

Paneth cells in the developing gut: when do they arise and when are they immune competent?

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BACKGROUND: Little is known about the perinatal development of Paneth cells (PCs) during gestation and the relation with necrotizing enterocolitis (NEC). We aimed to investigate when PCs arise and when they become immune competent during gestation.

METHODS: We included 57 samples of ileum tissue of fetuses/infants with a gestational age (GA) between 9 and 40 wk taken as part of a standard autopsy procedure. Hematoxylin-eosin staining and anti-human defensin 5 immunohistochemistry were performed. We performed a semi-quantitative assessment of (immune-competent) PC numbers per 10 crypts per tissue section per GA.

RESULTS: The number of PCs and the number of immune-competent PCs increased with increasing GA (Spearman's $\rho = 0.41$, $P = 0.002$ and $\rho = 0.61$, $P < 0.001$, respectively). Whereas significantly higher PC numbers were observed after 37 wk gestation (median 7, range 0–12) compared to preterm infants (median 0, range 0–15; $P = 0.002$), we counted higher numbers of immune-competent PCs already in infants with GA above 29 wk (median 6, range 0–18) compared to infants with GA under 29 wk (median 2, range 0–9; $P < 0.001$).

CONCLUSION: The significant increase of immune-competent PCs starting from a GA of 29 wk mimics the rise in incidence of NEC during a similar postmenstrual age in preterm infants.

Paneth cells (PCs) are specialized epithelial immune cells located in the base of the crypts of Lieberkühn located in the small intestine (1,2). PCs can be seen in the gut by the first trimester and are thought to mature during gestation (1,2). PCs protect the intestinal stem cells from pathogens by stimulating stem cell differentiation, shaping the intestinal microbiota, and assist in reparation of the gut (2). To this end, they secrete (among other things) human α -defensins (HD5/HD6) (3). These defensins protect the intestinal mucosa against bacterial invasion, and are thought to be associated with initiating and adapting immunity (3). How PCs develop after the first trimester and when PCs become immune competent remains uncertain.

PCs are thought to play a role in the development of NEC, a common and devastating disease most commonly observed in preterm infants (4–6). NEC involves the preterm intestine and has a complex pathophysiology in which bacterial invasion and an excessive inflammatory response is an important contributing factor (5,6). Unfortunately, the exact pathophysiology is incompletely understood.

It has been hypothesized that maturation of immune-competent PCs is crucial for NEC development (4,6–8). In the preterm gut, PCs secrete defensins that might contribute to an excessive inflammatory response as observed in NEC (4). This hypothesis is derived from the observation that extremely-low-birth-weight human infants generally have the longest interval before the onset of NEC—with a peak incidence at the postmenstrual age of 29–33 wk (4,6–8). Despite the possible role for PC-maturation in the pathophysiology of NEC, little is known about PC-maturation and functioning in the immature intestine. Therefore, we aimed to investigate when PCs arise and when they become immune competent in human tissue during gestation.

RESULTS

Patients

We included ileum tissue from 57 fetuses/infants born between June 2003 and June 2014. The fetuses/infants were divided into eight groups based upon gestational age (GA) in weeks (1: 9–12, 2: 13–16, 3: 17–20, 4: 21–24, 5: 25–28, 6: 29–32, 7: 33–36, 8: 37–40). Eight fetuses/infants were included per group, except for groups 1 ($n = 2$) and 5 ($n = 7$). Major causes for death were intra-uterine fetal death ($n = 17$; 30%) and clinically indicated termination of pregnancy for fetal or maternal disease ($n = 16$; 28%). Six infants died 2 d postpartum. We present the patient characteristics in **Table 1** and in the **Supplementary Table S1** online.

Histological PC Count

Figures 1 and **2** and **Table 2** show (total numbers of) PCs that stained positively with Hematoxylin-eosin (HE) staining. Intraobserver variability for the PC count was excellent (intra-class correlation coefficients (ICC) 0.95 (95% CI: 0.91–0.97)). GA correlated positively and strongly with the number of PCs

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($\rho = 0.41$, $P = 0.002$). We observed a significant difference between the eight groups in terms of PC numbers, using the Kruskal-Wallis test ($P = 0.02$). Significantly higher numbers of PCs were counted in infants with a GA above 37 wk (median 7, range 0–12) compared to all preterm infants (median 0, range 0–15; $P = 0.002$). No significant differences in total PC counts were found between the groups before the GA of 37 wk (values are depicted in **Table 2**). **Supplementary Figure S1** online and **Supplementary Table S1** online present detailed patient characteristics and PC count.

Table 1. Patient characteristics

Patients	57
Gestational age in weeks, median (range)	26 (9–40)
Sex (male), <i>n</i> (%)	32 (56)
Birth weight (grams), median (range)	627 (11–4756)
Survival period, <i>n</i> (%)	
0 d	37 (65)
1 d	14 (25)
2 d	6 (10)
Cause of death, <i>n</i> (%)	
Intrauterine fetal death	17 (30)
Clinically indicated termination	16 (28)
Respiratory/circulatory insufficiency	11 (19)
Live birth, other causes of death	13 (23)

Data are expressed as median (range) or as numbers unless specified otherwise.

Anti-HD 5 Immunohistochemistry PC Count

Figures 1 and **2** and **Table 2** show (total numbers of) immune-competent PCs. Intraobserver variability for the PC count was excellent (ICC 0.98 (95% CI: 0.98–0.99)). GA correlated positively and strongly with the number of immune-competent PCs ($\rho = 0.61$, $P < 0.001$). Kruskal-Wallis test demonstrated a significant difference between the eight groups in terms of immune-competent PC numbers ($P = 0.001$). In infants with a GA of 29 wk or greater had significantly more immune-competent PCs (median 6, range 0–18) when compared to infants with a GA of less than 29 wk (median 2, range 0–9; $P < 0.001$). When infants became term (GA of 37 wk) even higher numbers of immune-competent PCs (median 10, range 3–18) were observed when compared to preterm infants (GA <37 wk; median 3; range 0–17; $P = 0.003$). **Supplementary Figure S1** online and **Supplementary Table S1** online present detailed patient characteristics and PC count.

DISCUSSION

Although a role for PCs in the pathophysiology of NEC has been suggested, little is known about the PC number and functioning in the immature human gut and its relation with the development of NEC. The present study suggests that the number of PCs increases rapidly after a GA of 37 wk. However, starting from 29 wk of gestation, we observed a rapid increase in immune-competent PCs as demonstrated by the expression of defensin HD5. This corresponds with the peak incidence of NEC occurring at a postmenstrual age of 29–33 wk. When

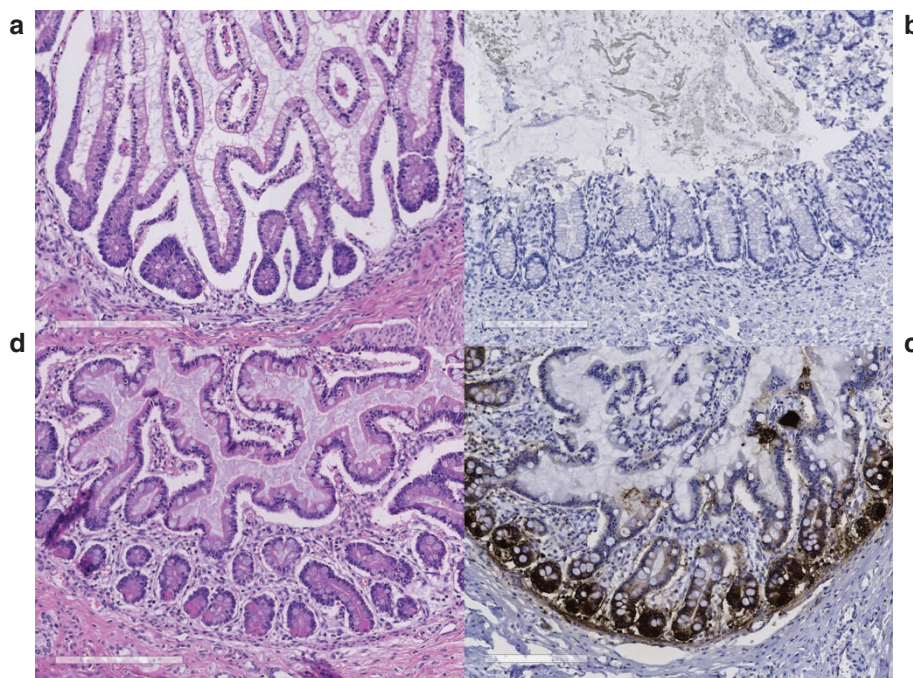


Figure 1. Paneth cells (PCs) hematoxylin-eosin (HE) staining and anti-HD 5 immunohistochemistry. PCs on HE staining are characterized by their rose color, eosinophilic granules, incident light fluorescence and their location in the crypts. Immune-competent PCs on anti-HD5 staining are characterized by the brown color and their location in the crypts. **a** and **b** show tissue of a patient with a gestational age (GA) of 20 wk. No PCs or immune-competent PCs are observed. **c** and **d** show tissue of a patient with a GA of 37 wk. Multiple PCs are observed (the arrow indicates a (immune-competent) PC). Magnification of 400 \times , scale bar represents 200 μ m.

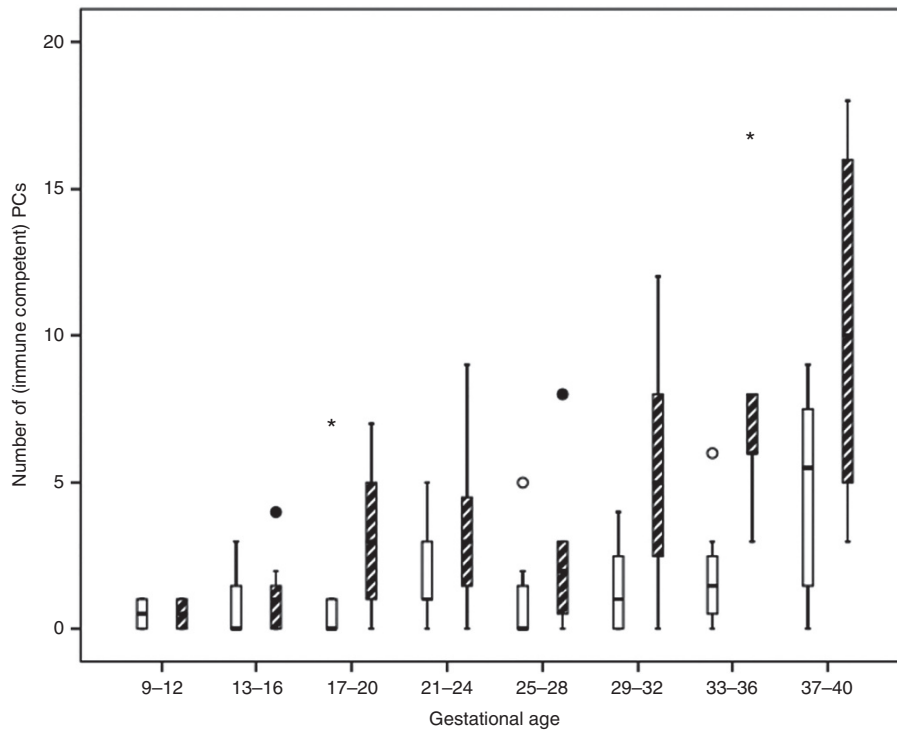


Figure 2. Boxplot of numbers of (immune-competent) Paneth cells (PCs). Y-axis represents number of (immune-competent) PCs per crypt, whereas □ represents numbers of PCs on hematoxylin-eosin staining, and ▨ represents numbers of (immune-competent) PCs on anti-HD5 staining. Numbers of PCs were scored per 10 crypts per tissue section in random order. The bars represent interquartile range (Q3-Q1); the whiskers represent the ranges; and the stars, open/closed circles represent all outliers.

Table 2. Differences in (immune-competent) PC count per GA

	GA 9-12	GA 13-16	GA 17-20	GA 21-24	GA 25-28	GA 29-32	GA 33-36	GA 37-40
PC numbers hematoxylin-eosin staining	0 (0-0)	0 (0-2)	0.5 (0-15)	0 (0-3)	0 (0-0)	2 (0-6)	2 (0-6)	6.5 (0-12)
Immune-competent PC numbers (HD5 expression)	0 (0-1)	1 (0-4)	3 (0-7)	3 (0-9)	2 (0-8)	5 (0-12)	6 (3-17)	10 (3-18)
GA 13-16	0.64**		0.10*	0.49*	0.39*	0.07*	0.16*	0.008*
GA 17-20	0.19**	0.24**		0.28*	0.05*	0.65*	0.80*	0.16*
GA 21-24	0.11**	0.07**	0.92**		0.20*	0.47*	0.41*	0.02*
GA 25-28	0.29**	0.36**	0.52**	0.38**		0.20*	0.47*	0.41*
GA 29-32	0.09**	0.02**	0.29**	0.22**	0.12**		0.57*	0.09*
GA 33-36	0.03**	0.002**	0.02**	0.02**	0.013**	0.18**		0.05*
GA 37-40	0.04**	0.002**	0.02**	0.011**	0.007**	0.09**	0.71**	

Data are expressed as median (range) or as numbers unless specified otherwise. Comparison between PC counts per GA group was performed using the Kruskal-Wallis test. The Mann-Whitney U-test was used to assess differences between (each) GA group(s) in case of a significant difference found using the Kruskal-Wallis test. Significance is shown by boldfaced values.

*P values represent GA vs. PC numbers. **P values represent GA vs. immune-competent PC numbers. GA, gestational age; PC, Paneth cells.

taken together, these observations suggest a putative role for immune-competent PCs in the pathophysiology of NEC.

Most insights on PC development and functioning are from murine studies. Their epithelium is immature at birth and undergoes extensive postnatal remodeling and crypt ontogeny after birth (9). These animal studies are not directly comparable with human tissue because human PCs are thought to develop during the first trimester of gestation (9). According to current laboratory studies using gene expression—and intestinal isografts, PCs develop during the first trimester of

gestation and increase in numbers by term gestation (1,9,10). The present study could not confirm the development of PCs during the first trimester, which is possibly the result of the only moderate sensitivity of HE staining (11). However, we did observe significantly higher numbers of PCs at term gestation.

It has been suggested that PCs start producing defensins, including HD5 around a GA of 13 wk (4). Both PC numbers and defensins expression are hypothesized to be lower in pre-term infants with a GA of 24 wk compared with term infants (1,12). According to our current understanding, the appearance

of HD5 coincides approximately with PC differentiation during intestinal crypt ontogeny (9,12). The present study suggests that HD5 expression coincides with PC development until a GA of 28 wk; in the period starting from 29 wk of GA, HD5 expression increases more rapidly than the number of PCs.

PCs with their defensin expression, such as HD5, play a key role in the intestinal innate immunity and development of diseases (13–15). PCs with their defensin expression are previously described in the pathophysiology of Crohn's disease (15). The present study hypothesizes associations between PC defensin expression and NEC. This hypothesis is based on the finding that the increase of HD5 expression by PCs equals the peak incidence of NEC at a postmenstrual age of 29–33 wk, if we assume that, after preterm birth, PCs follow the same developmental path as before birth.

The current hypotheses on the role of PCs in NEC development are based on either depletion of PCs, increased immune activity of PCs or PC dysfunction (6,8,16). In the first hypothesis, it is suggested that there is a relative deficiency of (immune-competent) PCs at a low GA (4,17). This deficiency could lead to a limited protection against opportunistic bacteria involved in NEC development (4,17). Our data is not consistent with this hypothesis, because we observed a significant increase of immune-competent PCs during gestation and not depletion. In the second hypothesis, the secretion of antimicrobial peptides by the PCs might be overactivated in the immature immune system leading to an overwhelming inflammatory response (1,18,19). This exaggerated inflammatory response could lead to increased intestinal damage, bacterial dysbiosis and reduced epithelial repair, which in turn would lead to the development of NEC (1,18,19). With the results of the present study, one could speculate that the rapid increase of defensin expression around a GA of 29 wk, in combination with colonization with opportunistic pathogens and a still premature intestine, triggers NEC development. The last hypothesis assumes a dysfunction of PCs by environmental stressors: dysfunction of PCs may be an early event that predisposes the preterm infant to NEC by inducing bacterial dysbiosis (1,20,21). This hypothesis could, however, not be tested in the present study and should be subject of future research.

We acknowledge several limitations in the present study. First, epithelial differentiation from common progenitor stem cells, including the differentiation into PCs, is controlled by transcription factors, such as Math 1, under the control of the Wnt and Notch signaling pathways starting during crypt morphogenesis within the first trimester of pregnancy (12,22). It is not yet known how this pathway further develops during gestation, e.g., when it reaches its maximum and if it influences HD5 expression of the PCs. We could therefore not relate the influence of the epithelial differentiation pathway to the results of the present study. Future studies should focus on elucidating signaling pathways mediating PC formation—such as the Wnt and Notch signaling pathways—and function in the premature intestine. Second, the population size is small. However, this study is to our knowledge the first study focusing on the emergence and immune competence of PCs per GA performed in human tissue. Third, we only studied anti-HD5, whilst there are more antimicrobial products

secreted by PCs that could be of interest (i.e., HD6, sPLA2, lysozymes, Reg3G) (17). However, of the antimicrobial expression of PCs, HD5 is responsible for the majority of total antimicrobial expression (17). In line with Shen *et al.* (21), the data of the present study indicates that HD5 immunohistochemistry is a more sensitive method for detecting PCs than routine HE staining that could have influenced our results. However, detection of PCs with HE staining gives valuable additional insight into the numbers of PCs, potentially without immune competency. Lastly, we did not use NEC tissue for our analysis, and therefore the exact relation between PCs and NEC development remains speculative. Unfortunately, NEC resection specimens were too damaged for PC analysis according to our methods and, thereby, only give insight at the end stage of the disease rather than in the early development stage of NEC.

A key deficiency in our understanding of PCs is still the mechanisms that underlie their formation during development. We also do not understand the signaling pathways that mediate PC formation and their behaviour during critical events during late fetal and perinatal development (23). Understanding of these pathways together with the knowledge of our study, will shed light on PC development in human intestinal tissue. The considerable increase of antimicrobial expression of the PCs starting from 29 wk GA could lead to an excessive inflammatory response which is seen in NEC.

In conclusion, with the present study, we were able to investigate when PCs arise and when they become immune competent in human tissue during gestation. We observed that while the number of PCs significantly increased when infants became term, the increase of immune-competent PCs increased significantly starting from 29 wk of gestation, which equals the peak incidence of NEC at a postmenstrual age of 29–33 wk. Whether this association between PCs and NEC is a causal one, remains to be seen.

METHODS

Patients

This retrospective study was conducted in a tertiary referral Neonatal Intensive Care Unit center. The archives of the Department of Pathology & Medical Biology were searched for fetal and neonatal ileum tissue taken as part of a standard autopsy procedure. Tissue samples from cases with a GA between 0 and 40 wk were selected. GA between 0 and 40 wk were divided in 4-wk intervals. Upon retrieval from the archive HE-stained sections were reviewed. Only those with an intact mucosa without injury or autolysis were selected for the study. We excluded autopsy material from patients who died 2 d or more postpartum to minimize the possible external influences on PC development. We attempted to include eight fetuses/infants per 4 wk interval of GA. Parental consent was not needed to utilize the tissue samples following the WMO Medical Research Involving Human Subjects Act requirements. This study was approved by the Medical Ethical Committee of the University Medical Center Groningen.

Tissue Modification and Immunohistochemistry

After sampling ileum tissue was fixed in 10% buffered formalin, embedded in paraffin, and 4- μ m-thick serial sections were prepared. Paraffin-embedded sections were stained with HE. Tissue morphology was qualitatively assisted by two trained observers (F.H.H. and A.T.).

To determine PC-specific expression of HD5, we performed immunohistochemistry. Deparaffinized sections (4 μ m) were subjected to heat-induced antigen retrieval by 15 min incubation in 1 mmol/l Ethylenediaminetetraacetic acid buffer (pH 8.0) at 95 °C. Endogenous peroxidase was blocked for 30 min with 0.075% H₂O₂ in

Phosphate-buffered saline (PBS). Primary antibody Cat. No. MABF31, anti-alpha Defensin 5, clone 8C8 (Merck Millipore, Billerica, MA) was diluted 1/100 in 1% Bovine Serum Albumin (BSA)/PBS, were incubated for 60 min at room temperature. Binding was detected using sequential incubations (30 min) with rabbit-anti-mouse peroxidase-labeled secondary antibody (DakoCytomation, Glostrup, Denmark) and goat-anti-rabbit peroxidase-labeled tertiary antibody (DakoCytomation) diluted in PBS with 1% BSA and 1% normal human serum. Peroxidase activity was developed using 3,3'-diaminobenzidine tetrachloride for 10 min. Sections were counterstained with hematoxylin. Evaluation of a positive control of immunohistochemistry was done in normal human transverse colon tissue.

PC Count

Ileum tissue samples were used for analysis to accomplish greater equality during the following analysis. We scored number of PCs per 10 crypts per tissue section with HE staining, in random order determined by the observer and blinded for GA, using microscopy at a magnification of 400x. PCs were identified based on their eosinophilic granules and incident light fluorescence (16). The numbers of PCs were scored starting from the first uninterrupted undamaged tissue at the lower right region of the tissue section for the length of 10 crypts. Next, we performed a semiquantitative assessment of the numbers of immune-competent PCs based on HD5-positive cells. Whereas, we have to point out that HD5 immunohistochemistry is a more sensitive method for detecting PCs than routine HE staining (11). PCs were scored as described above. All scores were given by one observer and validated by the second observer blinded for the results of the first observer, after which the intraobserver variability was assessed.

Analysis and Statistics

First, to investigate when PCs arise in the developing intestine, we analyzed PC numbers in the HE-stained sections per GA via PC count in each section. Secondly, to investigate immune competency (maturity) of PCs per GA, we counted all PCs in each section that was stained with the anti-human HD5 antibody per GA.

Interobserver variability was analyzed using ICC. Comparison between PC counts per GA group was performed using the Kruskal-Wallis test considering our data as non-normally distributed. The Mann Whitney *U*-test was used to assess differences between (each) GA group(s) in case of a significant difference found using the Kruskal-Wallis test. For comparison between two categorical variables the χ^2 analysis was used, the Spearman ρ -test for continuous variables and the Mann Whitney *U*-test was used for the combination of a categorical and a continuous variable, as appropriate. Statistical analyses were performed using the Statistical Package for the Social Sciences (IBM SPSS Statistics 21, IBM, Armonk, NY). Data were presented as median with range, unless specified otherwise. Two sided *P* values less than 0.05 were considered significant.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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AUTHOR CONTRIBUTIONS

F.H.H.: Conceptualized and designed the study, performed the study, drafted the initial manuscript, and approved the final manuscript as submitted. G.B.: Performed the study, edited the English language in the manuscript, and approved the final manuscript as submitted. M.L.C.B.: Assisted and supervised the immunohistochemistry, reviewed and revised the manuscript and approved the final manuscript as submitted. E.M.W.K.: Reviewed and revised the manuscript and approved the final manuscript as submitted. A.F.B.: Reviewed and revised the manuscript and approved the final manuscript as submitted. A.T.: Supervised the study, reviewed and revised the manuscript, and approved the final manuscript as submitted. J.B.F.H.: Supervised the study, reviewed and revised the manuscript, and approved the final manuscript as submitted.

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