Journal of Perinatology (2016) 00, 1–6

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ORIGINAL ARTICLE

# Identification of bacterial invasion in necrotizing enterocolitis specimens using fluorescent *in situ* hybridization

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**OBJECTIVE:** Investigation of bacterial invasion into the intestinal wall in necrotizing enterocolitis (NEC) specimens.
**STUDY DESIGN:** We compared 43 surgical NEC specimens with 43 age-matched controls. We used fluorescent *in situ* hybridization (FISH), a universal bacterial probe together with species-specific probes for *Clostridium* spp., *Enterobacteriaceae*, bacteroides and enterococci/lactobacilli. We used a FISH scoring system to reveal invasion of the intestinal wall, in which 1 represented no colonies and 4 invasion of the intestinal wall.
**RESULTS:** We observed invasion of the intestinal wall in 22/43 of the most affected NEC tissue samples as compared with 16/43 in the least affected NEC tissue samples ($P = 0.03$). A FISH score of 4 was reached in 7/43 control cases. *Enterobacteriaceae* dominated the NEC specimens. *Clostridium* spp. were detected occasionally in NEC samples.
**CONCLUSION:** Bacterial invasion of the intestinal wall is more present in most affected NEC tissue samples compared with least affected NEC tissue samples or controls. *Enterobacteriaceae* are prevalent in advanced NEC.

*Journal of Perinatology* advance online publication, 29 September 2016; doi:10.1038/jp.2016.165

## INTRODUCTION

Necrotizing enterocolitis (NEC) is a devastating inflammatory disorder found mostly in preterm infants. NEC characteristically occurs at a postmenstrual age of 30 to 33 weeks.[1] NEC is characterized by inflammation and necrosis of intestinal tissue. Mortality rates are high (20 to 30%), and approach 50% in infants who require surgery.[2,3] The underlying pathophysiology of NEC development and progression is still poorly understood.

Amiss bacterial colonization is one of the factors involved in the development of NEC.[2,3] However, the details of this association between NEC development and disease progression remain elusive.[3,4] Previous studies have suggested associations between the presence of microorganisms (such as *Clostridium* spp. (including *C. perfringens*, *C. neonatale*, *C. butyricum* and *C. parputrificum*)) and NEC development.[4–8] In a prospective study in our center we observed a link between colonization with *C. perfringens* and/or *Bacteroides dorei* and NEC development.[9]

The role of bacterial colonization during NEC progression has not yet been fully elucidated. Through an increase in intestinal wall permeability, bacteria may invade the intestine and further aggravate inflammation. In almost all previous studies searching for bacterial colonization processes in NEC, fecal samples are used as a surrogate for intestinal tissue. Analysis of fecal samples may not be representative because it does not give adequate information on the intestinal microbiota exclusively on the affected site. Few studies focused on the possibility to examine the microbiota at the affected intestinal site, that is, the intestinal wall. There are no data regarding the different bacterial composition on highly affected versus little affected intestinal sites.

We aimed to investigate the presence of bacterial invasion together with the identification of bacterial species within the intestinal lumen adhering to and invading into the intestinal wall in surgical NEC specimens compared with controls. Using fluorescent *in situ* hybridization (FISH)[10] we assessed the location and abundances of the bacteria within the intestine (for example, in the lumen, adhering to the mucosa or invading the intestinal wall) and identified specific bacterial species. We hypothesized that we would observe bacteria invading the intestinal wall more often in more severe NEC cases. In addition, we hypothesized to observe bacteria commonly associated with NEC (for example, bacteroides spp., *Clostridium* spp. and *Enterobacteriaceae*) in the severe NEC cases.

## MATERIALS AND METHODS

### Patients

We performed this retrospective study in a tertiary referral neonatal intensive care unit center. The institutional review board of the University Medical Center Groningen approved the study.

We included NEC patients who received surgery between July 2003 and December 2013. Only infants with proven NEC (Bell's stage $\geqslant$ 2) who needed surgery were included in the present study. Indications for surgery were bowel perforation (NEC 3b) or lack of improvement despite optimal conservative therapy. The decision to proceed to surgery was always a multidisciplinary decision by the neonatology and pediatric surgery team caring for the infant in combination with elaborate counseling of the parents.

Control patients included preterm infants who died because of cardio-respiratory pathology, neurological pathology and/or genetic disorders and underwent an autopsy procedure (Table 2). Control patients did not

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Received 27 February 2016; revised 11 August 2016; accepted 22 August 2016

**Table 1.** Probes used to analyze the bacterial identity and density in the intestinal sections

| *Target* | *Probe* | *Label* | *Sequence* | *Reference* |
|---|---|---|---|---|
| All bacteria | EUB338 | Cy3 | 5′-GCTGCCTCCCGTAGGAGT-3′ | Amann *et al.*[29] |
| Bacteroides/*Prevotella*[a] | Bac303 | FITC | 5′-CCAATGTGGGGGACCTT-3′ | Manz *et al.*[30] |
| *Clostridium* clusters I and II (*C. perfringens and relatives*)[a] | Chis0150 | FITC | 5′-TTATGCGGTATTAATCT(T/C)CCTTT-3′ | Franks *et al.*[31] |
| *Enterobacteriaceae*[a] | EC1531 | Cy3 | 5′-CACCGTAGTGCCTCGTCATCA-3′ | Poulsen *et al.*[32] |
| Enterococci/Lactobacilli[b] | Lab158 | FITC | 5′-GGTATTAGCA(C/T)CTGTTTCCA-3′ | Harmsen *et al.* [33] |

Abbreviation: FITC, fluorescein isothiocyanate. [a]Bacterial populations commonly associated with necrotizing enterocolitis (NEC). [b]Bacterial populations commonly observed in 'healthy' infants.

suffer from gastrointestinal diseases. We matched controls with NEC patients with regard to gestational age (GA), birth weight and postmenstrual age (ratio 1:1). We accepted a difference in postmenstrual age of maximal ± 4 days. Demographic data concerning sex, GA, birth weight, mode of delivery, antibiotic therapy, neonatal feeding regime and occurrence of positive blood cultures were obtained. The neonatal feeding regime included: start of enteral nutrition at 3 to 6 h postpartum, feeding with own mother's milk and/or feeding with formula feeding. Feeding was started on day 1 with 10 to 15 ml $kg^{-1}$ in 12 times per 24 h (body weight $<1200$ g) or 8 times a day (body weight $>1200$ g). Feeding was increased—when possible—with a maximum of 20 ml $kg^{-1}$ a day. Preferably own mother's milk was given, stimulating the mothers to express milk starting from birth. If own mother's milk was not available, preterm formula was given. Addition of breast milk fortifier was started after 100 ml $kg^{-1}$ breast milk, but from at least 1 week postmenstrual age onward.

### Resection and autopsy specimens

In NEC cases, the pathologist routinely examined the resection specimens and provided the confirmation of the diagnosis. Characteristic macroscopic findings in NEC include a distended bowel, hemorrhage, pneumatosis intestinalis and/or necrosis. Characteristic histological findings in NEC were mucosal edema, hemorrhage, inflammation, transmural bland necrosis, bacterial infiltration and/or collections of gas. Representative sections of normal and diseased bowel were resected and embedded in paraffin. Tissue sections of 4 μm were stained with hematoxylin and eosin using a standard staining protocol.

We used a previously established histological scoring system for NEC.[11] All samples were assigned a histological NEC severity score (0 to 4) based on the degree of epithelial and/or mucosal damage. When pneumatosis intestinalis and/or necrosis were present, scores of 3 to 4 were given. Both observers (FHH/AT) were blinded to the preliminary Bell's stages. We elected two sections of each specimen for further analysis based on the histological scoring: the section with the lowest score ($\leqslant 2$; least affected tissue) and the section with the highest score ($\geqslant 3$; most affected tissue).

In the controls intestinal resection was performed following the general autopsy procedure consisting of macroscopy and microscopy. After general macroscopic pathological examination the intestinal specimens were directly fixated with 10% buffered formalin solution and imbedded in paraffin using standard pathology procedures. Tissue sections of 4 μm were stained with hematoxylin and eosin using the standard staining protocol. As controls for the present study we only included patients in whom the intestinal tissue was considered as nonpathological.

### FISH analysis

We used FISH to analyze the location and abundances of bacteria and identify specific bacterial species in the intestinal tissue sections. The sections were deparaffinated by immersion for 2 times in xylol for 2 min after which the xylol was washed away by immersion for 10 min in 96% ethanol. Deparaffinated slides were dried at ambient air. Bacterial density in the samples was first characterized with a universal bacterial oligonucleotide targeting rRNA probe. We subsequently used the probes as described in Table 1 to detect bacterial populations commonly associated with NEC development.[3,4,8,10] All oligonucleotide probes were obtained from Eurogentec (Seraing, Belgium). For this the slides were hybridized at 50 °C overnight in hybridization buffer containing 5 ng $\mu l^{-1}$ fluorescently labeled probes. Slides were washed at 50 °C in hybridization buffer without SDS and mounded with Vectashield (Vector Laboratories, Burlingame, CA, USA) and a coverslip. We examined the slides using the Leica fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

### Analysis

We investigated the presence of bacterial invasion together with the identification of bacterial species within the intestinal lumen adhering to and invading into intestinal wall in surgical NEC specimens compared with controls using FISH. For analysis we used the most affected section and the least affected section of the NEC samples. We used the FISH score to determine the presence of bacterial invasion, adapted from Cilieborg *et al.*:[10] 1 = no/few colonies, 2 = average colonies in the intestinal lumen, 3 = abundant colonies adhering to the mucosa and 4 = abundant colonies invading the bowel wall. We subsequently investigated the density of the separate bacterial species (see Table 1). For both we used the following scoring system: 0 = not present, 1 = low density, 2 = moderate density and 3 = high density. During scoring, the assessor was blinded for two categorical groups (NEC versus controls). All scoring was performed within 10 crypts per tissue section (thus 2 scores per NEC case and 1 score for controls).

The $\chi 2$ test or Fisher's exact analysis was used for testing differences between categorical variables. For testing differences between two continuous variables Spearman's rho test was used, and for testing differences among three groups analysis of variance test was used. To assess differences between the combination of a categorical and a continuous variable, Mann–Whitney *U* or Kruskal–Wallis test was used. Statistical analyses were performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics 21, IBM, Armonk, NY, USA). All data are presented as median values with range, unless specified otherwise. Two-sided *P*-values of $<0.05$ were considered statistically significant.

## RESULTS

### Patients

We included 43 surgical NEC specimens and 43 controls. In the controls no evidence of intestinal disease was present (Table 2). Age at time of surgery/autopsy was 11 days (4 to 37) for NEC patients and 8 days (2 to 37) for the controls. Patient characteristics are presented in Table 2.

### Bacterial invasion

First we used the universal bacterial FISH probe. In 36/43 (84%) of the most affected NEC tissue samples, we observed bacteria adhering to the mucosa or invading the intestinal wall (FISH scores 3 and 4) compared with 30/43 (70%) in the least affected NEC tissue samples ($P=0.04$). In control cases we observed FISH scores 3 and 4 in 8/43 (19%) of the cases (both $P<0.001$ compared with the NEC cases). In all, 22/43 (51%) of the most affected NEC tissue samples scored the maximum FISH score of 4 compared with 16/43 (37%) of the least affected tissue samples ($P=0.03$). A maximum FISH score occurred in 7/43 (16%) of the controls (both $P<0.001$).

### Bacterial species

In Figure 1 we present the different bacterial species observed. *Enterobacteriaceae* were most commonly observed. We observed *Enterobacteriaceae* in most affected NEC tissue samples in 33/43 (77%) cases, in least affected NEC tissue samples in 32/43 (74%) cases and in controls in 20/43 (26%) cases. We observed high densities (density score 3) of *Enterobacteriaceae* (Figure 1)

**Table 2.** Patient characteristics

| *Patient characteristics* | *NEC*, n = *43* | *Controls*, n = *43* | P-*value* |
|---|---|---|---|
| Sex (male) | 25 (58%) | 27 (63%) | 0.66 |
| Gestational age, days | 28 (24–39) | 29 (24–39) | 0.41 |
| Birth weight, g | 1142 (650–2650) | 1235 (560–2830) | 0.52 |
| Cesarean section | 16 (37%) | 20 (47%) | 0.27 |
| Antibiotic therapy postpartum | 31 (72%) | 37 (86%) | 0.10 |
| Antibiotic therapy >48 h postpartum | 43 (100%) | 38 (89%) | 0.03[a] |
| Enteral feeding postpartum days | 0 (0–3) | 1 (0–3) | 0.25 |
| *Sort of feeding* | | | |
| Exclusively mother's milk[b] | 2 (4.6%) | 2 (4.6%) | 0.36 |
| Exclusively formula | 19 (44%) | 23 (53%) | 0.76 |
| Both | 22 (51%) | 18 (42%) | 0.43 |
| Accomplishment of full enteral feeding before intestinal resection | 23/38 (61%) | 6/34 (18%) | 0.013[a] |
| Days NPO before intestinal resection | 2 (0–12) | 1 (0–7) | 0.08 |
| Age at time of collection of intestinal resection material | 11 (4–37) | 8 (2–37) | 0.966 |
| NEC Bell's stage 3b | 33 (77%) | — | — |
| Intestinal perforation | 26 (60%) | — | — |
| *Removed tissue* | | | |
| Small intestine | 15 (35%) | 15 (35%) | 0.91 |
| Small intestine and large intestine | 22 (51%) | 22 (51%) | 0.91 |
| Large intestine | 6 (14%) | 6 (14%) | 0.91 |
| *Positive blood cultures 48 h before and after date of intestinal resection* | 3/37 (8.1%) | 8/43 (19%) | 0.11 |
| *Enterobacterieceae* | 0 | 1 (2.3%) | |
| *Staphylococci* | 2 (5.4%) | 5 (12%) | |
| *Clostridium* spp. | 0 | 0 | |
| Other | 1 (2.7%) | 2 (4.7%) | |
| *Positive peritoneal cultures* | 18/33 (55%) | — | — |
| *Enterobacterieceae* | 11/18 (61%) | | |
| Staphylococci | 3/18 (17%) | | |
| *Clostridium* spp. | 1/18 (5%) | | |
| Other | 3/18 (17%) | | |
| NICU stay, days (range) | 31 (4–153) | — | — |
| Mortality during the acute phase of NEC | 12 (28%) | — | — |
| Overall mortality | 16 (37%) | 43 (100%) | — |
| *Cause of death* | | | |
| NEC | 43 | 0 | |
| (Intra)cranial bleeding | | 6 | |
| Respiratory insufficiency | | 11 | |
| Circulatory insufficiency | | 3 | |
| Discontinuation of treatment because of complex congenital disorders | | 6 | |
| Lung hypoplasia | | 6 | |
| Meningitis | | 2 | |
| Other | | 9 | |

Abbreviations: NEC, necrotizing enterocolitis; NICU, neonatal intensive care unit; NPO, nil per os. Values are expressed as median (range) if applicable. [a]Statistically significant $P < 0.05$. [b]In this study cohort no patient received donor milk.

in the most and least affected NEC tissue samples (24/33 (73%) and 18/32 (56%) respectively) that were both significantly higher compared with the control tissue samples (7/20 (35%); both $P < 0.001$). A density score of 3 of *Enterobacteriaceae* together with a FISH score of 4 in the most and least affected NEC tissue samples was reached in 16/24 (67%) and 7/18 (39%) cases, respectively. We detected *Clostridium* spp. and enterococci/lactobacilli in 5/43 (12%) respectively 2/43 (4.7%) of the NEC samples. Both were detected only in low numbers (density score 1) and only in the presence of *Enterobacteriaceae*. *Clostridium* spp. and enterococci/lactobacilli were not observed in controls. Bacteroides were never detected, neither in NEC samples nor in controls.

## Relation between clinical variables and bacterial colonization

No relation between gestational groups (groups: extremely preterm <28 weeks, very preterm 28 to <32 weeks and moderate-to-late preterm 32 to <37 weeks) and bacterial density were observed ($P = 0.57$), nor for these gestational groups and abundance of *Enterobacteriaceae* ($P = 0.77$).

Bacterial density was not related with severity of disease (expressed in Bell's stage; $P = 0.92$), postmenstrual age ($P = 0.40$) and acute-phase morality (mortality within 30 days; $P = 0.09$). We did not observe a relation between GA and the accomplishment of full enteral feeding before death ($P = 0.82$). Accomplishment of full enteral feeding before NEC was not related with bacterial density, nor with high abundances of *Enterobacteriaceae* ($P = 0.39$ and $P = 0.75$). However, in control samples, increased bacterial density was associated with infants who accomplished full enteral feeding before they died ($P = 0.01$). In specimens of patients who received exclusively formula feeding before NEC ($n = 19$), we observed a higher density of bacteria in the most affected tissue compared with infants who were not exclusively fed with formula feeding ($P = 0.03$) and in the least affected tissue ($P = 0.04$). Conversely, the density of bacteria in the least affected NEC

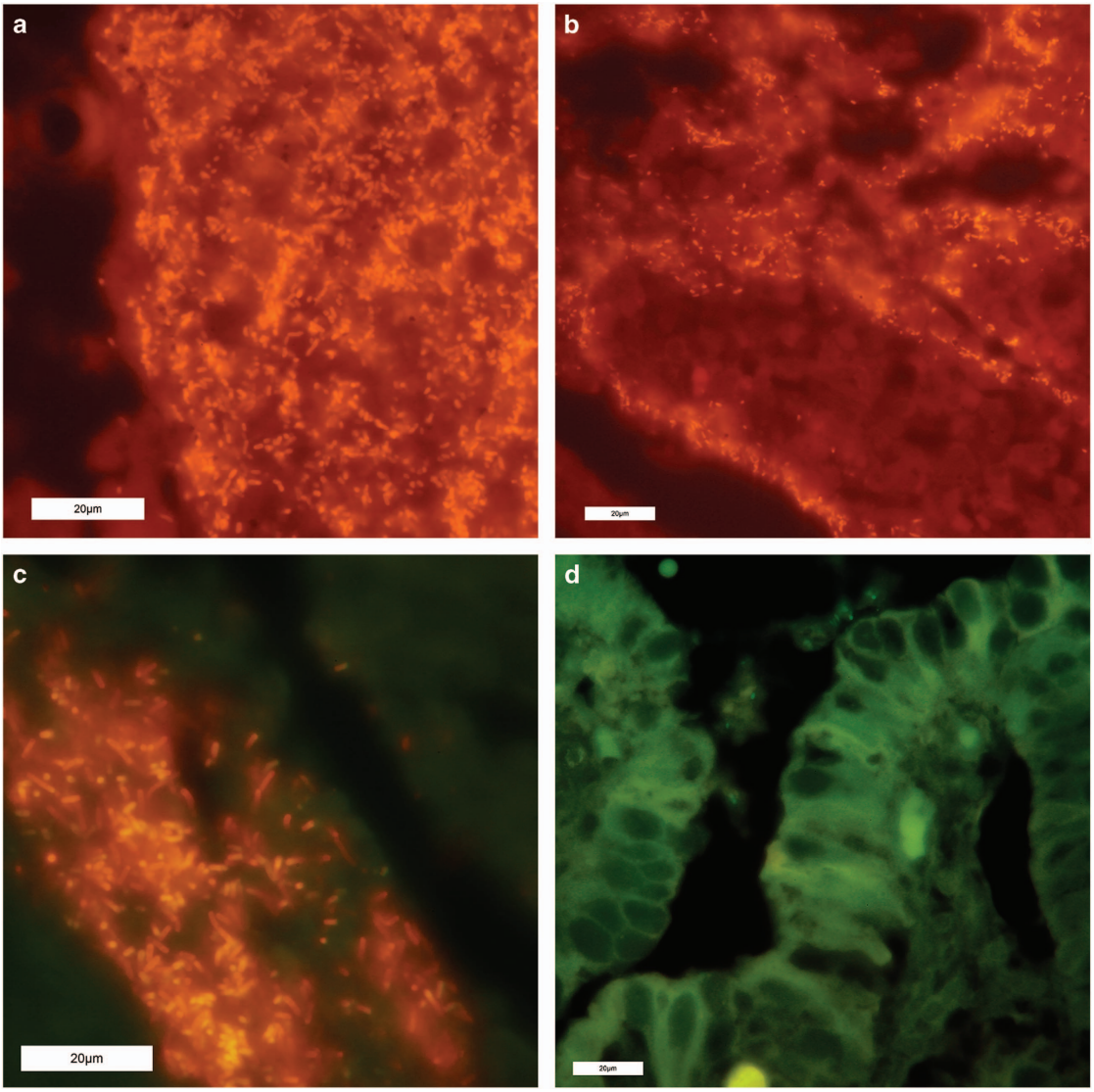


**Figure 1.** Epifluorescence micrographs of fluorescent *in situ* (FISH) hybridized tissue samples. (**a**) Bacteria invading the bowel wall (FISH score 4) in a necrotizing enterocolitis (NEC) sample hybridized (general bacterial probe (EUB338) tagged with Cy3 (red color)). (**b**) *Enterobacteriaeceae* invading the bowel wall (FISH score 4) in a NEC sample (*Enterobacteriaeceae* probe tagged with Cy3). (**c**) Clostridium spp. (yellow) adhering to the bowel wall (FISH score 3) in a NEC sample (merging of EUB338 probe (tagged with Cy3) and clostridium spp. probe (tagged with fluorescein isothiocyanate (FITC), green color)). (**d**) Sample with no bacterial colonization (FISH score 1) in the control specimens in the intestinal lumen (EUB338 tagged with FITC). Magnification ×630 to ×1000. The scale bars represent 20 µm in all the micrographs.

specimens was lower in patients who received exclusively their own mother's milk before NEC ($n = 2$; $P = 0.01$). The duration of nil per os (NPO) also influenced bacterial colonization in the intestinal wall. The duration of NPO was associated with lower bacterial densities in the NEC specimens ($P = 0.04$). The duration of NPO was related with higher densities of *Enterobacteriaceae* ($P = 0.03$). In control samples, no relation between duration of NPO and bacterial colonization was observed.

## DISCUSSION

This study investigated the presence of bacterial invasion together with the identification of bacterial species within the intestinal lumen adhering to and invading into the intestinal wall in surgical NEC specimens compared with controls. We hypothesized that we would observe bacteria within the intestinal wall more often in more severe NEC cases. Indeed, bacterial invasion in the intestinal wall (FISH score 4) occurred significantly more often in highly affected NEC tissue samples (51%), compared with least affected NEC tissue samples (37%) or controls (16%). This observation suggests that bacterial invasion is associated with the degree of intestinal wall injury. Second, in the NEC cases we observed high densities of *Enterobacteriaceae* suggesting an important role for *Enterobacteriaceae* in the progression of disease.

Higher densities of bacteria were observed in the most affected NEC tissue samples. Our results are in line with three other studies, namely the studies of Remon *et al.*,[12] Brower-Sinning *et al.*[13] and Smith *et al.*[14] These three studies also observed proteobacteria (to which *Enterobacteriaceae* belong) in mucosal tissue in NEC specimens. However, these studies were limited by sample size. Our study adds that we differentiated between highly affected NEC tissue samples and least affected NEC tissue samples, and thereby included control samples of patients without (proven) intestinal abnormalities.

Although we observed significantly more bacterial invasion in highly affected NEC tissue samples (51% of the cases) compared

with least affected, bacterial invasion was observed (37% of the cases) in still a noteworthy percentage of the latter. This observation suggests that bacterial invasion might be more prevalent during the more advanced stages, but can also be present in less affected tissue. We also detected, surprisingly, bacterial invasion into the intestinal wall in 16% of the control specimens. This finding could be due to the process of dying because of underlying cardiorespiratory, neurological and/or genetic disorders, even in the absence of pathological findings of the gastrointestinal tract. We do not believe that bacterial colonization within the intestinal wall occurs in the 'healthy' preterm infant, although literature on this issue is nonexistent. Therefore, with our findings it is plausible that bacterial invasion in the intestinal wall can be considered as a complication of the vulnerable preterm intestine, even in infants without evident gastrointestinal abnormalities. Whether this has any pathophysiological consequences is as yet unknown.

In a previous cohort from our center we identified *Clostridium* spp. and/or bacteroides to be associated with NEC development.[9] We were surprised that we did not observe *Clostridium* spp. and/or bacteroides in this cohort of patients conducted in the same center. Although *Enterobacteriaceae* have previously been associated with the pathophysiology of NEC before, we observed in the previous conducted cohort from our center no difference between abundances of *Enterobacteriaceae* in NEC cases versus controls.[9,15] McMurtry *et al.*[5] reported that abundance of clostridia decreased as the severity of NEC increased. This is in line with the present series, all consisting of severe, that is, surgical NEC cases. A possible explanation for this observation is the following. *Clostridium* spp. (including *C. perfringens*) might decrease gut wall integrity via the production of α-toxins. This in turn could lead to the inflammation leading to NEC.[9,16–18] As a result of the inflammation, oxidative stress is induced by reactive oxygen species, resulting in an environment in which *Clostridium* spp. can hardly survive.[16–18] *Enterobacteriaceae* include versatile species that derive energy for growth from aerobic or anaerobic nitrate respiration or from fermentation.[19,20] *Enterobacteriaceae* are therefore resistant against nutrient variation as well as oxidative stress and can survive in a highly inflamed and necrotic intestine that is seen during NEC.[21] This phenomenon is also described in the pathogenesis of Crohn's disease, where activated neutrophils infiltrate the intestinal wall and produce reactive oxygen species, leading to oxidative stress in which *Enterobacteriaceae* survive.[5,11,22] We speculate that *Enterobacteriaceae* invade the bowel wall during NEC development and further aggravate the already present inflammation. The reason that we did not observe lactobacilli is probably because lactobacilli have only been identified in samples from children with a GA of $>33$ weeks and our cohort was significantly younger than that.[23,24]

Although we observed high abundances of *Enterobacteriaceae* in the NEC samples, a fair percentage of the controls were also colonized with high densities of *Enterobacteriaceae* (35% with density score 3). We speculate that intensive antibiotic treatment in preterm infants might explain the abundances of *Enterobacteriaceae* in both groups. Tanaka *et al.*[25] demonstrated that antibiotics do not clear the intestinal microbiota in infants but reduce the overall diversity of bacterial species. A more intensive antibiotic use reduces the diversity of bacterial species and increase the domination of *Enterobacteriaceae*.[5,26] In a study previously conducted at our center,[9] we also observed high abundances of *Enterobacteriaceae* in fecal samples during the first days after birth in both preterm infants who developed NEC and controls.

The results of the current study emphasizes again that both early enteral nutrition after birth and own mother's milk are important in protecting the intestine against (harmful) bacterial colonization and secondarily against development of NEC. However, the actual numbers of patients who received exclusively mother's milk were small. The duration of NPO was negatively related with the bacterial density and with abundances of *Enterobacteriaceae* in NEC patients, whereas in control samples we did not observe this pattern. This finding suggests that NPO in patients with NEC is an important tool to limit progression of potentially harmful bacterial colonization in the intestinal wall. Although this study was a retrospective cohort study and causality is far from proven, further research should investigate the relation between the neonatal feeding regime and bacterial colonization within the intestinal wall.

Notably, we observed that timing of NEC in our cohort seemed to occur earlier compared with other cohorts, such as the MEDNAX cohort.[27] This is an important and interesting difference between the performed studies in two different countries. Earlier timing because of contamination with spontaneous intestinal perforation cases were ruled out by an experienced pathologist. In addition, we cannot prove the hypothesis that surgical NEC occurs earlier compared with medical NEC. In a previous study performed by Schat *et al.*,[28] we included medical and surgical patients, without significant differences in timing of NEC between the medical and surgical NEC patients. Although we acknowledge that there may be different entities between medical and surgical NEC patients, we cannot explain the difference in timing of NEC with the current data available in our center. A possible contributing factor could be the different practice of care of the preterm neonate.

Most studies focusing on bacterial colonization during NEC are performed with the use of fecal samples and/or animal experiments. Only three studies analyzed bacterial colonization within the intestine or the intestinal wall.[12–14] In the current study we differentiated between highly affected and least affected tissue of the NEC specimens to be able to study bacterial invasion within the extent of mucosal damage within the same child. We also included a control group matched to GA, birth weight and postmenstrual age. It would be helpful to investigate the bacterial population present in these tissue samples in more detail. Unfortunately, the formalin-fixed, paraffin-embedded NEC tissues did not allow us to perform 16S sRNA sequencing reliably. Therefore, we were able to assess only a limited selection of bacterial populations. Although *Enterobacteriaceae* dominated the specimens, it is possible that we missed other bacterial families that were not assessed in the study. Of note, the retrospective nature of this study, in which the intestinal resection material was exposed to oxygen before fixation and was preserved before usage for study purposes, could have influenced our results. Finally, this study was performed within a tertiary referral center, meaning that both inborn and outborn patients were included in our cohort. Although it is known that different centers have different common flora within their neonatal intensive care unit, we did not include this as a parameter because of small numbers of outborn infants, and this can be seen as a limitation of this study. Besides data regarding accomplishment of full enteral feeding and type of feeding, we did not include the detailed data regarding the exact amount of feeding per kg per day for each individual infant. Thereby, for outborn infants the detailed information regarding time of full enteral feeding was not always available.

In conclusion, we observed bacterial invasion into the intestinal wall in the majority of the most affected NEC tissue samples. Bacterial invasion also occurred in the least affected NEC tissue samples and, to a lesser extent, in the control samples. We observed high densities of *Enterobacteriaceae*. Although other pathogenic bacteria (such as *C. perfringens* and *B. dorei*) might also be associated with NEC development, *Enterobacteriaceae* might have an important role in disease deterioration by invading the intestinal wall and aggravating the inflammation. Further research should investigate the exact relation between invasion with *Enterobacteriaceae* and NEC progression. When indeed invasion

with *Enterobacteriaceae* is linked with NEC progression, there might be an important role for targeted antibiotics during NEC.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

This study was part of the research program of the Graduate School of Medical Sciences, Research Institute GUIDE, University of Groningen. FH Heida was financially supported by a grant from the Junior Scientific Master Class of University of Groningen.

## AUTHOR CONTRIBUTIONS

FH Heida conceptualized and designed the study, performed the study, drafted the initial manuscript and approved the final manuscript as submitted; HJM Harmsen assisted and supervised the study, reviewed and revised the manuscript and approved the final manuscript as submitted; A Timmer, EMW Kooi and AF Bos reviewed and revised the manuscript and approved the final manuscript as submitted; JBF Hulscher supervised the study, reviewed and revised the manuscript and approved the final manuscript as submitted.

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