# Paneth Cell Hyperplasia and Metaplasia in Necrotizing Enterocolitis

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**ABSTRACT:** Paneth cell dysfunction has been suggested in necrotizing enterocolitis (NEC). The aim of this study was to i) study Paneth cell presence, protein expression, and developmental changes in preterm infants with NEC and ii) determine Paneth cell products and antimicrobial capacity in ileostomy outflow fluid. Intestinal tissue from NEC patients ($n = 55$), preterm control infants ($n = 22$), and term controls ($n = 7$) was obtained during surgical resection and at stoma closure after recovery. Paneth cell abundance and protein expression were analyzed by immunohistochemistry. RNA levels of Paneth cell proteins were determined by real-time quantitative RT-PCR. In ileostomy outflow fluid, Paneth cell products were quantified, and antimicrobial activity was measured *in vitro*. In acute NEC, Paneth cell abundance in small intestinal tissue was not significantly different from preterm controls. After recovery from NEC, Paneth cell hyperplasia was observed in the small intestine concomitant with elevated human alpha-defensin 5 mRNA levels. In the colon, metaplastic Paneth cells were observed. Ileostomy fluid contained Paneth cell proteins and inhibited bacterial growth. In conjunction, these data suggest an important role of Paneth cells and their products in various phases of NEC. (*Pediatr Res* 69: 217–223, 2011)

Paneth cells, named after Joseph Paneth (1), play a significant role in the innate immune response. Localized at the base of the crypts of Lieberkühn, Paneth cells are abundant in the ileum and are only occasionally found in the proximal colon (2). Paneth cells have defensive functions including 1) protection of stem cells in response to invading microbes (3,4); 2) eradication of ingested pathogens (5–7); 3) regulation of the composition, distribution, and number of commensal bacteria in the intestinal lumen (8,9); and 4) induction of cytokine secretion, immune cell recruitment, and chloride secretion to flush the intestinal crypts of pathogens (10,11).

Paneth cells execute their functions by production of antimicrobial proteins/peptides such as lysozyme (12,13), secretory phospholipase-A2 (sPLA2) (14), and human $\alpha$-defensins (HD5 and $-6$) (15,16). In the human fetal intestine, Paneth cells are present from 20 wk of gestation; however, HD5 and $-6$ mRNAs are expressed from 13.5 wk onward (17,18). Both Paneth cell numbers and HD5 and $-6$ mRNA expression are lower in premature infants at 24 wk of gestation compared

with term infants, and up to 200-fold lower than in adults (18). In the premature infant, who is often exposed to nosocomial pathogens and has delayed colonization with beneficial commensals, this phenomenon could result in higher susceptibility to bacterial infection and inflammation.

Although Paneth cell dysfunction in necrotizing enterocolitis (NEC) has been suggested (19), little is known about Paneth cell abundance and function in preterm infants at risk for NEC. NEC is the most common gastrointestinal disease in premature infants with mortality rates up to 50% for infants needing surgery (20,21). Risk factors for NEC are prematurity, very low birth weight, enteral formula feeding, and bacterial colonization (22–24). However, the underlying etiology and the impact of the innate immune system on the development of NEC require further investigation.

We hypothesized that preterm infants with acute NEC have fewer Paneth cells compared with control patients, and that Paneth cell numbers are up-regulated during recovery from NEC, thereby enhancing the innate immune response. Our aim was i) to study Paneth cell presence, Paneth cell protein expression, and disease-related changes in Paneth cell numbers over time in premature infants with NEC compared with control patients and ii) to measure Paneth cell products in ileostomy outflow fluid during NEC recovery and to determine the bactericidal activity of ileal outflow fluid.

## METHODS

*Study population.* Premature infants who underwent bowel resection for NEC between August 2003 and September 2009 were eligible for the study. NEC was diagnosed and staged according to the criteria of Bell *et al.* (25). Diagnosis was confirmed during surgery and by histopathological evaluation of resected intestinal tissue. Samples of both ends of the resected intestine represented macroscopically vital tissue and were collected for histology and real-time quantitative RT-PCR (real time qRT-PCR).

Approximately 3–5 wk after surgery, when infants received an enteral intake of at least 100 mL · kg · d, enterostomy outflow fluid was collected

**Abbreviations: A-NEC,** acute phase of necrotizing enterocolitis; **CFU,** colony forming units; **HD,** human defensins; **LGG,** *Lactobacillus rhamnosus* GG; **NEC,** necrotizing enterocolitis; **NEC-R,** stoma reanastomosis after necrotizing enterocolitis; **post-NEC stricture,** stricture formation after acute phase of necrotizing enterocolitis; **preterm CO,** preterm control patients; **preterm CO-R,** stoma reanastomosis in preterm control patient; **qRT-PCR,** real-time quantitative PCR; **sPLA2,** secretory phospholipase-A2; **term CO,** term control patient

every 3 h for 24 h and immediately stored at −20°C. After full recovery, patients underwent a second surgical procedure when eligible for reanastomosis (*i.e.* stoma closure). To allow proper reanastomosis, tissue was resected, which was collected for histology.

Patients who underwent surgery for resection of post-NEC strictures, as a result of obstructive fibrotic intestinal tissue that developed during nonsurgical therapy for NEC, were also included. These samples were not subjected to surgical manipulation or exposed to the extra-abdominal environment and are thus representative for the effects of NEC only. Moreover, samples were taken from both the proximal and distal part of the resected intestine.

Finally, both preterm and term neonates who underwent resection for developmental defects or diseases other than NEC were included as control patients, and intestinal tissue was collected as described earlier. Infants diagnosed with cystic fibrosis were excluded. The study protocol was approved by the "Central Committee on Research involving Human Subjects" (The Hague, the Netherlands), and written informed consent was obtained from the parents.

*Histology and immunohistochemistry.* Intestinal tissues were fixed in 4% (wt/vol) paraformaldehyde in PBS for 24 h at 4°C and processed for light microscopy. Five micrometer-thick sections were cut and deparaffinized through a graded series of xylol-ethanol. For histology, tissue samples were stained with hematoxylin and eosin. To determine Paneth cell-specific expression of lysozyme, trypsin, and HD5, immunohistochemistry was performed as described previously (26,27). Antibodies used were anti-human lysozyme (Dako, Glostrup, Denmark), anti-human trypsin (MAB 1482; Millipore, Billerica, MA), and anti-human HD5 (HyCult Biotechnology, Uden, the Netherlands). Secondary antibodies applied were biotinylated horse anti-mouse IgG diluted (Vector Laboratories, Burlingame, England) and biotinylated goat anti-rabbit IgG (Vector Laboratories). Detection was performed using the ABC-PO complex (Vectastain Elite Kit, Vector Laboratories). Staining was developed using diaminobenzidine.

Collected tissue samples were assigned to intestinal segments according to their origin: jejunum, ileum, coecum, colon ascendens, colon transversum, colon descendens, and rectosigmoid. When samples originated from the same intestinal segment, only one sample per segment was analyzed. Samples from NEC and control infants were matched and analyzed according to their segment of origin. Tissue morphology was qualitatively assessed by two trained observers (P.J.P. and I.B.R.). A semi-quantitative assessment of Paneth cell abundance based on lysozyme and HD5 positive cells was performed. Paneth cells within 10 crypts per tissue sample were scored blinded as follows: 0, no Paneth cells; 1, 0–1 Paneth cells per crypt and at least 1 Paneth cell per 10 crypts; 2, 1–4 Paneth cells per crypt; and 3, >4 Paneth cells per crypt. Scores were given by one observer and validated by the second observer.

*Real-time Quantitative RT-PCR.* RNA was isolated from snap-frozen ileal tissue (RNeasy Midi kit; Qiagen Benelux, Venlo, the Netherlands) and used for cDNA synthesis. Expression levels of *DEFA5* (HD5), *LYZ* (lysozyme), *TRY2* (anionic trypsin), and *PLA2G2A* (sPLA2) were quantified using qRT-PCR analysis based on the intercalation of SYBR Green on an ABI prism 7900HT Fast real-time PCR system (PE Applied Biosystems, Foster City, CA) as described previously (28). The mRNA levels were expressed relative to the epithelial-specific housekeeping gene *VIL1* (Villin). Primer combinations are given in Table 1.

*Protein isolation and Western blot analyses.* To isolate proteins from the small intestinal outflow fluid, cesium-chloride density gradient ultracentrifugation was performed as described previously (29,30). Fractions with a buoyant density >1.35 g/mL, containing high-density proteins, were discarded. Fractions with a buoyant density <1.35 g/mL were pooled, dialyzed, and used to quantify lysozyme, trypsin, and sPLA2 by Western blot. The used dialysis membrane had a cut-off size of 10 kD and therefore did not allow analysis of HD5 as HD5 is smaller than 10 kD. Primary antibodies used were rabbit anti-lysozyme (Dako), rabbit anti-sPLA2 (H-74; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-α-trypsin (MAB 1482; Chemicon International). Bound antibodies were detected using HRP-conjugated goat anti-rabbit or anti-mouse antibodies and Luminol Enhancer (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL). Western blots were analyzed using densitometry.

*Antimicrobial assay. Escherichia coli* (*E. coli*), *Lactococcus lactis* (*L. lactis*), and *Lactobacillus rhamnosus* (*LGG*) were grown overnight in LB medium, GM17 medium, and MRS medium (BD, Franklin Lakes, NJ), respectively, at 37°C. The pooled antimicrobial protein fractions (100 $\mu$g) were added to 200 $\mu$L of bacterial cultures of $2 \times 10^7$ colony forming units (CFUs)/mL and incubated for 1h at 37°C. A $10^{-4}$ dilution was prepared, and 100 $\mu$L of the suspension was plated. After overnight incubation at 37°C, CFUs were counted. Bacterial growth inhibition was analyzed by calculating the number of CFUs in comparison with untreated bacteria.

*Statistical analysis.* Comparisons between patient groups were made using ANOVA with a post hoc Tukey *t* test for normally distributed data or the Kruskal-Wallis test followed by Dunn's Multiple Comparison test for not-normally distributed data. Protein and mRNA levels of antimicrobial proteins were determined using the Mann Whitney test. Analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Significance was defined at $p < 0.05$.

## RESULTS

*Study population.* A total of 84 infants were included in the study. Patient characteristics are shown in Table 2. Forty-nine preterm infants underwent bowel resection for stage III acute NEC, further referred to as A-NEC. After resection of necrotic tissue, primary anastomosis was performed in four patients and an enterostomy was created in 40 infants; in five patients, NEC lesions were too extensive to allow further surgical treatment compatible with life. In total, 13 infants (27%) died of complications of NEC. After recovery, 31 of the 40 enterostomy patients were eligible for reanastomosis (further referred to as NEC-R), allowing repeated tissue collection. In addition, six premature infants were included, who had received nonsurgical treatment for NEC but developed post-NEC strictures requiring surgery (post-NEC stricture).

Twenty-two premature infants were included as control (preterm CO). These patients were diagnosed with small intestinal atresia ($n = 8$), milk curd obstruction ($n = 3$), perforation caused by herniation ($n = 2$), solitary perforation ($n = 1$), ileus ($n = 1$), Meckel's diverticulum ($n = 1$), volvulus ($n = 2$), gastroschisis ($n = 3$), and cloacal malformation ($n = 1$). After intestinal resection, six infants received a primary anastomosis and 16 received an enterostomy. Post-surgically, one patient died of additional clinical complications. After full recovery, 13 infants were eligible for stoma closure (preterm CO-R), allowing repeated tissue collection. Seven term infants who were resected for small intestinal atresia ($n = 3$), intestinal perforation ($n = 3$), and volvulus ($n = 1$) were included as term controls (Term CO). Figure 1 depicts the corrected GA at the time of tissue sampling during surgery for all patient groups.

**Table 1.** *Primer sequences used for real-time qRT-PCR*

| Gene | Forward primer | Reverse primer |
|------|----------------|----------------|
| *LYZ* | 5′- TTT GCT GCA AGA TAA CAT C -3′ | 5′- GAC GGA CAT CTC TGT TTT G -3′ |
| *DEFA5* | 5′- TGC AGG AAA TGG ACT CTC -3′ | 5′- GCC ACT GAT TTC ACA CAC -3′ |
| *PLA2G2A* | 5′- TGG CAC CAA ATT TCT GA -3′ | 5′- GCA GCC TTA TCA CAC TCA -3′ |
| *TRY2* | 5′- GCT CCA AGG AAT TGT CTC -3′ | 5′- GGG GCT TTA GCT GTT G -3′ |
| *VIL1* | 5′- CTG CCT TCT CTG CTC TG -3′ | 5′- ATC GGT GAG AAA ATG AGA C -3′ |

Primers for *DEFA5* (HD5), *LYZ* (lysozyme), *TRY2* (anionic trypsin), and *PLA2G2A* (secretory phospholipase A2) were designed using OLIGO 6.22 software (Molecular Biology Insights, Cascade, CO).

**Table 2.** *Patient demographics*

| | NEC | Post-NEC stricture | Preterm CO | Term CO | *p* |
|---|---|---|---|---|---|
| No. patients | 49 | 6 | 22 | 7 | |
| Demographics | | | | | |
| Sex, % male | 61 | 67 | 50 | 57 | |
| GA (wk) | 29.5 ± 3 | 31.8 ± 3.7 | 32.9 ± 3.9 | 38.2 ± 1.2 | a, c, i, k |
| Birth weight (g) | 1239 ± 501.8 | 1618 ± 507.5 | 2051 ± 874.8 | 2946 ± 738.9* | a, c, h, j |
| Apgar score 1 min | 7 (4–9) | 7.5 (2.9–9) | 8 (5–9)* | 9 (8–9)† | NS |
| Apgar score 5 min | 9 (8–10) | 9.5 (8.3–10) | 9 (8–9)* | 9 (9–10)† | NS |
| Clinical and surgical outcome | | | | | |
| Postnatal age at 1st surgery (d) | 11 (7–17.5) | 40 (35.8–51) | 1.5 (0–16) | 2 (0–3) | b, d, e, g, h |
| GA at 1st surgery (wk) | 31.7 (29.2–34) | 38 (35.8–40.5) | 34.4 (31–36.1) | 38 (37.6–40.3) | b, c, f |
| Primary anastomosis (%) | 8 | 100 | 27 | 42.3 | |
| Jejunostomy (%) | 12 | — | 23 | 14.3 | |
| Ileostomy (%) | 61 | — | 45 | 42.3 | |
| Colostomy (%) | 8 | — | 4 | — | |
| Deaths (%) | 27 | — | 4 | — | |
| Patients eligible for reanastomosis (%) | 63 | — | 59 | — | |
| Postnatal age at reanastomosis (d) | 95 (57–125) | — | 55 (44–102) | — | NS |
| GA at reanastomosis (wk) | 43 (40.1–46.6) | — | 41 (39.5–46.4) | — | NS |

Data provided are percentages or means ± SD. Medians (interquartile range) are provided for variables that are not normally distributed.

* Data on one patient were not recorded.

† Data on two patients were not recorded.

[a] *p* < 0.001 for comparisons between NEC and preterm controls.

[b] *p* < 0.05 for comparisons between NEC and preterm controls.

[c] *p* < 0.001 for comparisons between NEC and term controls.

[d] *p* < 0.05 for comparisons between NEC and term controls.

[e] *p* < 0.05 for comparisons between NEC and post-NEC strictures.

[f] *p* < 0.001 for comparisons between NEC and post-NEC strictures.

[j] *p* < 0.001 for comparisons between post-NEC strictures and preterm controls.

[h] *p* < 0.001 for comparisons between post-NEC strictures and term controls.

[i] *p* < 0.01 for comparisons between post-NEC strictures and term controls.
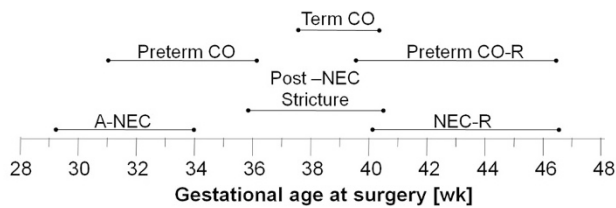
[j] *p* < 0.05 for comparisons between preterm controls and term controls.

[k] *p* < 0.01 for comparisons between preterm controls and term controls.



**Figure 1.** Time frame representing an overview of intestinal tissue sampling according to GA. *Bars* represent interquartile ranges. A-NEC patients are significantly younger than preterm CO (Mann-Whitney *p* < 0.01), but corrected GA is comparable during reanastomosis. Corrected GA during reanastomosis is in both groups not different from patients with post-NEC strictures and term controls during surgery.

**Table 3.** *Sample evaluation of intestinal tissue obtained during acute surgery and reanastomosis*

| | Patient groups | | | | | |
|---|---|---|---|---|---|---|
| | Small intestine | | | Colon | | |
| | Total | Excluded | Evaluated | Total | Excluded | Evaluated |
| Acute surgery | | | | | | |
| NEC | 46 | 16 | 30 | 18 | 8 | 10 |
| Post-NEC stricture | 0 | 0 | 0 | 5 | 0 | 5 |
| Preterm CO | 21 | 4 | 17 | 2 | 1 | 1 |
| Term CO | 7 | 1 | 6 | 1 | 0 | 1 |
| Reanastomosis | | | | | | |
| NEC | 29 | 3 | 26 | 13 | 2 | 11 |
| Preterm CO | 12 | 1 | 11 | 3 | 0 | 3 |

Tissue samples were obtained from both ends of resected intestine and might originate from multiple intestinal segments. Therefore, patient numbers do not correlate with sample numbers.

***Sample evaluation.*** In most infants, the resected intestine covered multiple intestinal segments. Samples were cut from both ends of the resected material and therefore sample numbers do not correlate with the number of patients included. Samples from jejunum, ileum, and colon were used for morphological analysis (*n* = 157). Samples that showed complete mucosal erosion were excluded from further analyses (*n* = 36). The remaining samples were stained for lysozyme, HD5, and trypsin to analyze Paneth cell-specific protein expression. Scoring was based on both lysozyme and HD5 staining. Table 3 demonstrates the number of samples that were obtained and evaluated for each patient group.

***Paneth cell hyperplasia during NEC recovery.*** Paneth cells were present in small intestinal tissue of NEC patients and preterm CO and were positive for lysozyme (Fig. 2*A–D*), HD5 (Fig. 2*E–H*), and trypsin (not shown). Abundance of Paneth cells, determined by lysozyme and HD5 staining, was not significantly different between A-NEC and preterm CO (Fig. 3). In reanastomosis samples, no differences in Paneth cell abundance between NEC-R and preterm CO-R were observed (Fig. 3). However, small intestinal samples in both NEC-R and preterm CO-R showed increased numbers of Paneth cells, determined by lysozyme and HD5 staining, when compared with A-NEC and preterm CO samples, respectively (Fig. 3).
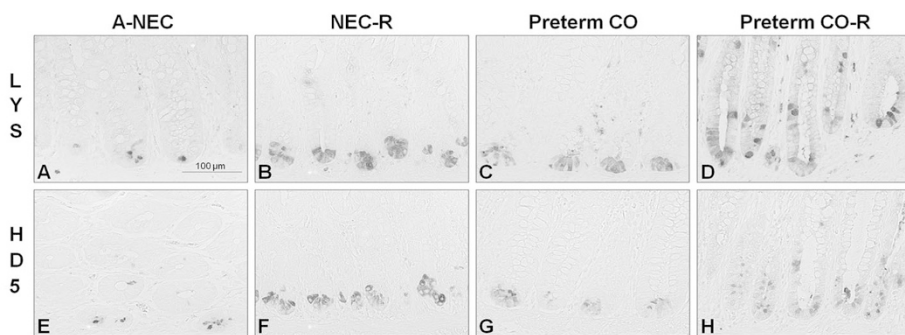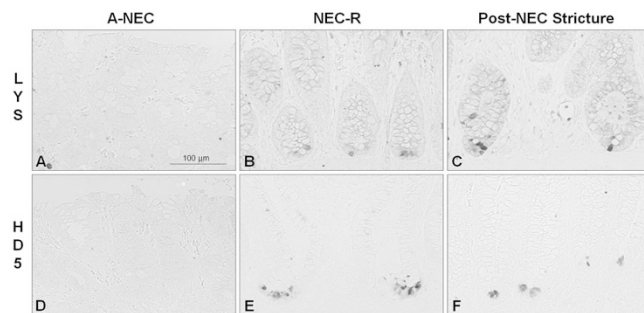
**Figure 2.** Immunohistochemistry for lysozyme-(LYS) (*A–D*) and HD5-positive (*E–H*) Paneth cells in ileal tissue from NEC patients and preterm CO. (*A, E*) A-NEC; (*B, F*) NEC-R; (*C, G*) preterm CO; and (*D, H*) preterm CO-R. Images are representative of 19 A-NEC, 21 NEC-R, nine preterm CO, and six preterm CO-R specimens derived from the ileum.
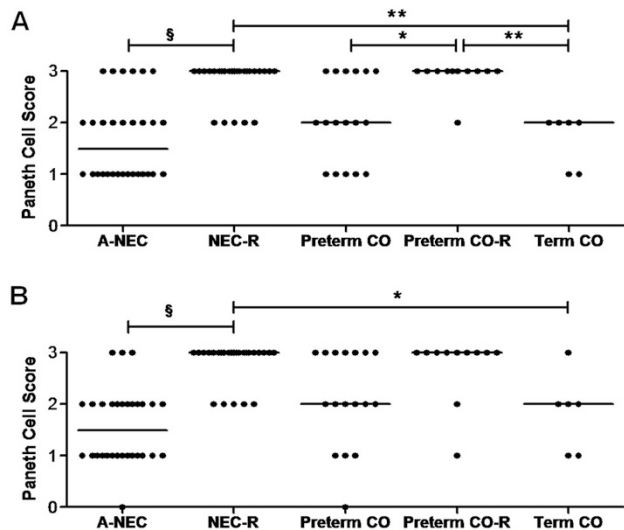


**Figure 3.** Paneth cell abundance in small intestinal tissue from A-NEC and NEC-R patients, preterm CO and preterm CO-R, and term CO obtained during acute surgery and reanastomosis. Scoring of HD5-positive (*A*) and lysozyme-positive (*B*) Paneth cells, based on the number of stained cells per crypt from an average of 10 crypts (see Methods). Lines represent medians. Statistics were performed using the Kruskal-Wallis—Dunn's Multiple Comparison test. *$p < 0.05$; **$p < 0.01$; §$p < 0.001$.

To determine whether this increase in Paneth cell numbers was age- or disease-related, we compared term CO samples with NEC-R and preterm CO-R samples, as the corrected GA at time of surgery was not significantly different between these groups (Fig. 1). Interestingly, Paneth cell abundance in small intestinal NEC-R and preterm CO-R tissue was increased compared with term CO indicating a disease-related effect.

*Paneth cell metaplasia during NEC recovery.* In nine of 10 samples, no Paneth cells were found in colonic tissue from A-NEC patients (Figs. 4 and 5). Unexpectedly, at the time of reanastomosis, nearly all proximal and distal colon samples showed metaplastic Paneth cells, determined by lysozyme and HD5 staining (Fig. 4). This finding was confirmed by the post-NEC stricture colon samples, all of which displayed metaplastic Paneth cells (Fig. 4). Figure 5 presents the scoring of Paneth cell abundance in the colon and demonstrates the induction of metaplastic Paneth cells, assessed by staining for HD5 (Fig. 5*A*) and lysozyme (Fig. 5*B*), in NEC-R and post-NEC stricture. In preterm CO-R, only few colon samples ($n = 3$) were obtained; however, all samples showed Paneth cell metaplasia (not shown).



**Figure 4.** Representative immunohistochemistry for lysozyme (LYS; *A–C*) and HD5 (*D–F*) in metaplastic Paneth cells in colonic tissue from NEC patients. (*A, D*) A-NEC; (*B, E*) NEC-R; and (*C, F*) post-NEC stricture. Images are representative of 10 A-NEC, 11 NEC-R, and five post-NEC stricture specimens.
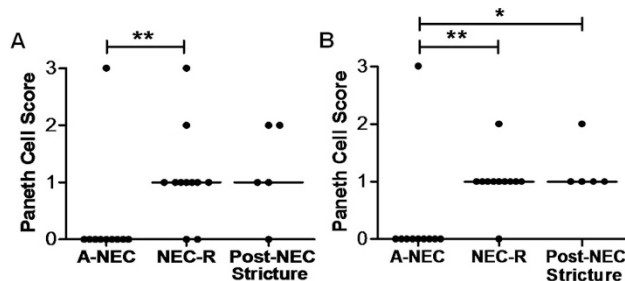


**Figure 5.** Scoring of Paneth cell abundance in colonic tissue from NEC A-NEC, NEC-R, and post-NEC stricture patients. Scoring of HD5-positive (*A*) and lysozyme-positive (*B*) Paneth cells, based on the number of stained cells per crypt from an average of 10 crypts (see Methods). *Lines* represent medians. Statistics were performed using the Kruskal-Wallis—Dunn's Multiple Comparison test. *$p < 0.05$ and **$p < 0.01$.

*LYZ, TRY2, PLA2G2A, and DEFA5 gene expression levels in NEC.* Analyses of ileal samples from 21 NEC patients by qRT-PCR showed that *LYZ*, *TRY2*, and *PLA2G2A* expression levels were not different in tissue from A-NEC ($n = 8$) *versus* NEC-R ($n = 13$; Fig. 6). However, expression of *DEFA5* was significantly higher in NEC-R compared with A-NEC (Fig. 6*A–D*). Although sample numbers obtained from preterm control infants were limited, expression of *LYZ*, *TRY2*, *PLA2G2A*, and *DEFA5* tended to be higher in preterm CO-R samples ($n = 5$) compared with preterm CO ($n = 4$; Fig. 6*E–H*).

*Antimicrobial products in the ileostomy outflow fluid and antimicrobial activity.* Ileostomy outflow fluid from 12 patients was collected during the regenerative phase of NEC (Table 4). Isolates of the outflow fluid samples contained
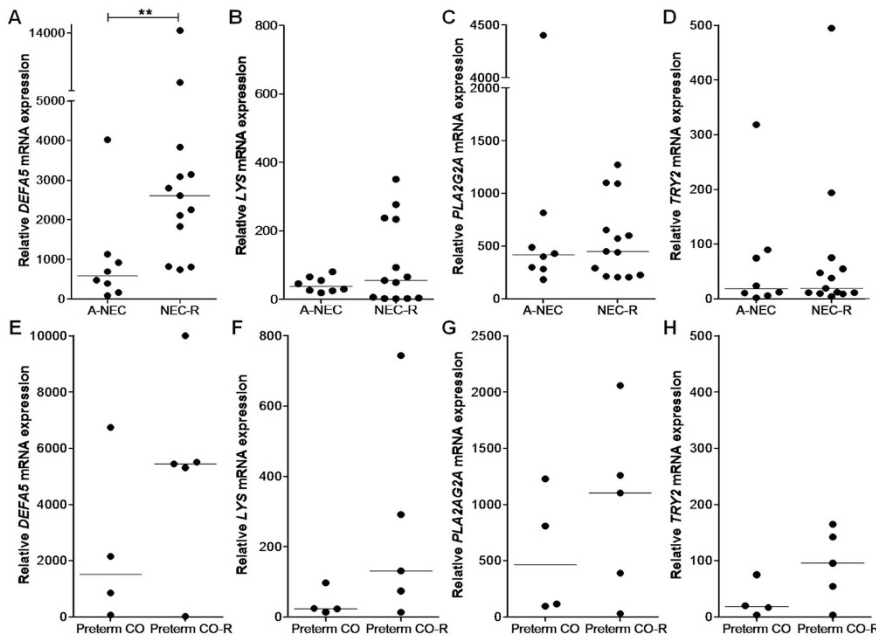
**Figure 6.** Expression of antibacterial peptide genes in A-NEC and NEC-R (*A–D*) and preterm CO and preterm CO-R (*E–H*): (*A, E*) *DEFA5*, (*B, F*) *LYS*, (*C, G*) *PLA2G2A*, and (*D, H*) *TRY2*. *DEFA5* expression was up-regulated in NEC reanastomosis; Mann Whitney test $p = 0.013$. *Lines* represent medians.

**Table 4.** *Baseline characteristics of NEC patients sampled for ileostomy outflow fluid*

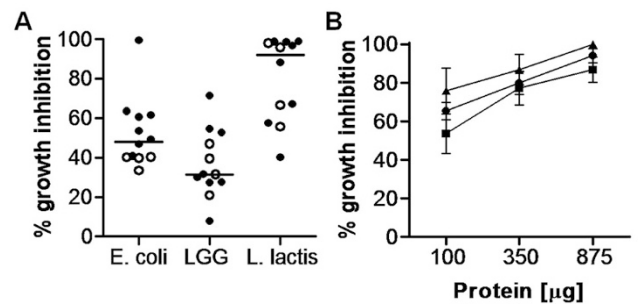| | |
|---|---|
| No. patients | 12 |
| Demographics | |
| Sex, % male | 92 |
| GA (wk) | $29.1 \pm 3.1$ |
| Birth weight (g) | $1210 \pm 524$ |
| Postnatal age at 1st surgery (d) | 11.5 (7.8–13.5) |
| Time of enterostomy outflow collection postsurgery (d) | 22 (18.3–25) |
| Patients receiving breast milk (%) | 50 |
| Patients receiving antibiotic treatment during sampling (%) | 33 |

Data provided are percentages or means ± SD. Medians (interquartile range) are provided for variables that are not normally distributed.



**Figure 7.** (*A*) Antimicrobial activity of protein isolates from ileostomy outflow fluid samples ($n = 12$) on *E. coli*, *LGG*, and *L. lactis*. Antibacterial activity is expressed as % growth inhibition compared with nontreated controls. *Open symbols* denote antibiotic-treated patients. *Lines* represent means. (*B*) Antibacterial activity showing up to 100% growth inhibition of *E. coli* ◆, *LGG* ■, and *L. lactis* ▲ with increasing doses of protein isolates ($n = 7$). *Bars* represent means ± SEM.

lysozyme, trypsin, and sPLA2. Because lysozyme and trypsin are present in breast milk, we analyzed whether levels of these antimicrobial products would differ between infants receiving breast milk ($n = 6$) or formula ($n = 6$). There was no statistically significant difference between the groups (data not shown).

Bactericidal activity of the outflow fluid isolates was demonstrated by growth inhibition of *E. coli* with 52% (±18%), *L. lactis* with 81% (±20%), and *LGG* with 43% (±17%; Fig. 7A). Increasing concentrations of protein isolates ($n = 7$) were used to determine bacterial growth inhibition capacity. Growth inhibition up to 100% was reached as presented in Fig. 7B. To evaluate whether antibiotic treatment influenced the observed effect, we compared bacterial growth inhibition from isolates of antibiotic-treated patients ($n = 4$) with isolates from patients without antibiotics but no difference was observed (Fig. 7).

## DISCUSSION

Paneth cells play a key role in the innate immune response and host defense. Although Paneth cell dysfunction has been suggested, little is known about Paneth cell presence and function in the immature gut and gastrointestinal complications of prematurity such as NEC. In this study, we analyzed Paneth cell presence, function, and disease-related changes in Paneth cell abundance over time in preterm infants with NEC. Our results show Paneth cell hyperplasia and metaplasia in premature infants recovering from NEC. Furthermore, Paneth cell products from ileostomy outflow fluid of NEC patients exhibited strong bactericidal activity.

First, Paneth cell presence and protein expression in small intestinal samples of A-NEC patients *versus* preterm CO were investigated. We found that Paneth cells were present in both A-NEC and preterm CO. Coutinho *et al.* (19) observed an absence of lysozyme-positive Paneth cells in acute NEC and suggested a developmental defect in Paneth cells, *i.e.* delayed maturation of Paneth cells, leading to lack of antimicrobial agents such as lysozyme and defensins. Besides the fact that 40% of their NEC-patients were term newborns in whom the disease is thought to be different from premature infants

(31–33), the absence of lysozyme-positive Paneth cells might be explained by enhanced secretion of lysozyme. Nevertheless, our study demonstrates Paneth cell-specific lysozyme and HD5 mRNA and protein in A-NEC contradicting Paneth cell deficiency in preterm infants.

Similarly to our results, Salzman *et al.* (34) showed presence of Paneth cells in acute NEC patients. Semi-quantitative analysis of the six preterm acute NEC infants investigated in that study revealed an increase in Paneth cell numbers and HD5 mRNA levels in infants with NEC compared with (near) term control infants. In our study, we did not observe a difference between A-NEC and term CO with respect to Paneth cell abundance; however, we did when we compared NEC-R infants to term CO. Another point of interest is that in the study of Salzman *et al.*, intracellular peptide levels in NEC did not coincide with the observed rise in mRNA. Although we did not quantify intracellular peptide levels per Paneth cell, low HD5 mRNA levels coincided with low numbers of HD5-positive Paneth cells, whereas high HD5 mRNA levels correlated with the hyperplasia of HD5-positive Paneth cells during reanastomosis. Therefore, our study is the first to report small intestinal Paneth cell hyperplasia after recovery from NEC and strongly implies an up-regulation of Paneth cell abundance. We did not detect differences in lysozyme, sPLA2, and trypsin mRNA levels in A-NEC *versus* NEC-R. However, in contrast to HD5, these products are not restricted to Paneth cells for their production and therefore do not necessarily reflect changes in Paneth cell abundance.

In concordance to the hyperplasia observed in NEC, Paneth cell numbers in preterm CO-R were also up-regulated compared with preterm CO. Moreover, both preterm CO-R and NEC-R tissue showed higher numbers of Paneth cells compared with term CO. Most likely, severe or prolonged intestinal inflammation, which to a lesser extend was also seen in preterm control infants, might explain the observed hyperplasia. However, the effect of increased postnatal age in this preterm population compared with term CO cannot be excluded.

A novel finding in our study is the occurrence of Paneth cell metaplasia in colon samples during NEC-recovery. The expression of lysozyme, trypsin, and HD5 indicated the presence of well-differentiated cells able to exert Paneth cell-defensive functions. Although Paneth cell metaplasia has not been described in NEC, this phenomenon has been reported in inflammatory bowel disease (35). Metaplasia can occur through restitution or regeneration of tissue after intestinal mucosal surface loss, either because of resection and inflammation or both (36). However, as our post-NEC stricture patients had not undergone resection during acute NEC, but did show Paneth cell metaplasia, we suggest that inflammation caused Paneth cell metaplasia.

During recovery, lysozyme, trypsin, and sPLA2 were present in intestinal outflow fluid and outflow fluid inhibited bacterial growth *in vitro*. Similar outcomes were found in *in vitro* studies performed with human and mouse peptide isolates (37–39). A caveat is that these antibacterial products are not Paneth cell specific. However, the presence of Paneth cells in A-NEC and the presence of these peptides in the ileal

outflow fluid imply that Paneth cells in preterm infants are present and at least partially functional by secreting antimicrobial products. Therefore, Paneth cell deficiency, *i.e.* a lack of Paneth cells, in preterm infants with NEC seems unlikely. However, we cannot exclude that there are abnormalities in Paneth cells that limit their functioning in infants with NEC.

A limitation of this study is that interpretation of our findings is difficult because preterm controls also exhibit signs of inflammation, numbers of age-matched control patients are limited, and collecting material from a healthy control group is not feasible. Nevertheless, our results indicate the presence of Paneth cells in preterm infants with NEC and imply that Paneth cell hyperplasia and metaplasia is most likely caused by inflammation. Subsequently, increased Paneth cell numbers suggests enhanced secretion of active antimicrobial products and might be indicative of an enhanced innate defense response during prolonged inflammation that might contribute to NEC recovery. However, it still remains to be elucidated, which cell signaling and regulatory pathways are involved in these processes to target improvement of therapy and clinical outcome.

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