Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Dig Dis Sci. Author manuscript; available in PMC 2023 March 01.

Published in final edited form as:

Dig Dis Sci. 2022 March ; 67(3): 863–871. doi:10.1007/s10620-021-06929-z.

Galectin-4 as a novel biomarker of neonatal intestinal injury

Jennifer B. Fundora, MD,
Department of Pediatrics, Division of Neonatology, Johns Hopkins University School of Medicine, 1800 Orleans St, Suite 8534, Baltimore, MD, 21287,

Jie Zhu, BS,
Department of Pediatrics, Division of Pediatric Cardiology, Johns Hopkins University School of Medicine, 720 Rutland Ave. Ross Building 1129, Baltimore, MD, 21205,

Lisa R. Yanek, MPH,
Department of Medicine, Johns Hopkins University School of Medicine, 1830 E Monument St 1830 Building, 8024, Baltimore, MD, 21287,

Mitzi Go, MD,
Maternal, Fetal and Neonatal Institute, Division of Neonatology, Johns Hopkins All Children's Hospital, 501 6th Ave S, St. Petersburg, FL, 33701,

Fauzia Shakeel, MD,
Maternal, Fetal and Neonatal Institute, Division of Neonatology, Johns Hopkins All Children's Hospital, 501 6th Ave S, St. Petersburg, FL, 33701,

Sandra S. Brooks, MD,
Maternal, Fetal and Neonatal Institute, Division of Neonatology, Johns Hopkins All Children's Hospital, 501 6th Ave S, St. Petersburg, FL, 33701,

Jun Yang, PhD,
Department of Pediatrics, Division of Pediatric Cardiology, Johns Hopkins University School of Medicine, 720 Rutland Ave. Ross Building 1129, Baltimore, MD, 21205,

David J. Hackam, MD PhD,
Department of Surgery, Division of Pediatric Surgery, Johns Hopkins University School of Medicine, 1800 Orleans St, Suite 7310, Baltimore, MD, 21287,

Allen D. Everett, MD,
Department of Pediatrics, Division of Pediatric Cardiology, Johns Hopkins University School of Medicine, 720 Rutland Ave. Ross Building 1129, Baltimore, MD, 21205,

Corresponding author: Jennifer Fundora, Department of Pediatrics, Division of Neonatology, Johns Hopkins Hospital Bloomberg Children's Center, 1800 Orleans St, Suite 8534, Baltimore, MD, 21287. Fax: 410-955-0298. jfundor1@jhmi.edu.

Conflict of Interest: The authors declare they have no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Parental informed written consent was obtained for all infants enrolled in various prospective cohort studies included. A waiver of consent was granted for some infants who were enrolled at time of diagnosis of intestinal injury, as only remnant samples from routine clinical tests were used to determine Gal-4 concentration.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Darla R. Shores, MD PhD
Department of Pediatrics, Division of Pediatric Gastroenterology, Johns Hopkins University School of Medicine, 600 N Wolfe St, Baltimore, MD, 21287

Abstract

Background: Neonates are at risk of gastrointestinal emergencies including necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (SIP). Identifying biomarkers to aid in diagnosis is imperative. We hypothesized that circulating intestinal-specific protein concentrations would distinguish infants with intestinal injury from controls.

Aims: To identify serum concentrations of intestinal-specific protein(s) in infants with intestinal injury and controls.

Methods: We used an *in-silico* approach to identify intestinal-specific proteins. We collected serum from control infants and infants with NEC or SIP, and measured protein concentrations using ELISA. If baseline concentrations were near the detection limit in initial control assays, we proceeded to assess concentrations in a larger cohort of controls and infants with injury. Control infants were frequency matched to infants with injury and compared with non-parametric and mixed-effects models analysis.

Results: We evaluated four proteins with high intestinal expression: Galectin-4 (Gal-4), S100G, Trefoil Factor-3, and alanyl aminopeptidase. Only Gal-4 demonstrated consistent results near the lower limit of quantification in controls and was studied in the larger cohorts. Gal-4 concentration was low in 111 control infants (median 0.012 ng/ml). By contrast, Gal-4 was significantly increased at diagnosis in infants with surgical NEC and SIP (n=14, $p \leq 0.001$ and n=8, p=0.031) compared to matched controls, but not in infants with medical NEC (n=32, p=0.10).

Conclusions: Of the intestinal-specific proteins evaluated, circulating Gal-4 concentrations were at the assay detection limit in control infants. Gal-4 concentrations were significantly elevated in infants with surgical NEC or SIP, suggesting that Gal-4 may serve as a biomarker for neonatal intestinal injury.

Keywords

Galectin-4; Biomarker; Neonate; Necrotizing enterocolitis; Spontaneous Intestinal Perforation

Introduction

Necrotizing enterocolitis (NEC) is a life-threatening disease affecting premature infants with no reliable test to readily detect it early in its course. Symptoms at disease onset are often non-specific and may resemble other diseases of prematurity such as infection and dysmotility, highlighting the necessity to identify a reliable biomarker for both diagnosis and intestinal recovery. Biomarkers for diagnosis have been studied, but no unifying single or collection of biomarkers has been adopted universally in clinical practice. Serum and urine intestinal fatty acid binding protein (i-FABP) and fecal calprotectin are among two of the more studied markers[1,2]. Identifying reliable biomarkers is imperative to establish the diagnosis and understand the optimal time for feeding post-injury as these infants are at risk for further feeding intolerance and additional gastrointestinal complications.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

The etiology of NEC is complex and multi-factorial[3,4]. Abnormal intestinal barrier function and immune responses have been shown to be involved in the pathophysiology of NEC [3,5]. Spontaneous intestinal perforation (SIP) is another gastrointestinal emergency in preterm neonates that is a distinct entity from NEC[6], as it is a focal lesion without extensive necrosis [7,8].

We hypothesized that circulating intestinal-specific proteins could be a biomarker of intestinal injury in neonates. Our objectives were to 1) identify an optimal circulating protein from a panel of intestinal-specific proteins, 2) evaluate the natural history of this circulating intestinal-specific protein across gestational age and post-menstrual age in control infants without intestinal injury, and 3) to evaluate the same protein in infants with intestinal injury, NEC and SIP, at diagnosis and longitudinally following injury.

Methods

In-silico intestinal epithelial protein discovery and preliminary assays

To identify intestinal epithelial-specific proteins, we used an *in-silico* discovery approach using Human Protein Atlas (https://www.proteinatlas.org/)[9]. Filtering for intestinal epithelial specificity by mRNA expression and by immunochemistry identified Galectin-4 (Gal-4), S100G, Trefoil Factor-3 (TFF-3), and alanyl aminopeptidase (ANPEP). Commercial ELISA assays for Gal-4 (Novus Biologicals NBP2-60568), S100G (R&D Systems DCALD0), TFF-3 (R&D Systems DTFF30), and ANPEP (R&D Systems DY3815) were used to determine baseline concentrations. We tested each assay in cord blood of healthy term infants, who presumably had no intestinal inflammation at birth to determine baseline detection limits and used two different dilutions for each sample. From these four proteins, we then chose the optimal protein that was at or below the level of detection to evaluate further in a larger sample size of control infants and in infants with intestinal injury.

Infant Population

Infants were recruited from January 2015 to August 2019 from the neonatal intensive care units (NICU) of two tertiary children's hospitals within the same healthcare system. Data and samples were used from multiple existing cohorts enrolling infants across gestational ages. Three prospective neonatal cohorts were used that enrolled infants shortly after birth. Two studies enrolled preterm and term infants, and one study enrolled only preterm infants. Regular biospecimen collection began shortly after birth. All prospective cohort studies from time of birth collected samples within the first week of life, and two of the prospective cohort studies had longitudinal samples available, which included some infants with intestinal injury. We have previously published a study evaluating the natural history of two biomarkers using these cohorts[10]. A fourth prospective study enrolled infants with intestinal injury, and biospecimen collection began at time of diagnosis and continued for up to six weeks after diagnosis. All studies were approved by the Institutional Review Board at Johns Hopkins Hospital and/or Johns Hopkins All Children's Hospital. Parental informed written consent was obtained for all infants enrolled in the three prospective cohort studies that enrolled infants after birth. A waiver of consent was granted for infants only in the intestinal injury study, as only remnant samples from routine clinical tests were used.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Relevant clinical and demographic information was collected including gestational age, birth weight, sex, race, Apgar scores, maternal chorioamnionitis (diagnosed either clinically or via placental pathology), intestinal injury diagnosis (medical NEC, surgical NEC, SIP), diagnosis of recurrence of NEC (defined by clinical team), and feeding type at time of sample, if available (maternal or donor milk, formula, or combination).

Control Infants

For the control group, infants with gestational ages 24–41 weeks with at least one serum or plasma sample available within the first week of life were included. Excluded infants had a diagnosis of intestinal injury or culture positive early- or late-onset sepsis. Samples from the first week of life, predominantly days of life four to seven were used in the main control analysis. We also included a longitudinal control cohort with two preterm gestational age groups (24–26 weeks and 27–29 weeks), as they are at higher risk of developing intestinal injury than older infants. Infants had samples available at serial timepoints from the first week of life and had a minimum of three additional samples throughout hospital stay (post-menstrual age range 25.7–37.6 weeks). Serial samples were chosen between 29–34 weeks post-menstrual age, as this is a general range when preterm infants are most likely to develop NEC[11].

Infants with Intestinal Injury

Infants with intestinal injury included a diagnosis of NEC or SIP. NEC was defined as medical or surgical NEC (Bell Stage 2 or 3, respectively)[12,13]. Infants with Stage I NEC were not included. SIP was defined as an isolated intestinal perforation by the clinical team. Diagnosis samples were generally obtained from available remnant clinical specimens (serum and plasma) within 24–48 hours following diagnosis, and if necessary up to 72 hours after diagnosis pending sample availability. Following intestinal injury, longitudinal samples were evaluated at serial timepoints over 6 weeks of hospital stay and at time of NEC recurrence, if applicable.

Sample Processing

Blood samples were processed by the central lab into aliquot tubes, typically within 72 hours of sample collection, and the serum or plasma was frozen and stored at −80°C until assayed. All ELISA assays were performed at the Johns Hopkins Pediatric Proteome Center after determining optimal sample dilution. For samples with concentrations less than the lower limit of detection, the value was imputed as half of the lower limit of detection.

Statistical Analysis

Our primary outcome was the serum concentration of Gal-4 (ng/ml). Given the relatively small sample size and non-normal distribution, control data across gestational ages was analyzed with a Kruskal Wallis test and longitudinal samples were analyzed with a linear mixed effects model for repeated measures. For comparison of each intestinal injury cohort (medical NEC and surgical NEC), infants with injury were frequency matched to controls (1:2) by gestational age within two weeks and sex. As there were an insufficient number of young controls for the SIP group for a successful gestational age match, infants with

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

SIP were frequency matched (1:1) by gestational age within one week and sex. Groups were compared using Wilcoxon rank-sum tests for continuous traits and Fisher's exact tests for categorical variables. Serial samples were analyzed with a linear mixed effects model for repeated measures. Given the varying presentation and underlying mechanisms of NEC in certain populations, sensitivity analyses were performed with relevant infants excluded from the analysis (infants with gastroschisis, congenital heart disease, and term gestation). Significance was set at $p < 0.05$. SAS (Version 9.4, SAS Institute Inc., Cary, NC) and GraphPad Prism (Version 8.2.1, GraphPad Software, San Diego, CA) software were used for the statistical analysis and figures.

As this was an exploratory study, we estimated needing a minimum of ten subjects per gestational age group in the control analysis. Our intestinal injury cohort was limited to the number of infants enrolled. With our sample size of 111 control infants and 56 infants with intestinal injury, we had 81% power to detect a two point difference in Gal-4 concentrations between groups with the Wilcoxon rank-sum test.

Results

Identifying Gal-4 as Target Protein

Using intestinal-specific filtering of Human Protein Atlas, S100G, TFF-3, Gal-4, and ANPEP were all found to have intestinal epithelial specific expression by mRNA and immunohistochemistry (https://www.proteinatlas.org/)[9]. To determine serum concentrations of these proteins, we evaluated each protein in four cord blood samples. Of these, only S100G and Gal-4 showed detectable, but low baseline concentrations (Supplemental Figure 1) which would facilitate easier discrimination of healthy infants from those with intestinal injury. In a repeat assay of S100G and Gal-4 (data not shown), only Gal-4 demonstrated consistent results at, or below the lower limit of quantification in the controls. Therefore, Gal-4 was selected as our target protein for further study.

Infant Demographics

We identified 111 control infants with available samples within the first week of life. Nineteen of these infants were identified with longitudinal samples throughout their hospital stay (nine infants 24–26 weeks gestational age and ten infants 27–29 weeks gestational age). A total of 91 samples were evaluated at serial timepoints during their hospitalization, with infants contributing up to six samples over the course of 72 days.

There were 56 infants with intestinal injury, of whom 46 had NEC. Of the infants with NEC, 32 (70%) were diagnosed with medical NEC and 14 (30%) surgical NEC. There were ten infants with SIP. Of note, two infants had a diagnosis of SIP and later in their NICU course, a diagnosis of NEC. For these two infants, the samples from diagnosis of SIP were used in the main analysis. There were nine infants with surgical NEC and 31 infants with medical NEC that had at least one sample available after diagnosis.

The characteristics of both controls and infants with intestinal injury are listed in Table 1. The median gestational age and birth weight of the infants in the overall control cohort was higher compared to the intestinal injury cohorts. The SIP cohort had the smallest gestational

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

age and birth weight. Most of the infants with NEC were male (72%). The predominant race and ethnicity in both the control and NEC cohorts was white and non-Hispanic, respectively. Of the known enteral nutrition types at time of diagnosis of injury (n=39), 51% of infants were receiving exclusively human milk.

Gal-4 concentrations in Control Infants

The overall median Gal-4 concentration in the control cohort was 0.012 ng/ml (IQR 0.012–0.22). The Gal-4 concentrations in gestational age groups are shown in Figure 1. There was no difference in Gal-4 concentrations during the first week of life across gestational ages (p=0.33). Gal-4 concentrations did not differ based on sex, race, ethnicity, 5-minute Apgar score, or maternal chorioamnionitis. Limited data on feeding was available for control infants (n=22); no differences were seen between feeding types (p=0.50). In infants with longitudinal samples, the linear mixed effects model found no significant difference in Gal-4 over time by either gestational age (p=0.14) or post-menstrual age (p=0.46). Supplemental Figure 2 shows the Gal-4 concentrations of these infants across post-menstrual age.

Gal-4 concentrations in Infants with Intestinal Injury

Figure 2 shows Gal-4 concentrations by injury group compared to its matched control group (See Supplemental Table 1 for characteristics of matched cohorts). There was a significant difference in Gal-4 concentration between those with surgical NEC (median 2.54 ng/ml, IQR 0.2–5.15, p≤0.001) and SIP (median 0.91 ng/ml, IQR 0.31–2.69, p=0.031), and their respective matched control infants (median 0.012 ng/ml, IQR 0.012–0.21 and median 0.21 ng/ml, IQR 0.012–0.46, respectively). There was no statistically significant difference in Gal-4 concentration between those with medical NEC and matched controls (median 0.022 ng/ml and 0.012 ng/ml, respectively, p=0.10). Given the small sample sizes, we are not able to adjust for race, presence of chorioamnionitis, or Apgar score in evaluating Gal-4 concentrations amongst injury groups. The receiver operator curve for surgical NEC at diagnosis and selected cut point values is shown in Figure 3, with an area under the curve of 0.84. Similarly, the receiver operator curve for infants with SIP shows an area under the curve of 0.83 (not shown, p=0.03) and a cut point of 0.55 ng/ml was determined to have an 75% sensitivity and 88% specificity.

We additionally compared the values of Gal-4 concentration in infants with SIP and surgical NEC and found no significant differences (median 2.54 ng/ml vs 1.32 ng/ml, p=0.35).

Longitudinal Gal-4 Concentrations after NEC

As shown in Figure 4, changes in Gal-4 after diagnosis in infants with surgical NEC were variable. In a linear mixed effects model with repeated measures in these infants, there was a maximum of eight repeated weekly measures and there was no significant difference in Gal-4 over time by either gestational age (p=0.26) or post-menstrual age (p=0.36). However, four of the six infants with detectable concentrations at diagnosis had decreased Gal-4 concentrations within the first one to two weeks post-diagnosis. There was one infant with persistent elevation in Gal-4 after diagnosis who had no reported feeding intolerance or recurrent NEC. Another infant with persistently elevated Gal-4 concentrations was one of the five infants with a NEC recurrence.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

In 31 infants with medical NEC and at least one sample post-diagnosis, a linear mixed effects model was run to model repeated measures with a maximum of seven repeated measures and found no significant difference in Gal-4 concentrations over time by gestational age (p=0.15) or PMA (p=0.30).

Gal-4 concentrations with NEC recurrence

Five infants had NEC recurrence; three of these infants had an original diagnosis of medical NEC and two had an original diagnosis of surgical NEC. The median Gal-4 concentration of these infants at diagnosis was 3.12 ng/ml (Range 0.02–6.7 ng/ml) and at recurrence was 0.02 ng/ml (Range 0.006–5.86 ng/ml). When evaluating type of original NEC, at diagnosis the range of Gal-4 for infants with surgical NEC was 0.02–3.12 ng/ml and for infants with medical NEC was 1.64–6.7 ng/ml.

Sub-Analysis of Gal-4 concentrations in specific populations

There were two term infants with medical NEC. In a sensitivity analysis of only preterm infants with medical NEC, similar results were found with no significant difference in Gal-4 concentrations (n=30, p=0.20).

There were four preterm infants with gastroschisis (a mix of surgical and medical NEC diagnoses) and three infants with congenital heart disease who also had NEC (all medical NEC diagnoses). Sensitivity analyses with each group excluded showed similar results to the main analysis. Again, there were significantly higher Gal-4 concentrations in infants with surgical NEC compared to matched controls with infants with gastroschisis excluded (n=36, p=0.001). And, there was no significant difference in Gal-4 concentrations in infants with medical NEC compared to matched controls when those with gastroschisis or congenital heart disease were excluded (n=72, p=0.25 and n=69, p=0.12 respectively).

Discussion

We evaluated four candidate proteins (Gal-4, S100G, TFF-3, ANPEP) based on intestinal epithelial specific expression. We consistently identified low concentrations of circulating Gal-4 in neonates without intestinal injury, with minimal variability across gestational and post-menstrual ages. In those with perforating intestinal injury, there was a significant elevation of Gal-4. There was no significant difference in Gal-4 concentrations in those with medical NEC, though several outliers did have elevated Gal-4 concentrations.

Gal-4 belongs to the galectin protein family, which bind carbohydrates and are involved in a large range of biological functions including inflammation, immune responses, and cell signaling[14]. Gal-4 is expressed almost exclusively in the gastrointestinal tract[15] and is involved on the enterocyte cell surface at the brush border via cross-linking of glycoproteins and glycolipids, stabilizing lipid rafts[16,17], and cell trafficking[18]. Intracellular Gal-4 has also been shown *in vitro* to be involved in cell proliferation, apoptosis, and differentiation[19] and extracellular Gal-4 is also been shown to be involved in cell-cell adhesion[15,20]. It has been studied in several diseases including cancers, such as colorectal[21,22] and breast cancer[15,23], and inflammatory bowel disease[24,25]. To date, Gal-4 is unexplored in neonates.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Additionally, Gal-4 has been shown to have a role in intestinal inflammation[19,26], a defining feature of NEC. Our findings of elevated Gal-4 in infants with surgical NEC also support the role of Gal-4 in intestinal inflammation. It is noteworthy that in studying Gal-4 involvement in inflammatory bowel disease, both pro- (via IL-6 production) and anti-inflammatory mechanisms have been suggested [27,28] and the conclusive mechanism of Gal-4 has yet to be elucidated. Interestingly, an elevated Gal-4 was also observed in our study in infants with SIP despite distinct pathological differences between SIP and NEC[29] and a posited lesser role of inflammation in SIP compared to NEC. The elevated Gal-4 in patients with SIP could be due to unrecognized commonalities in the pathophysiology of SIP compared to NEC. Conversely, there is evidence that Gal-4 is involved in intestinal healing[30], and it is possible Gal-4 a role in intestinal recovery after perforation in both diseases.

In the control infants in our cohort, the serum concentrations of Gal-4 were all low in the first week of life, irrespective of gestational age. We continued to see low circulating concentrations of Gal-4 over time in our longitudinal samples. This is particularly important in a disease such as NEC that often occurs after the first week of life. Our results support the hypothesis that Gal-4 is not circulating at high concentrations in the absence of intestinal injury. Knowing the circulating concentration remains low with increasing PMA is of value in distinguishing intestinal injury from a developmental change in the maturing intestine of preterm infants. For example, in a study of i-FABP in control infants with longitudinal samples, we showed that i-FABP concentrations slowly increase with postnatal age[10], providing valuable insight into for choosing an appropriate cut off for diagnosis of NEC.

Many other biomarkers for diagnosis of NEC have been studied. In a review of serum biomarkers for the diagnosis of NEC by Terrin et al[31], it was stated that serum calprotectin (S100 A8/A9), Apolipoprotein CII, Interleukin-10, Interleukin-1ra, i-FABP and ischemia modified albumin may increase diagnostic accuracy but they concluded that studies were highly variable and more well-designed, larger cohort studies were needed. Specifically looking at studies of i-FABP, a meta-analysis of plasma i-FABP that included three studies evaluating i-FABP in diagnosing surgical NEC showed the pooled sensitivity and specificity was 71 and 76%, respectively[32].

In our study, we also found the Gal-4 concentrations were not significantly different in infants with medical NEC, though there were important outliers. The spectrum of medical NEC varies widely[7] and this may account for the range of circulating Gal-4 amongst infants with medical NEC (range 0.0055–35.9 ng/ml). These findings also support the concept that in general medical NEC reflects a lower degree range of intestinal injury (thus lower Gal-4 concentrations) as compared with surgical NEC, where greater degrees of intestinal injury (and thus higher Gal-4 concentrations) are expected. While Gal-4 concentrations may not aid in making the diagnosis of medical NEC, it is possible it plays a role in grading the severity or post-NEC complications. For example, infants diagnosed with medical NEC and a later recurrence tended to have a higher Gal-4 concentration at original diagnosis than those who did not go on to have a recurrence (median 4.34 ng/ml (IQR 1.64–6.7) and 0.02 ng/ml (IQR 0.014–0.56), respectively).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Few studies have evaluated biomarkers in intestinal recovery after NEC. Kuik et al.[33] showed that in a small cohort of patients with NEC (n=27), intestinal tissue oxygen saturation and urinary i-FABP measured after refeeding predicted intestinal recovery or post-NEC complications. In our study, while there was no significant change in concentrations of Gal-4 after diagnosis of surgical NEC, it is noteworthy that Gal-4 concentrations appear to decrease within the first 7–14 days following injury and then remain low afterward. Among the five infants who had recurrence of NEC, four had an elevated Gal-4 concentration at the time of initial diagnosis. Of these four, three had continued elevation of Gal-4 following injury, despite the concentration being overall lower at time of recurrence diagnosis (data not shown) compared to original diagnosis. Further studies with a larger cohort are needed to evaluate how Gal-4 changes in infants with complications after initial intestinal injury.

This study has demonstrated a unique method of identifying a potential novel biomarker for neonatal intestinal injury. Identifying that the intestinal-specific protein, Gal-4, is elevated at time of intestinal injury compared to control infants, where it remains low, is an important step in identifying a potential biomarker for injury. Given the timing of sample collection in this study, we cannot definitively determine whether Gal-4 plays a role in the mechanism of intestinal injury or recovery in infants with perforation. However, we have shown that in the absence of NEC or SIP, circulating Gal-4 is low, suggesting that elevated Gal-4 is associated with severe injury of the intestinal epithelial cells.

There are limitations in our study. Because of inherent difficult in obtaining blood from neonates and accruing sufficient sample sizes in a rare disease, we utilized several cohorts that collected blood samples from various times and clinical data collection was not uniform. Additionally, our sample size of infants with intestinal injury was relatively small, which we attempted to mitigate by including infants from multiple cohorts from two centers. Our sample size is comparable to that of other similar studies, but it is possible that a larger cohort could detect greater variability in both control infants and infants with injury, including medical NEC. We also recognize that in current clinical practice, the use imaging techniques (X-ray or ultrasound) can often be used to diagnose surgical abdominal emergencies, highlighting the importance of prospectively evaluating the trends in Gal-4 in future studies. We also excluded infants with culture positive sepsis in our control group, which will be important to ultimately evaluate if circulating Gal-4 is elevated in these infants.

In conclusion, Gal-4 has the potential to be used among a panel of biomarkers to diagnose intestinal injury in neonates. In our cohort, the circulating concentrations of Gal-4 are significantly elevated in infants with intestinal perforation, including both surgical NEC and SIP compared to control infants. Additional studies are needed to evaluate Gal-4 prospectively in infants that develop intestinal injury and to longitudinally evaluate concentrations through intestinal recovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Acknowledgements

We would like to acknowledge Dr. Rachel Troch and Dr. Brooke Krbec for assisting with data entry. We would also like to acknowledge the Biostatistics, Epidemiology, and Data Management (BEAD) Core for statistical support and the Johns Hopkins Medicine All Children's Hospital Biorepository and the Johns Hopkins Clinical Chemistry laboratory for sample processing support.

Statement of Financial Support:

JF is supported by the NIH T32 Institutional Training Grant for Pediatricians (T32HD044355); DS is supported by the All Children's Hospital Institutional Research Grant, Thomas Wilson Sanitarium award. AE, SB, JZ are supported by NIH NICHD R01HD086058. DJH is supported by RO1DK121824, RO1DK117186, and RO1GM078238.

References

1. Wang K, Tao G, Sun Z, Sylvester KG. Recent Potential Noninvasive Biomarkers in Necrotizing Enterocolitis Gastroenterology Research and Practice. 2019;2019:1–9.
2. Goldstein GP, Sylvester KG. Biomarker Discovery and Utility in Necrotizing Enterocolitis Clin Perinatol. 2019;46:1–17. [PubMed: 30771811]
3. Kasivajjula H, Maheshwari A. Pathophysiology and current management of necrotizing enterocolitis Indian Journal of Pediatrics. 2014;81:489–497. [PubMed: 24652270]
4. Rich BS, Dolgin SE. Necrotizing Enterocolitis Pediatrics in Review. 2017;38:552–559. [PubMed: 29196510]
5. Bazacliu C, Neu J. Pathophysiology of Necrotizing Enterocolitis: An Update Curr Pediatr Rev. 2019;15:68–87. [PubMed: 30387398]
6. Vongbhavit K, Underwood MA. Intestinal perforation in the premature infant J Neonatal Perinatal Med. 2017;10:281–289. [PubMed: 28854518]
7. Neu J Necrotizing Enterocolitis: The Future Neonatology. 2020:1–5.
8. Pumberger W, Mayr M, Kohlhauser C, Weninger M. Spontaneous localized intestinal perforation in very-low-birth-weight infants Journal of the American College of Surgeons. 2002;195:796–803. [PubMed: 12495312]
9. Pontén F, Jirström K, Uhlen M. The Human Protein Atlas—a tool for pathology The Journal of Pathology. 2008;216:387–393. [PubMed: 18853439]
10. Shores DR, Fundora J, Go M et al. Normative values for circulating intestinal fatty acid binding protein and calprotectin across gestational ages BMC Pediatrics. 2020;20.
11. Llanos AR, Moss ME, Pinzòn MC, Dye T, Sinkin RA, Kendig JW. Epidemiology of neonatal necrotising enterocolitis: a population-based study Paediatr Perinat Epidemiol. 2002;16:342–349. [PubMed: 12445151]
12. Bell MJ, Ternberg JL, Feigin RD et al. Neonatal necrotizing enterocolitis. Therapeutic decisions based upon clinical staging Annals of surgery. 1978;187:1–7. [PubMed: 413500]
13. Walsh M, Kliegman R. Necrotizing Enterocolitis: Treatment Based on Staging Criteria Pediatric Clinics of North America. 1986;33:179–201. [PubMed: 3081865]
14. Johannes L, Jacob R, Leffler H. Galectins at a glance J Cell Sci. 2018;131.
15. Huflejt ME, Leffler H. Galectin-4 in normal tissues and cancer Glycoconjugate Journal. 2003;20:247–255.
16. Brewer CF, Miceli MC, Baum LG. Clusters, bundles, arrays and lattices: novel mechanisms for lectin-saccharide-mediated cellular interactions Curr Opin Struct Biol. 2002;12:616–623. [PubMed: 12464313]
17. Danielsen EM, Hansen GH. Lipid raft organization and function in the small intestinal brush border Journal of Physiology and Biochemistry. 2008;64:377–382. [PubMed: 19391463]
18. Stechly L, Morelle W, Dessein A-F et al. Galectin-4-Regulated Delivery of Glycoproteins to the Brush Border Membrane of Enterocyte-Like Cells Traffic. 2009;10:438–450. [PubMed: 19192249]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

19. Cao Z-q Guo X-l. The role of galectin-4 in physiology and diseases Protein & Cell. 2016;7:314–324. [PubMed: 27017379]
20. Huflejt ME, Jordan ET, Gitt MA, Barondes SH, Leffler H. Strikingly Different Localization of Galectin-3 and Galectin-4 in Human Colon Adenocarcinoma T84 Cells Journal of Biological Chemistry. 1997;272:14294–14303.
21. Watanabe M, Takemasa I, Kaneko N et al. Clinical significance of circulating galectins as colorectal cancer markers Oncol Rep. 2011;25:1217–1226. [PubMed: 21369702]
22. Rechreche H, Mallo GV, Montalto G, Dagorn JC, Iovanna JL. Cloning and expression of the mRNA of human galectin-4, an S-type lectin down-regulated in colorectal cancer Eur J Biochem. 1997;248:225–230. [PubMed: 9310382]
23. Barrow H, Guo X, Wandall HH et al. Serum galectin-2, −4, and −8 are greatly increased in colon and breast cancer patients and promote cancer cell adhesion to blood vascular endothelium Clin Cancer Res. 2011;17:7035–7046. [PubMed: 21933892]
24. Papa Gobbi R, De Francesco N, Bondar C et al. A galectin-specific signature in the gut delineates Crohn's disease and ulcerative colitis from other human inflammatory intestinal disorders Biofactors. 2016;42:93–105. [PubMed: 26891020]
25. Yu TB, Dodd S, Yu LG, Subramanian S. Serum galectins as potential biomarkers of inflammatory bowel diseases PLoS One. 2020;15:e0227306. [PubMed: 31929564]
26. Hokama A, Mizoguchi E, Mizoguchi A. Roles of galectins in inflammatory bowel disease World J Gastroenterol. 2008;14:5133–5137. [PubMed: 18777589]
27. Hokama A, Mizoguchi E, Sugimoto K et al. Induced reactivity of intestinal CD4(+) T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation Immunity. 2004;20:681–693. [PubMed: 15189734]
28. Paclik D, Danese S, Berndt U, Wiedenmann B, Dignass A, Sturm A. Galectin-4 controls intestinal inflammation by selective regulation of peripheral and mucosal T cell apoptosis and cell cycle PLoS One. 2008;3:e2629. [PubMed: 18612433]
29. Kubota A, Yamanaka H, Okuyama H et al. Focal intestinal perforation in extremely-low-birth-weight neonates: etiological consideration from histological findings Pediatr Surg Int. 2007;23:997–1000. [PubMed: 17653555]
30. Paclik D, Lohse K, Wiedenmann B, Dignass AU, Sturm A. Galectin-2 and −4, but not galectin-1, promote intestinal epithelial wound healing in vitro through a TGF-beta-independent mechanism Inflamm Bowel Dis. 2008;14:1366–1372. [PubMed: 18484670]
31. Terrin G, Stronati L, Cucchiara S, De Curtis M. Serum Markers of Necrotizing Enterocolitis: A Systematic Review J Pediatr Gastroenterol Nutr. 2017;65:e120–e132. [PubMed: 28379923]
32. Yang G, Wang Y, Jiang X. Diagnostic Value of Intestinal Fatty-Acid-Binding Protein in Necrotizing Enterocolitis: A Systematic Review and Meta-Analysis Indian Journal of Pediatrics. 2016;83:1410–1419. [PubMed: 27272048]
33. Kuik SJ, Kalteren WS, Mebius MJ, Bos AF, Hulscher JBF, Kooi EMW. Predicting intestinal recovery after necrotizing enterocolitis in preterm infants Pediatr Res. 2020;87:903–909. [PubMed: 31649338]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

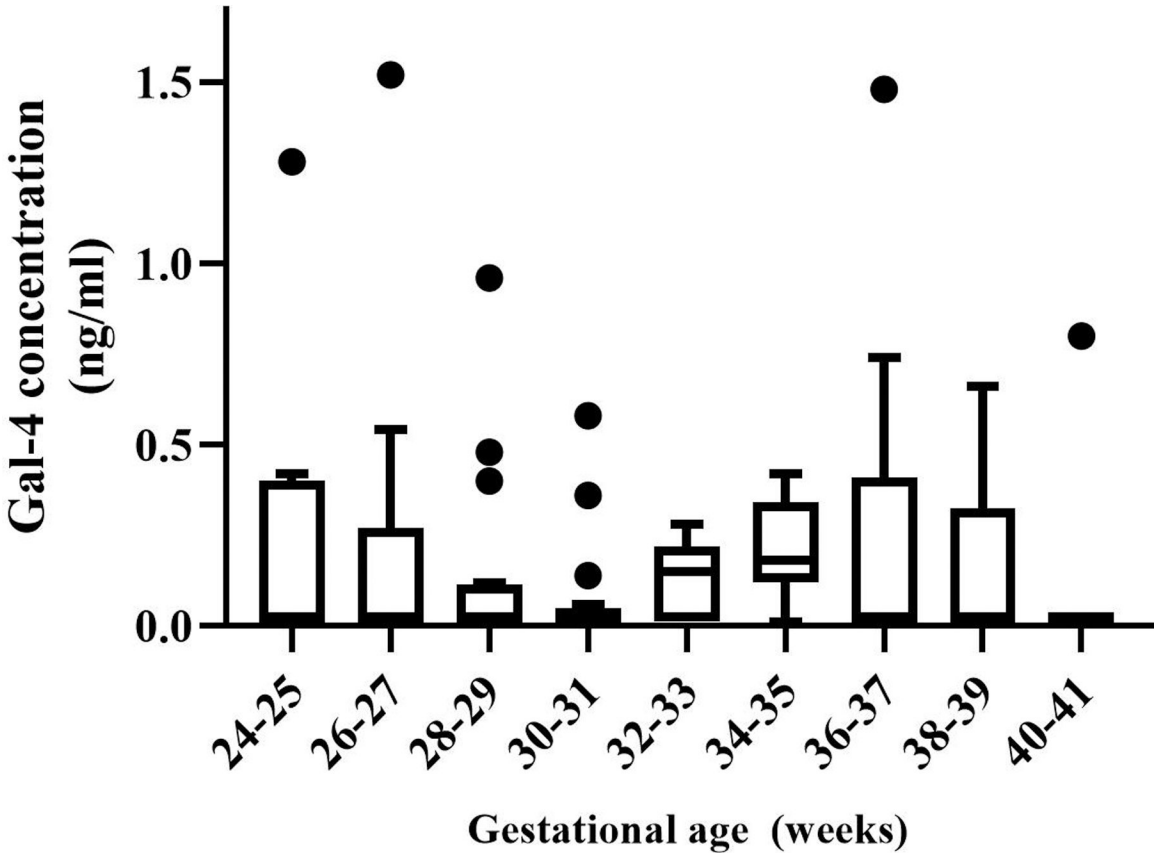


Figure 1:
Box plot of Gal-4 concentrations in the first week of life across gestational ages. n=111. p=0.33.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

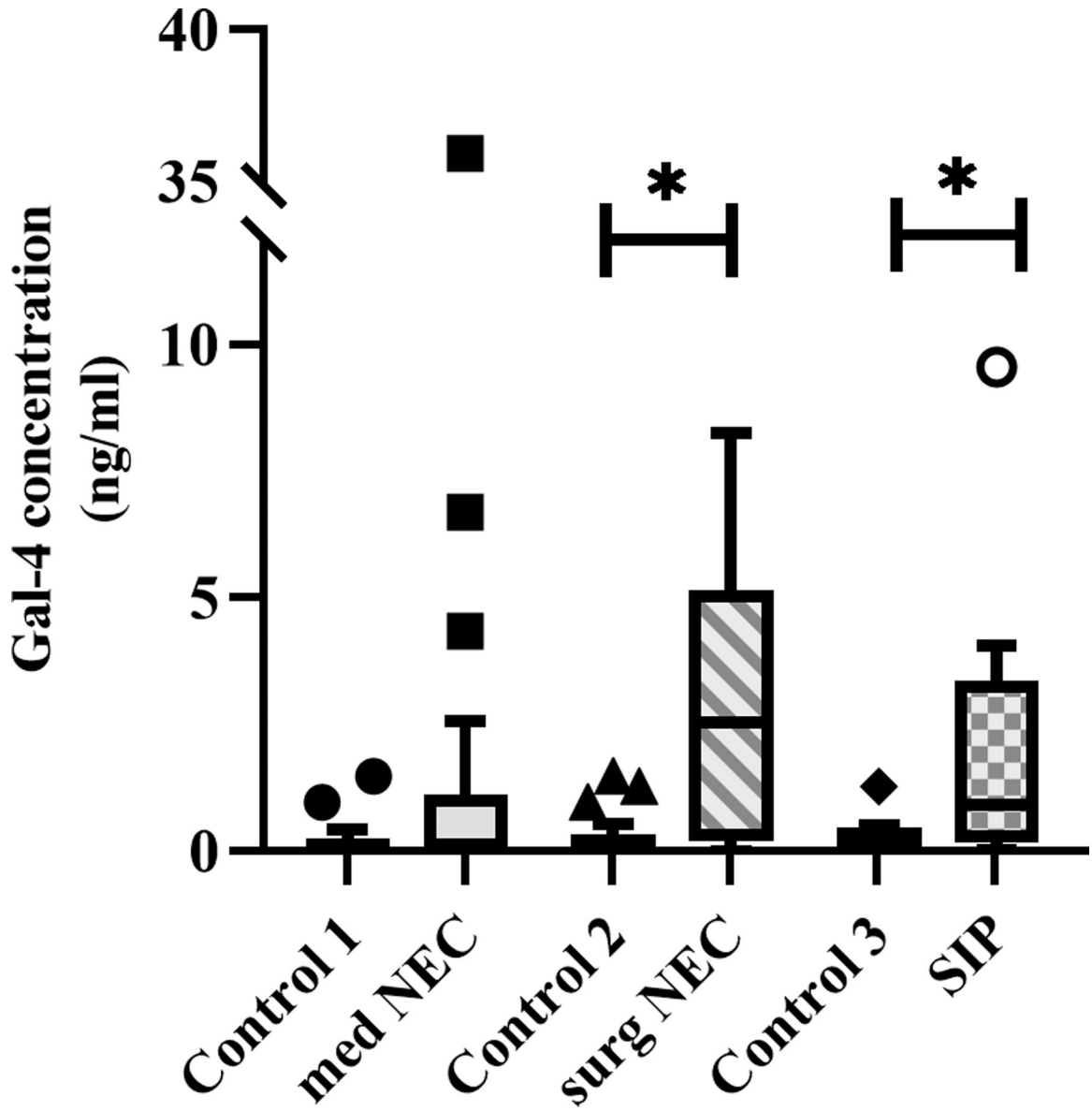
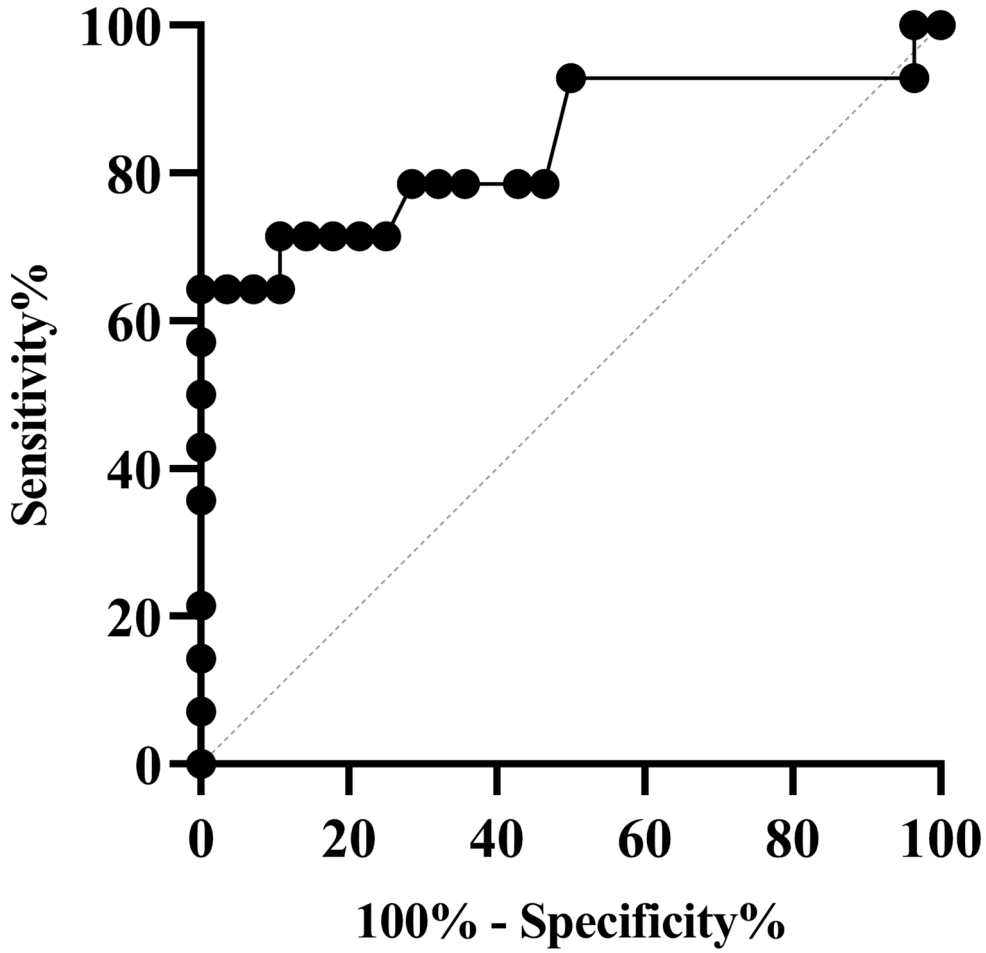


Figure 2:

Box plot of intestinal injury cohorts vs. group-specific matched controls; med NEC: medical NEC (n=42); surg NEC: surgical NEC (n=14), SIP: spontaneous intestinal perforation (n=8). *p<0.05.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript



Cutoff (ng/ml)	Sensitivity	Specificity
> 0.7	71%	89%
>1.38	64%	96%

Figure 3:
Receiver operator curve and selected cut-off values for Gal-4 and diagnosis of surgical NEC.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

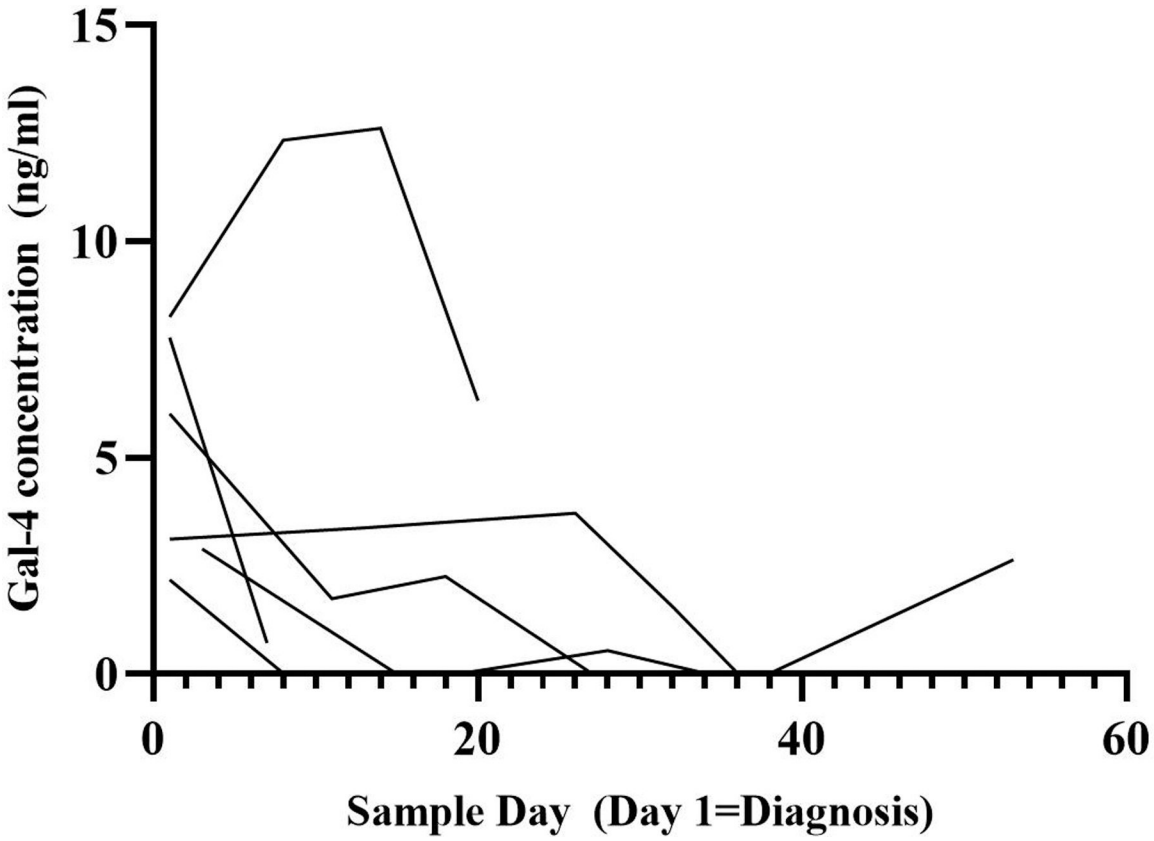


Figure 4:
Spaghetti plot of surgical NEC patients with serial samples after diagnosis; Sample Day 1 = day of diagnosis.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Table 1:

Infant characteristics in infants without intestinal injury (controls, non-matched) and intestinal injury groups. Note: Any NEC includes medical and surgical NEC patients. IQR: Interquartile range; PMA: Post-menstrual age

	Controls (n=111)	Any NEC (n=46)	Medical NEC (n=32)	Surgical NEC (n=14)	SIP (n=10)
Median gestational age (range)	31.3 (24–41)	29.9 (23–39)	30.3 (23–39)	29.4 (24.3–35.9)	24.4 (22.3–26.9)
Median birth weight, grams (IQR)	1420 (1030–2540)	1100 (735–1980)	1100 (730–2110)	1090 (725–1940)	700 (659–1010)
Small for gestational age (%) [a]	14 (13)	10 (22)	8 (25)	2 (14)	0
Sex, male (%)	56 (50)	33 (72)	24 (75)	9 (64)	6 (60)
Race (%)					
Caucasian	55 (49)	25 (54)	17 (53)	8 (57)	5 (50)
Black	44 (39)	16 (35)	11 (34)	5 (36)	5 (50)
Other	13 (12)	5 (11)	4 (13)	1 (7)	0 (0)
Ethnicity (%)					
Hispanic	8 (7)	5 (11)	3 (9)	2 (14)	1 (10)
Chorioamnionitis (%)					
Yes	19 (17)	7 (15)	5 (16)	2 (14)	3 (30)
No	45 (40)	34 (74)	25 (78)	9 (64)	7 (70)
Unknown	49 (43)	5 (11)	2 (6)	3 (22)	0
5-minute Apgar score					
Median	6	7	7	7	6
Unknown (%)	29 (26)	2 (4)	1 (3)	1 (7)	0
Enteral nutrition (%) [b]					
Nothing by mouth	7 (6)	1 (2)	1 (3)	0 (0)	2 (20)
Breast milk (Maternal or Donor Milk)	8 (8)	13 (28)	9 (28)	4 (29)	7 (70)
Formula	7 (6)	8 (18)	4 (12)	4 (29)	0 (0)
Combination breast milk/formula	0 (0)	7 (15)	5 (16)	2 (14)	1 (10)
Unknown	89 (80)	17 (37)	13 (41)	4 (28)	0 (0)
Median PMA (IQR) [b]	Not applicable	34 (30.4–38.9)	35.4 (30.6–39.1)	32 (30.3–37.1)	25.2 (24.9–26.4)
Median day of life (IQR) [b]	Not applicable	27 (15.5–40)	29 (19–39.5)	23.5 (9.8–43.3)	7.5 (5.8–10.3)

[a] Small for gestational age, defined as birth weight ≤ 10th percentile.

[b] At time of sample collection (first week of life for control samples or diagnosis of intestinal injury in study samples).