The pathophysiology of necrotizing enterocolitis in preterm infants

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CHAPTER 3

PANETH CELLS IN THE DEVELOPING GUT: WHEN DO THEY ARISE AND WHEN ARE THEY IMMUNE COMPETENT?

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Contents

Abstract

Background: Little is known about the perinatal development of paneth cells (PCs) during gestation and the relation with 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 – 40 weeks 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). Whilst significantly higher PC numbers were observed after 37 weeks' 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 weeks (median 6, range 0 – 18) compared to infants with GA under 29 weeks (median 2, range 0 – 9; p<0.001).

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

Introduction

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 alpha-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 contribute to 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 (ELBW) human infants generally have the longest interval before the onset of NEC - with a peak incidence at the postmenstrual age of 29-33 weeks.[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.

Methods

Patients

This retrospective study was conducted in a tertiary referral NICU 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 gestational age (GA) between 0 and 40 weeks were selected. GA between 0 and 40 weeks were divided in four- week intervals. Upon retrieval from the archive Hematoxylin-eosin (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 two days or more postpartum to minimize the possible external influences on PC development. We attempted to include eight fetuses/infants per four-week 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 µm-thick serial sections were prepared. Paraffin-embedded sections were stained with HE. Tissue morphology was qualitatively assisted by two trained observers (FH and AT).

To determine PC-specific expression of HD5, we performed immunohistochemistry. Deparaffinized sections (4 µm) were subjected to heat-induced antigen retrieval by 15 min incubation in 1 mM EDTA buffer (pH 8.0) at 95°C. Endogenous peroxidase was blocked for 30 min with 0.075% H_2O_2 in PBS. Primary antibody Cat. No. MABF31, Anti-alpha Defensin 5, clone 8C8 (Merck Millipore, Billerica, MA, USA) was diluted 1/100 in 1% 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 tertiairy antibody (DakoCytomation) diluted in PBS with 1% BSA and 1% normal human serum. Peroxidase activity was developed using 3,3'-diaminobenzidine tetrachloride (DAB) for 10 min. Sections were counterstained with HE. 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.[9] 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 semi-quantitative 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.[10] 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 intra-observer 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.

Inter-observer variability was analyzed using intraclass correlation coefficients (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 $\chi 2$ analysis was used, the Spearman rho-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 Corp., Armonk, New York, USA). Data were presented as median with range, unless specified otherwise. Two sided p-values less than 0.05 were considered significant.

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 two days post partum. We present the patient characteristics in Table 1 and in the Supplemental table S1 (supplements).

TABLE 1:

Patient characteristics

Patients	**57**
Gestational age in weeks, median (range)	26 (9 - 40)
Sex (male), n (%)	32 (56)
Birth weight (grams), median (range)	627 (11 - 4756)
Survival period, n (%)	
Zero days	37 (65)
One day	14 (25)
Two days	6 (10)
Cause of death, n (%)	
Intra-uterine 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.*

Histological PC count

Figures 1 and 2 and Table 2 show (total numbers of) PCs that stained positively with HE staining. Intra-observer variability for the PC count was excellent (ICC 0.95 (95% CI: 0.91 - 0.97)). GA correlated positively and strongly with the number of PCs (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 weeks (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 weeks (values are depicted in Table 2). Supplemental figure S1 and supplemental table S1 present detailed patient characteristics and PC count.

FIGURE 1:

Paneth cells (PCs) 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. Figure A and B show tissue of a patient with a GA of 20 weeks. No PCs or immune competent PCs are observed. Figure C and D show tissue of a patient with a GA of 37 weeks. Multiple PCs are observed (the arrow indicates a (immune competent) PC). Magnification of 400x, scale bar represents 200 µm.

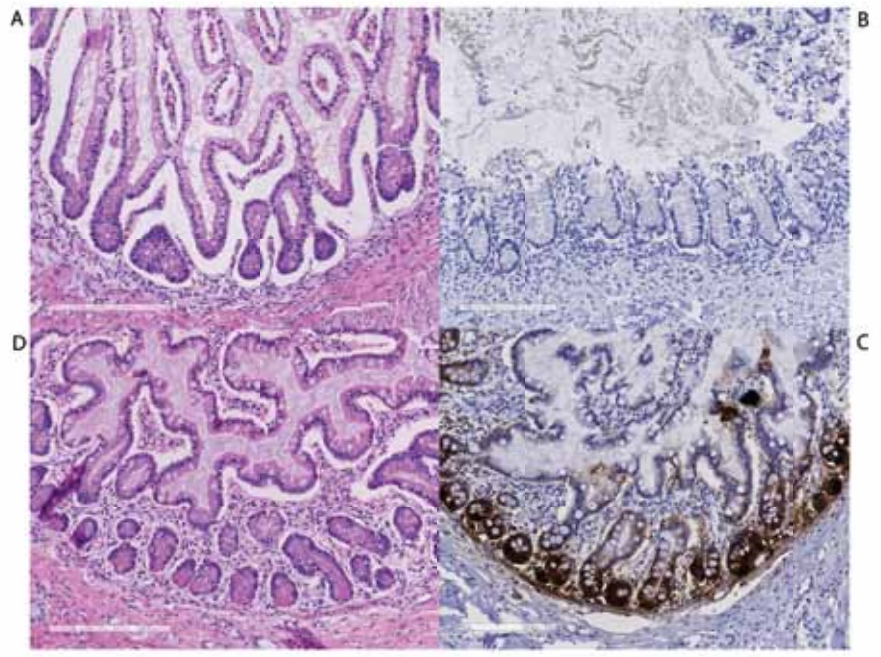

FIGURE 2:

Boxplot of numbers of (immune competent) PCs

Y-as represents number of (immune competent) PCs per crypt, whereas □ represents numbers of PCs on HE 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.

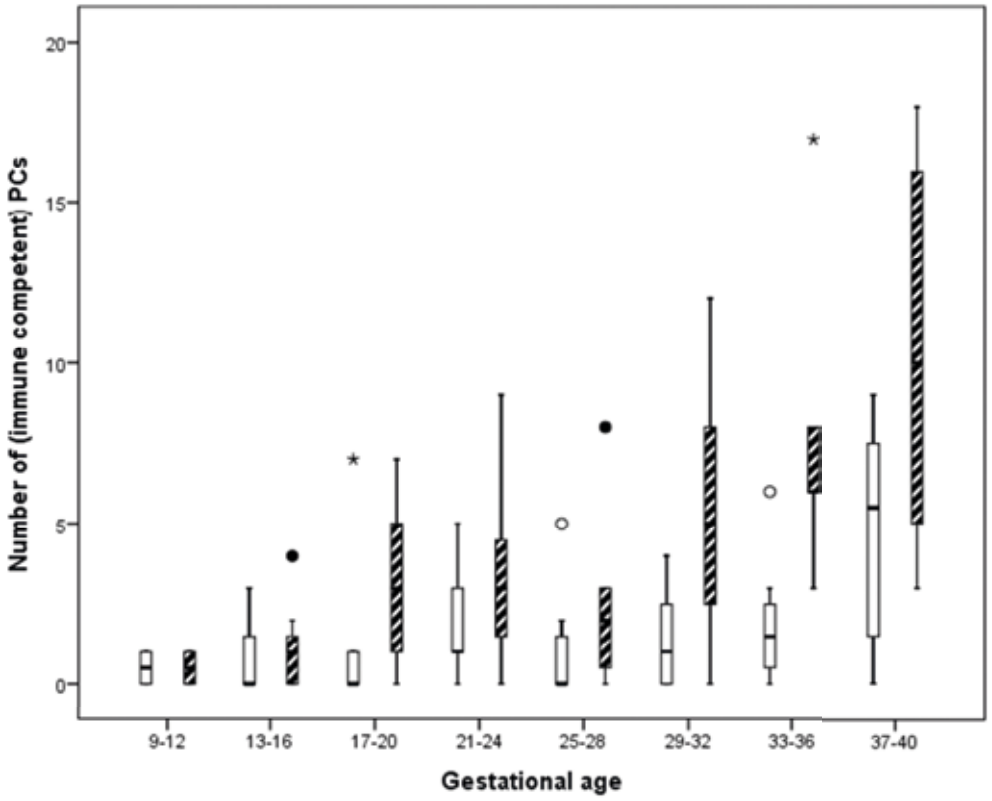

TABLE 2:

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 HE staining	0 (0-0)	0 (0-2)	.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 versus PC numbers. ** p-values represent GA versus immune competent PC numbers.*

Anti-HD 5 immunohistochemistry PC count

Figures 1 and 2 and Table 2 show (total numbers of) immune competent PCs. Intra-observer 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 weeks or greater had significantly more immune competent PCs (median 6, range 0 – 18) when compared to infants with a GA of less than 29 weeks (median 2, range 0 – 9; p<0.001). When infants became term (GA of 37 weeks) even higher numbers of immune competent PCs (median 10, range 3 – 18) were observed when compared to preterm infants (GA <37 weeks; median 3; range 0 – 17; p=0.003). Supplemental figure S1 and supplemental table S1 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 weeks. However, starting from 29 weeks 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 weeks. When 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.[11] These animal studies are not directly comparable with human tissue because human PCs are thought to develop during the first trimester of gestation.[11] 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.[1,11,12] 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.[10] 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 weeks.[4] Both PC numbers and defensins expression are hypothesized to be lower in preterm infants with a GA of 24 weeks compared with term infants.[1,13] According to our current understanding, the appearance of HD5 coincides approximately with PC differentiation during intestinal crypt ontogeny.[1113] The present study suggests that HD5 expression coincides with PC development until a GA of 28 weeks; in the period starting from 29 weeks 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.[14–16] PCs with their defensin expression are previously described in the pathophysiology of Crohn's disease.[16] 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 weeks, 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[6,8,9] 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 and/or pathogenic 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 over-activated 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 weeks, in combination with colonization with opportunistic and/or pathogenic 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.[13,22] 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 weeks' GA could lead to an excessive inflammatory response which is seen in NEC.

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 weeks of gestation, which equals the peak incidence of NEC at a postmenstrual age of 29-33 weeks. Whether this association between PCs and NEC is a causal one remains to be seen.

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Supplements

Supplemental table S1: Detailed patient characteristics

	GA (weeks)	Gender	Weight (grams)	Nature of specimen	Number of PCs HE staining	Number of PCs anti-HD5 staining
1	9	Male	11	Termination of pregnancy	0	0
2	12	male	15	Termination of pregnancy	0	1
3	14	Male	32	Intra-uterine death	0	1
4	15	Male	94	Intra-uterine death	0	2
5	15	Male	55	Termination of pregnancy	0	1
6	15	Unknown	unknown	Intra-uterine death	0	0
7	16	Unknown	unknown	Termination of pregnancy	0	0
8	16	Unknown	unknown	Termination of pregnancy	2	0
9	16	Female	82	Termination of pregnancy	0	0
10	16	Male	75	Termination of pregnancy	0	4
11	17	Male	164	Intra-uterine death	0	0
12	19	Male	210	Intra-uterine death	1	3
13	19	Male	19	Termination of pregnancy	0	3
14	19	Female	80	Termination of pregnancy	0	2
15	19	Female	532	Termination of pregnancy	0	0
16	20	Female	218	Intra-uterine death	3	7
17	20	Female	226	Termination of pregnancy	3	4
18	20	Female	302	Intra-uterine death	2	4
19	21	Male	410	Termination of pregnancy	15	6
20	21	Female	312	Termination of pregnancy	0	4
21	22	Female	438	Termination of pregnancy	0	1
22	22	Male	610	Respiratory/circulatory insufficiency	0	9
23	23	Male	610	Termination of pregnancy	3	5
24	24	Female	570	Intra-uterine death	0	2
25	24	Male	588	Respiratory/circulatory insufficiency	2	2
26	24	Female	336	Live birth, other causes of death	0	6
27	25	Male	550	Intra-uterine death	6	0
28	25	Male	644	Intra-uterine death	0	3
29	26	Male	556	Intra-uterine death	0	2
30	26	Male	946	Intra-uterine death	0	8
31	26	Male	870	Intra-uterine death	0	1
32	28	Female	318	Intra-uterine death	0	0
33	28	Female	600	Respiratory/circulatory insufficiency	0	3
34	30	Unknown	1656	Live birth, other causes of death	0	3
35	30	Male	2046	Live birth, other causes of death	4	10
36	31	Female	2200	Live birth, other causes of death	0	5
37	32	Male	1092	Live birth, other causes of death	6	12
38	32	Male	2120	Live birth, other causes of death	0	0

39	32	Male	1850	Live birth, other causes of death	0	2
40	32	Male	2124	Live birth, other causes of death	4	6
41	32	Male	1558	Termination of pregnancy	4	5
42	33	Male	1165	Live birth, other causes of death	0	3
43	33	Male	2450	Live birth, other causes of death	6	17
44	34	Female	2554	Respiratory/circulatory insufficiency	2	6
45	34	Male	3750	Live birth, other causes of death	3	6
46	34	Male	1720	Intra-uterine death	0	6
47	34	Male	1570	Respiratory/circulatory insufficiency	2	6
48	36	Male	2486	Respiratory/circulatory insufficiency	0	8
49	36	Female	2172	Intra-uterine death	0	8
50	37	Male	3359	Respiratory/circulatory insufficiency	0	6
51	37	Male	3820	Live birth, other causes of death	3	5
52	39	Female	2110	Respiratory/circulatory insufficiency	12	18
53	40	Female	4756	Respiratory/circulatory insufficiency	7	18
54	40	Female	4236	Respiratory/circulatory insufficiency	10	14
55	40	Female	3800	Intra-uterine death	0	3
56	40	Female	3456	Respiratory/circulatory insufficiency	6	14
57	40	female	3000	Live birth, other causes of death	12	5

SUPPLEMENTAL FIGURE S1:

Gestational age versus number of (immune competent) PCs