confidence interval) of the bowel disorder listed at the top of each column among children who had a concentration in the top quartile (for gestational age and day specimen was obtained) of the protein(s) listed on the left on one day or two or more days relative to that of children who did not have an elevated concentration of the same protein(s) (n=881). The models adjust for gestational age (23-24, 25-26, 27 weeks). **Bold** items are statistically significantly greater than 1.0

Protein	NEC*		Isolated Perforation	
	1 day	2 days	1 day	2 days
Systemic				
CRP	3.1 (1.3, 7.2)	3.8 (1.5, 9.4)	1.6 (0.5, 4.8)	6.1 (2.4, 16)
SAA	1.7 (0.7, 3.9)	3.4 (1.5, 8.0)	1.7 (0.6, 4.4)	3.9 (1.5, 10)
MPO	0.8 (0.3, 1.7)	0.9 (0.4, 2.1)	1.2 (0.5, 3.0)	1.3 (0.5, 3.4)
Cytokines				
IL-1 β	0.7 (0.3, 1.7)	1.3 (0.6, 3.0)	1.4 (0.6, 3.6)	1.7 (0.6, 4.6)
IL-6	1.1 (0.5, 2.5)	2.5 (1.1, 5.6)	2.5 (0.98, 6.4)	2.7 (0.9, 7.8)
IL-6R	0.8 (0.4, 2.0)	0.9 (0.4, 2.1)	1.2 (0.5, 2.9)	0.6 (0.2, 1.9)
TNF- α	2.0 (0.9, 4.5)	2.2 (0.9, 5.3)	1.7 (0.7, 4.1)	1.6 (0.6, 4.3)
TNF-R1	1.0 (0.5, 2.3)	1.2 (0.5, 3.0)	1.7 (0.7, 4.2)	1.6 (0.6, 4.5)
TNF-R2	1.8 (0.8, 3.9)	1.3 (0.5, 3.4)	2.0 (0.8, 5.0)	2.4 (0.9, 6.2)
Chemokines				
IL-8	3.0 (1.3, 7.1)	3.8 (1.5, 9.9)	0.9 (0.3, 2.3)	2.5 (1.01, 6.2)
MCP-1	1.2 (0.5, 2.6)	1.7 (0.7, 3.9)	1.3 (0.5, 3.3)	1.5 (0.6, 4.2)
MCP-4	1.5 (0.7, 3.3)	1.0 (0.4, 2.6)	1.9 (0.8, 4.5)	1.3 (0.5, 3.6)
MIP-1B	1.4 (0.6, 2.9)	0.7 (0.3, 2.0)	1.3 (0.5, 3.0)	0.9 (0.3, 2.7)
RANTES	1.1 (0.5, 2.2)	0.4 (0.1, 1.3)	0.5 (0.2, 1.4)	0.5 (0.2, 1.6)
I-TAC	0.9 (0.4, 1.9)	0.7 (0.3, 1.9)	1.6 (0.7, 3.6)	0.6 (0.2, 2.1)
Adhesion				
ICAM-1	0.9 (0.4, 2.1)	1.4 (0.7, 3.2)	1.4 (0.6, 3.2)	0.8 (0.3, 2.5)
ICAM-3	0.4 (0.1, 1.04)	0.7 (0.3, 1.6)	1.4 (0.6, 3.5)	1.2 (0.4, 3.4)
VCAM-1	0.6 (0.2, 1.4)	0.5 (0.2, 1.2)	1.1 (0.5, 2.7)	0.6 (0.2, 1.9)
E-SEL	1.3 (0.6, 2.7)	0.8 (0.3, 2.1)	0.6 (0.2, 1.6)	0.9 (0.3, 2.4)
MMPs				
MMP-1	0.9 (0.4, 2.1)	0.7 (0.3, 1.7)	0.6 (0.2, 1.9)	0.7 (0.3, 2.0)
MMP-9	0.5 (0.2, 1.2)	0.6 (0.2, 1.7)	1.6 (0.6, 3.8)	2.1 (0.8, 5.7)
Growth factor				
VEGF	1.0 (0.4, 2.2)	0.9 (0.4, 2.1)	0.8 (0.3, 2.1)	0.8 (0.3, 2.2)
VEGF-R1	1.0 (0.4, 2.4)	1.6 (0.7, 3.7)	0.7 (0.3, 1.7)	0.6 (0.2, 1.8)
VEGF-R2	1.4 (0.7, 3.2)	1.1 (0.4, 2.6)	1.5 (0.6, 3.7)	1.3 (0.5, 3.4)
IGFBP-1	1.1 (0.5, 2.4)	1.7 (0.7, 4.1)	1.9 (0.8, 4.4)	1.0 (0.3, 3.7)

* Bell stage IIIb

Table 5

Odds ratio (and 95% confidence interval) of the bowel disorder listed at the top of each column among children who had a concentration in the top quartile (for gestational age and day specimen was obtained) of the protein listed on the left relative to that of children who did not have an elevated concentration of the same protein collected at the specified postnatal ages and adjusted for gestational age (23-24, 25-26, 27 weeks).

Bold items are statistically significantly greater than 1.0. The sample is restricted to infants who were ventilated on postnatal day 7.

Protein	NEC*		Isolated Perforation	
	Day 7	Day 14	Day 7	Day 14
CRP	3.5 (1.6, 7.7)	6.4 (2.7, 15)	3.8 (1.3, 7.3)	9.5 (3.3, 27)
SAA	2.3 (1.04, 5.0)	5.4 (2.3, 12)	2.1 (0.9, 5.0)	5.7 (2.1, 15)
IL-1beta	2.0 (0.9, 4.4)	2.2 (0.98, 5.1)	1.8 (0.7, 4.5)	1.3 (0.9, 5.9)
IL-6	3.7 (1.7, 8.2)	2.8 (1.2, 6.3)	1.6 (0.6, 3.8)	2.4 (0.9, 6.3)
IL-8	2.4 (1.1, 5.2)	2.4 (1.1, 5.4)	1.9 (0.8, 4.5)	2.1 (0.8, 5.4)
TNF-alpha	2.5 (1.1, 5.4)	1.3 (0.5, 3.0)	1.6 (0.7, 4.0)	2.2 (0.5, 1.7)
ICAM-1	1.2 (0.5, 2.7)	2.6 (1.1, 5.9)	0.5 (0.2, 1.7)	1.5 (0.6, 4.0)

* Bell stage IIIb