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A Time-Based Analysis of Inflammation in Infants at Risk of Bronchopulmonary Dysplasia

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Abbreviations: BPD: Bronchopulmonary dysplasia, IL: interleukin; GCSF: Granulocyte colony-stimulating factor

ABSTRACT

Objective: To precisely delineate the timing and contribution of inflammation to bronchopulmonary dysplasia (BPD) in preterm infants during the neonatal period.

Study Design: Longitudinal study of blood inflammatory biomarkers (IL-6, IL-8 and GCSF) measured between birth and 42 days of age, at high temporal (daily) resolution, in infants born at or below 30 weeks of gestation. Cytokine predictors of BPD at 36 weeks post-menstrual age were adjusted for infant-specific and time-dependent factors, using hierarchical mixed effects regressions models.

Results: A total of 1518 data points were obtained in 62 infants (mean GA 27 weeks). Infants who developed BPD later on presented increased inflammation after birth compared to infants without BPD. Inflammation was sustained, with gradual attenuation over three weeks (IL-8: OR: 6.5 [95%CI: 1.8 – 24]; GCSF: 3.3 [1.5 – 7.6]) and was higher in boys and in infants of lower birth weight. This inflammation preceded the clinical increased requirement in supplemental oxygen characteristic of BPD, and preceded the peak occurrence of neonatal sepsis or necrotizing enterocolitis.

Conclusion: Systemic inflammation occurs early in the neonatal period and precedes clinical symptoms in infants with BPD. These data provide a discrete vulnerability window period, supporting a role for targeted intensive care interventions during the early phase of BPD.

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a serious and common neonatal complications affecting up to 35% of infants born below 32 weeks gestation; representing about 15,000 infants in North America each year. These infants are at increased risk of respiratory and neurodevelopmental impairments later in life [1]. Despite advances in neonatal care, few interventions have proven to be effective, and although the severity of BPD has improved, its overall incidence has not substantially decreased over the past two decades [2], warranting more human studies to better understand its pathophysiology, in infants.

A large body of animal experimental and human biomarker studies, support a major role for inflammation in BPD [3]. Previously, we demonstrated the occurrence of high subclinical inflammation during the neonatal period, in infants born very prematurely [4, 5]. However, how this inflammation temporally relates to the development of BPD remains to be determined. This question is important in order to best understand when to focus intervention studies. In preterm infants, episodes of sepsis and the ongoing use of supplemental oxygen [6, 7], mechanical ventilation [8] and parenteral nutrition [7, 9] have all been associated with both local as well as systemic inflammatory responses. To appreciate the relative contribution of these factors, previous studies have reported focal measures of inflammatory biomarkers in blood or in tracheal samples in these infants, over time [10-14]. However, while these studies provide important insights into risk factors, their inherent design make it impossible to exclude selection biases; for example, in studies reporting only on tracheal samples performed in mechanically ventilated infants, or to exclude kinetic effects due to the limited number of measures performed over time. This, in turn, limits our ability to design effective strategies to prevent BPD [15].

To address this question, we measured validated inflammatory biomarkers in a longitudinal cohort of infants at risk for BPD, at high temporal resolution. Based on our earlier observations [4], we

hypothesized that inflammation would occur early in the neonatal period and mainly in infants who subsequently develop BPD. Using this unique study design, we were able to temporally delineate a discrete, post-natal high inflammatory early window period predictive of BPD.

PATIENT and METHODS

Study population: Preterm infants born at or below 30 weeks of gestation at the Children's & Women's Health Centre of British Columbia (Vancouver, Canada) were eligible after admission to the Neonatal Intensive Care unit (NICU), and recruited following written consent from their parent or legal guardian. The Children's & Women's Health Centre is the only pediatric hospital in British Columbia and main provincial tertiary care referral centre. Exclusion criteria were an early anticipated demise (<72 hours) and lack of informed consent. Infants who died before 36 weeks of causes unrelated to BPD, were also excluded. Infants were followed until discharged home. However, for this study, daily longitudinal data were included up to the first 42 days of age. The study was approved by the University of British Columbia and Children's & Women's Research Ethics Boards (H07-00157; H10-01571). Details of this cohort, including other analyses unrelated to BPD have been published [4].

Clinical definitions: Data were prospectively collected by a single investigator (CW), and verified by PML. Infants with BPD were defined as cases according to a requirement for respiratory support or supplemental oxygen at 36 weeks post-menstrual age (PMA) in order to maintain oxygen saturations between 88% and 92%, as we described earlier [16]. All infants were systematically assessed for BPD at 36 weeks PMA. Controls were infants without BPD. Histological chorioamnionitis was defined as maternal or fetal stage 1 or greater scored blindly by a pathologist, using previously published criteria [17]. Sepsis was defined as a positive blood culture in the context of suspicious clinical signs (e.g. cardiorespiratory, temperature instability, gastrointestinal intolerance) as per the attending

neonatologist. Necrotizing enterocolitis (NEC) was defined based on clinical signs of acute gastrointestinal deterioration or grossly bloody stools, and radiological evidence of pneumatosis, free or portal air, or signs of fixed bowel dilatation with bowel wall thickening (modified Bell's staging criteria stage II or higher) [18]. Sepsis, NEC, maximal use of supplemental oxygen (FiO_2), mode of respiratory support, administration of hydrocortisone, dexamethasone and indomethacin were collected daily. Dexamethasone and hydrocortisone were the only two corticosteroids used during the study period, mainly for clinical indications of BPD treatment (either as a rescue in very ill infants, or to facilitate extubation in infants who required low supplemental oxygen) and hypotension, respectively. During the study period, indomethacin was largely used for symptomatic treatment of a patent ductus arteriosus (PDA), and its use as a prophylactic treatment was extremely uncommon. If the infant was transferred to another hospital facility, data were systematically obtained at 36 weeks PMA (for BPD status or death) and daily during the first 42 days by contacting the referral centre by phone, to obtain a written copy of the medical records. For validation, analyses were repeated using daily FiO2 at a fixed time (midnight) rather than maximal FiO2, and trends were identical (not shown).

Inflammation biomarkers: Infants were sampled during daily during routine clinical testing, in the morning, between birth and 42 days of age. To limit invasiveness analyses were carried out only from residual (left-over) serum whenever available from the hospital lab. To avoid a sample selection bias, parents or legal guardians of these infants were approached as early as possible after birth (usually <72 hours). The number of cytokine data points over time confirmed similar distribution between infants with or without BPD in the first 2 weeks (**Figure 1**, online). To minimize protein degradation, our hospital lab employed strict protocols where blood collected in lithium heparin BD Microtainers (Becton Dickenson, Mississauga ON, Canada) was spun down within 1 hour to remove the cell fraction, and left over plasma was immediately stored at -80°C. Cytokines (IL-6, IL-8 and GCSF) were

measured in a single batch, in a 1:2 dilution using a Procarta® multiplex assay on a Luminex analyzer (BioRad, Mississauga ON, Canada). These three cytokines were pre-selected from a larger array of 26 cytokine/chemokines, based on their strongest Bonferroni-adjusted association with culture-proven sepsis in newborns in our previous study [4]. Variability in cytokine measurements were generally ≤20% based on duplicate samples (not shown).

Statistical analyses: The sample size in this analysis was fixed by the number of subjects in the parent study [4]. Clinical characteristics were analyzed descriptively, using proportions (qualitative variables), and medians plus interquartile ranges (quantitative variables). Cytokines levels were transformed to normalize the data, by log (x + 1), where 1 was added to 0 cytokine values. Log-transformed cytokines were modeled using hierarchical mixed effects regression models to account for repeated measures over time, within infants. Non-linear effects were represented using cubic splines, with 95% confidence intervals. Multivariate models were separately adjusted for birth weight or gestational age at the individual (infant) level. Post-natal corticosteroid therapy (combining hydrocortisone and dexamethasone) and indomethacin were also adjusted at the sample level, taking into account the timing of administration (days). A sensitivity analysis was performed examining trends in cytokines over time in infants classified according to a severity-based BPD definition [19]. Subgroup analyses were performed on infants who did not receive dexamethasone or hydrocortisone using a similar approach. Significance levels were established at $p<0.05$. Statistical analyses were performed using R v3.3.2 [20].

RESULTS

Clinical characteristics of infants

During the entire study period, 154 infants were eligible and 65 were enrolled. Three infants died before 36 weeks PMA and were therefore excluded in the analysis: one infant died of bacterial sepsis,

one of necrotizing enterocolitis and another one from a severe intra-ventricular hemorrhage. Thirty-two infants of the 62 infants in the study had BPD (incidence ~50%). Clinical characteristics were similar between infants with and without BPD, except for gestational age, birth weight, and use of post-natal corticosteroids (**Table 1**). The clinical characteristics of enrolled infants did not differ from the eligible population during the study period with respect to the average gestational age (27.0 ± SD2.2 weeks), birth weight (1006 ± SD305 g) and sex distribution (54% males) of the latter (using 95% confidence intervals; data not shown).

Systemic inflammation in BPD

Altogether, 1518 data points were collected for all three biomarkers: IL-6, IL-8 and GCSF between birth and 42 days of age. **Figure 2A** presents the systemic inflammation detectable over time in plasma of infants with or without BPD. Levels of GCSF, IL-8 and to a lesser extent IL-6 were increased shortly after birth in infants with BPD, with a progressive attenuation over the first three weeks of age. Unadjusted odds ratios indicating statistically significant differences between infants with or without BPD, were significant for IL-8 (OR: 6.5 [95%CI: 1.8 to 24]) and GCSF (3.3 [1.5 to 7.6]), but not quite for IL-6 (2.4 [0.84 to 7.0]). The inflammation preceded the requirement for oxygen characteristically observed in the infants with BPD (**Figure 2B**). Highest inflammation also occurred before the peak incidence of sepsis, NEC or intestinal perforations in this cohort (**Figure 2C**). As a validation, we detected a statistically significant cytokine exposure-effect on BPD reclassified using a severity based standard definition (**Figure 3**, online).

In univariate analyses, IL-6, IL-8 and GCSF were higher in boys and in infants of lower birth weight. On the other hand, no statistically significant effect was detected for gestational age or chorioamnionitis, on cytokine levels (**Table 2**, online). In multivariate analyses, the effects of sex and

birth weight, on inflammation, remained significant after co-variable adjustments, indicating independent effects (**Table 3**). This was also the case for the effect of birth weight on IL-6, for the effect of sex and birth weight on IL-8 and for the effect of sex on GCSF (**Table 3**). Moreover, the effect of birth weight on inflammation was more significant in the earlier weeks, for all three cytokines, adjusting over time ($p \leq 0.005$, not shown). Finally, we detected an independent effect of inflammation on BPD after adjusting for gestational age, birth weight, sex and chorioamnionitis, and over time. This inflammation gradually attenuated for IL-6 and GCSF in days-adjusted regression models (**Table 4**).

Effect of post-natal corticosteroids

In our cohort, 20 (32%) infants received post-natal corticosteroids (either hydrocortisone or dexamethasone) and 27 (44%) infants received indomethacin. During the 42-day study period, infants were exposed to hydrocortisone for a median of 11 days [IQ range 5 to 18 days]) and to dexamethasone for a median of 27 days [IQ range 17 to 35 days]). The median time of indomethacin administration in this cohort was 7 days [IQ range 5 to 11 days]). For hydrocortisone, levels of GCSF were significantly attenuated during exposure to this drug (-4.8% per day of exposure, $p=0.015$). For dexamethasone or indomethacin, we detected no effects of these drugs on cytokine levels during exposure. Moreover, re-analysis of cytokine levels, over time, in infants excluding the relatively small proportion who did not receive corticosteroids yielded similar cytokine trends between groups, suggesting little masking effect from these drugs (not shown). Importantly also, there was an independent effect of inflammation on BPD after adjusting for exposure to these drugs (**Table 5**, online).

DISCUSSION

In this study, we conducted a detailed temporal analysis to precisely delineate when inflammation occurs in infants with BPD. Our data reveal that inflammation occurred shortly after birth and was

sustained, with gradual attenuation over the early neonatal period. This inflammation preceded clinical symptoms of BPD, which directly supports a role of inflammation in the BPD lung insult. The analysis of markers of BPD at high temporal resolution provides a clearer representation of inflammation in relation to exposures and clinical symptoms. This is of major importance to exclude kinetics effects. To the best of our knowledge, this is the first study that precisely delineate when inflammation occurs in infants with BPD.

Our study has a number of strengths. The use of longitudinal measures of inflammation at high density of blood sampling in a well-characterized infant cohort, combined with hierarchical mixed effects regression models adjusted for repeated measures, allow a robust comparison of infant groups over time, independent of differences due to a more frequent sampling schedule in sicker infants. This is important in order to minimize a selection bias. In a sensitivity analysis conducted, we detected a statistically significant exposure-effect of inflammation on BPD severity, in the first week, which helps validate our conclusions. Our cohort is representative, which facilitates generalization of the data. Finally, measuring cytokines in blood (as opposed to tracheal fluid) minimizes selection biases by targeting all infants regardless of their requirement for endotracheal ventilation and likely more accurately reflects the systemic impact of inflammation on neonatal morbidities.

Our data, while also consistent with previous studies that reported focal measures of inflammation in infants at risk of BPD, add considerably to our current understanding of BPD. Paananen tested an extended panel of immune markers in plasma on cord blood, day 1 and day 7, from a cohort of 128 very low gestational age infants. Of 11 biomarkers included in their initial screening, IL-6, IL-8, IL-10 and GCSF were the only markers detectable and significantly associated with BPD, and were higher on day 1 compared to day 7 in infants with BPD [10]. Contrary to this study [10], we did not detect an effect of

chorioamnionitis on IL-8 during the first week of life, which may be related to the limited number of infants exposed. However, we did observe a trend towards persistently increased IL-8 over the first week of age in infants with chorioamnionitis (not shown). Moreover in another study, IL-6, IL-8 were significantly associated with BPD, of a series of 7 immune biomarkers obtained on days 1, 3 and 5; these markers were higher and decreased over time in blood spots from a large cohort of 1067 extremely low birth weight preterm infants [11]. IL-6 has been directly implicated in a murine hyperoxia model of BPD [21]. GCSF increases bone marrow neutrophil production [22]. IL-8 is a powerful chemoattractant for neutrophils which play an important role in the early stages of BPD [23]. Therefore, we speculate that these cytokines play a direct role in the BPD lung injury in infants.

Our data have potential clinical implications. The observation of a sustained systemic inflammation in infants prior to clinical symptoms of BPD suggests that anti-inflammatory interventions during this period may be more effective. Consistent with this notion, improvement due to a treatment with an IL-1 receptor antagonist occurred on day 1 in a mouse therapeutic model of BPD lung disease, supporting a benefit for early interventions [24]. In preterm infants, the cumulative adverse effect of supplemental oxygen on BPD becomes negligible beyond 14 days, presumably through an oxidative stress [25]. Based on clinical trials, the reduction in BPD conferred by post-natal corticosteroids is greater when these drugs are used early on in infants [26]. However, this approach raises concerns of adverse long-term neurodevelopmental effects especially when these drugs are used early on in infants at low risk of BPD where negligible therapeutic benefits are anticipated [27]. In this case, the use of alternate systemic (e.g. hydrocortisone [28] or betamethasone [29]) or inhaled (e.g. budesonide [30, 31]) steroids has been studied in clinical trials, but even those interventions are not entirely without risks. For example in the *Neonatal European Study of Inhaled Steroids* (NEUROSIS) trial, mortality was increased in infants in the corticosteroid arm [30], whereas in the *Early Low-Dose Hydrocortisone to Improve Survival without*

Bronchopulmonary Dysplasia in Extremely Preterm Infants (PREMILOC) trial, the risk of sepsis was increased in infants 24-25 weeks [28]. Overall, our data support the timing of interventions in these trials. However, the use of early inflammatory biomarkers may help minimize the risks of these treatments by helping to identify infants at higher risk of BPD in whom corticosteroids may be more beneficial.

The therapeutic mechanism of action of corticosteroids in BPD is likely to be multifaceted [32]. On the other hand, the reduced inflammation observed in our cohort during exposure to hydrocortisone is consistent with an anti-inflammatory effect of this drug. For dexamethasone, the lack of a detectable effect on cytokine levels is more surprising and likely explained by the small number of infants exposed to this drug in our cohort at a relatively late age when differences in cytokine levels are more modest; thereby limiting statistical power. For indomethacin, the lack of apparent effect of this drug on inflammation (despite an early exposure, in a sizeable proportion of babies) may be more expected in light of the difference in mechanism and support a lack of clinical effect of this drug on BPD [33].

Infants with and without BPD also mainly differed by gestational age, birth weight and in the use of corticosteroids (see **Table 1**). Higher inflammation was detected in boys of smaller birth weight, after co-variable adjustments. This is consistent with clinical observations of an increased risk of BPD in low birth weight and male infants [34]. The sex-related difference is also observable in animal models of BPD [35] and may suggest an increased inflammatory response inherent to male infants, although this requires further study. Of note, we have previously not detected any major differences in immune reactivity between preterm boys and girls [36], on cord blood, which may indicate that post-natal factors are required for differences to be expressed. Previous data also showed skewed neonatal cytokine responses towards IL-6 and IL-8, which may suggest that the differences in cytokine levels represent a developmental aspect that could play an important role in the etiology of BPD [37]. However, the lack of

effect of gestational immaturity on cytokine levels and independent effect of inflammation on BPD after adjusting for gestational age and birth weight, argues against this possibility.

Our study has some intrinsic limitations. To limit the invasiveness of the study, we did not measure cytokine levels in tracheal aspirates, and it is important to mention that while blood measures present some advantages (as mentioned above), it may not necessarily reflect inflammation in the lung. Also, due to the relatively small sample size (including the availability of adequate amounts of scavenged blood samples) and due to the observational nature of our study, we were unable to determine the clinical factors driving the early inflammation in BPD. Interestingly, the inflammation in our cohort preceded the peak incidence of infections [38], suggesting that sepsis is unlikely to play a major role. Also, we did not detect an association between histological chorioamnionitis and BPD, which is consistent with other larger studies [39, 40]. We speculate, however, that the inflammation is driven by antenatal factors other than chorioamnionitis, combined to an early post-natal exposure to intensive care interventions in the context of limited anti-inflammatory/anti-oxidant defenses mechanisms in these infants. In conclusion, while data from this observational study should not be directly interpreted to guide clinical interventions, it provides valuable insights which may be used in trials to design intervention strategies targeting inflammation in preterm infants at risk of BPD.

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LEGENDS to FIGURES

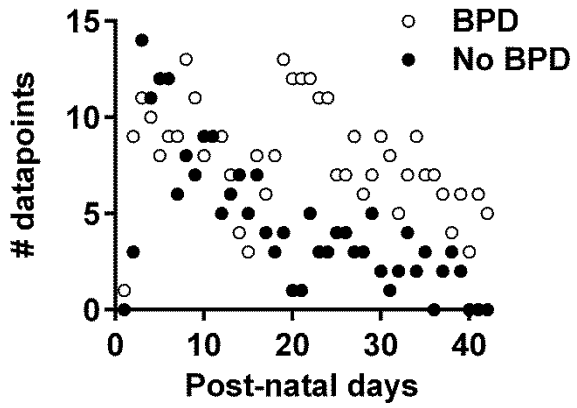

Figure 1 (online): Distribution of cytokine data points in infants with or without BPD. Note that the distribution of data points was comparable in the first two weeks of age, but diverged later, as infants became sicker and more symptomatic of their lung disease.

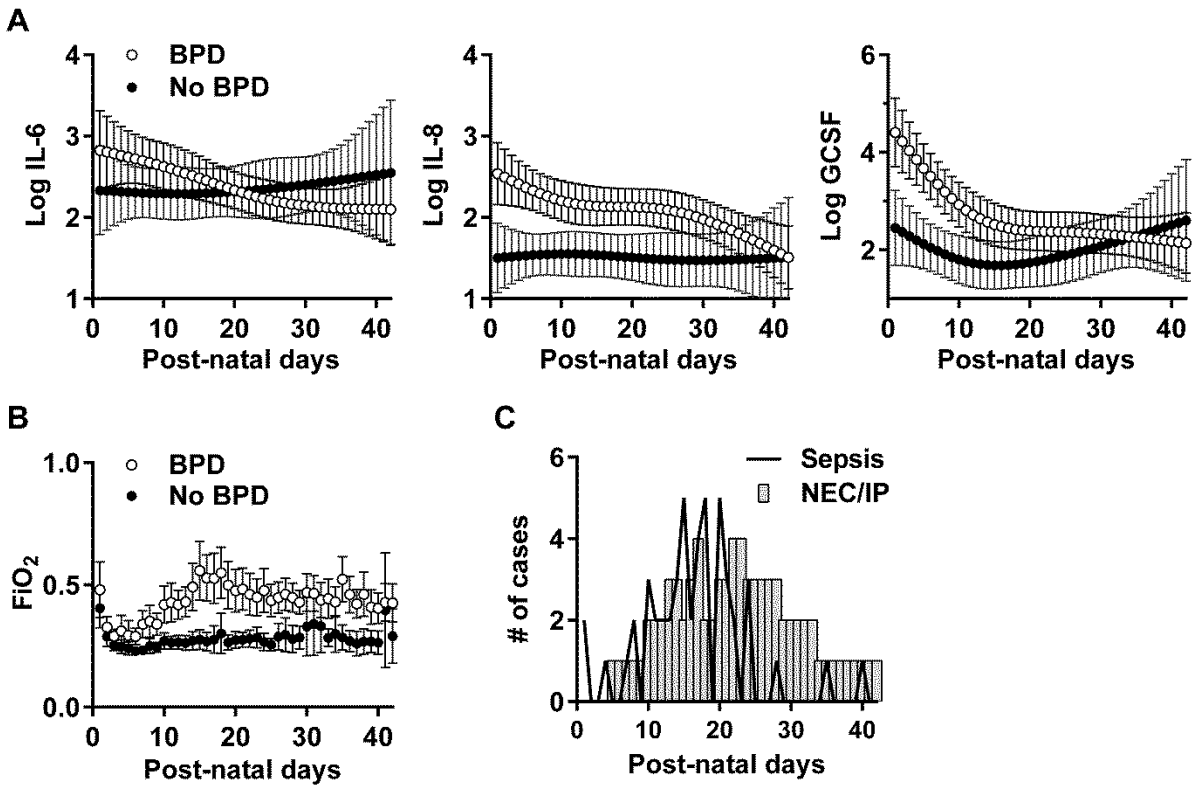

Figure 2: Inflammation in infants over time. **(A)** Mixed effects model-based spline fits each cytokines comparing infants with (open circles) or without (black circles) BPD; **(B)** Maximal (mean) supplemental oxygen requirement (FiO_2) on each day of age in infants with (open circles) or without (black circles) BPD. Bars represent 95%CI; **(C)** Daily cases of (culture-positive, bacterial or fungal) sepsis or necrotizing enterocolitis (NEC)/intestinal perforation (IP) in the entire cohort of infants.

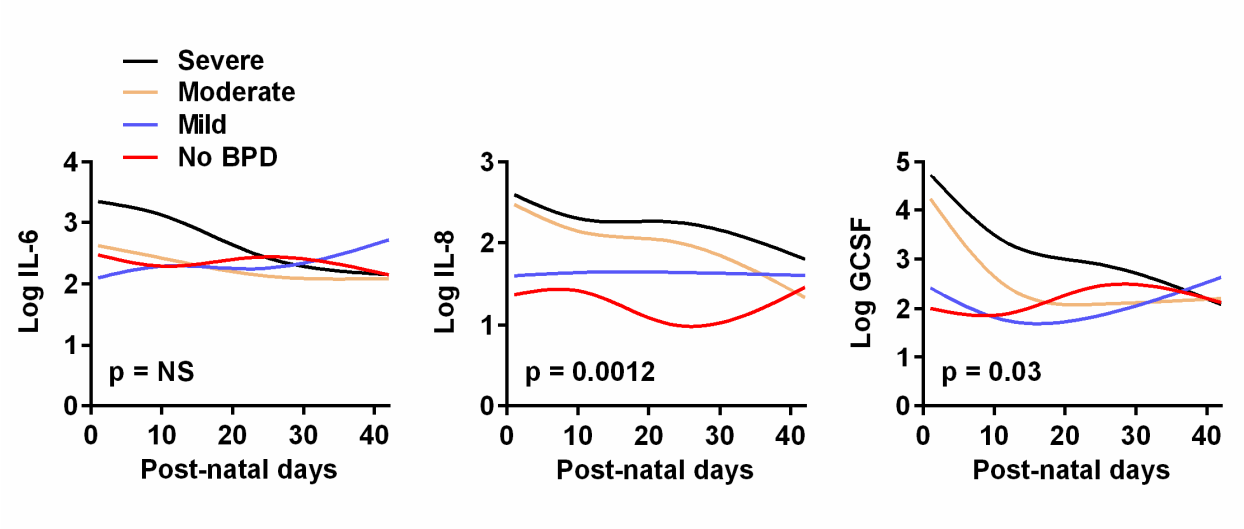

Figure 3 (online): Cytokine measures over time according to BPD severity. Spline curves of averaged log-transformed cytokine values over time (post-natal days) in each group of infants classified according to the National Institute of Child Health and Human Development (NICHD) severity-based definition of BPD. P values represent the significance of interaction of the differences in mean log-transformed cytokine over time between all four groups.

Table 1: Clinical characteristics of infants

Clinical characteristics	**No BPD (n = 30)**	**BPD (n = 32)**
Gestational age, mean ± SD (weeks)	27.9 ± 1.5	26.0 ± 1.5
Sex, n (% male)	16 (53)	18 (56)
Birth weight, mean ± SD (weeks)	1126 ± 235	833 ± 215
Small-for-gestational-age, n (%)	2 (6.7)	7 (22)
Histological chorioamnionitis, n (%)[&]	10 (38)	7 (26)
Endotracheal ventilation, n (%)	22 (73)	31 (97)
Culture-proven sepsis, n (%)*	6 (20)	10 (30)
Hydrocortisone, n (%)*	4 (13)	12 (38)
Hydrocortisone, median [IQR] (days)	7 [6 to 9]	7 [4 to 17]
Dexamethasone, n (%)*	0	10 (31)
Dexamethasone, median [IQR] (days)	-	5 [4 to 8]
Indomethacin, n (%)*	9 (30)	18 (56)
Indomethacin, median [IQR] (days)	3 [3 to 4]	3 [3 to 5]

[&]placental histology data was missing in 9 infants; *in the first 42 day of age; Shaded rows indicate significantly different variables ($p<0.05$); Note that all infants were exposed to parenteral nutrition, and therefore these data have been excluded from the table.

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Table 2: Effect of gestational age, sex, birth weight and chorioamnionitis on inflammation

Variable	IL-6		IL-8		GCSF	
	Estimates	**p value**	**Estimates**	**p value**	**Estimates**	**p value**
Gestational age (wks)	-0.073	0.117	-0.091	0.079	-0.111	0.158
Sex (boys/girls)	0.330	0.025*	0.382	0.021*	0.747	0.001**
Birth weight (per 100 g)	-0.074	0.014*	-0.086	0.010*	-0.092	0.074
Chorioamnionitis (yes/no)	0.009	0.953	0.019	0.898	0.130	0.585

Sex, birth weight, gestational age and chorioamnionitis were included in separate mixed effects models for each variable, adjusting for days of life using splines.*p<0.05, **p<0.01.

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Table 3: Independent effects of sex and birth weight on inflammation after adjustments for co-variables

Variable	IL-6		IL-8		GCSF	
	Estimates	**p value**	**Estimates**	**p value**	**Estimates**	**p value**
Gestational age (wks)	-0.048	0.307	-0.094	0.075	-0.096	0.257
Sex (boys/girls)	0.247	0.092	0.341	0.039*	0.723	0.002**
Birth weight (per 100 g)	-0.061	0.047*	-0.081	0.018*	-0.082	0.136
Chorioamnionitis (yes/no)	0.036	0.824	0.127	0.420	0.367	0.149

Sex, birth weight, gestational age, and chorioamnionitis were included jointly in a mixed effects model with adjustment for each co-variables, and days of life using splines; *p<0.05; **p<0.01.

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Table 4: Effect of inflammation on BPD after adjusting for infant-specific co-variables

Variable	IL-6		IL-8		GCSF	
	Estimates	p value	Estimates	p value	Estimates	p value
BPD (yes/no)	0.238	0.070	0.369	0.002**	0.651	0.002**
Gestational age (wks)	0.047	0.586	0.090	0.316	-0.031	0.815
Sex (boys/girls)	0.323	0.069	0.274	0.140	0.61	0.025*
Birth weight (per 100 g)	-0.101	0.060	-0.069	0.215	-0.067	0.417
Chorioamnionitis (yes/no)	0.069	0.699	0.103	0.516	0.410	0.119
BPD over time (days)	-0.016	0.006**	-0.005	0.336	-0.024	0.013*

Sex, birth weight, gestational age, chorioamnionitis and BPD, and its linear interaction with days of life were included jointly in a mixed effects models with adjustment for days of life using splines; *p<0.05; **p<0.01.

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Table 5: Effect of inflammation on BPD, adjusting for drug exposure

Variable	IL-6		IL-8		GCSF	
	Estimates	**p value**	**Estimates**	**p value**	**Estimates**	**p value**
BPD (yes/no)	0.233	0.015*	0.312	0.001**	0.737	7.25E-06**
Use of either dexamethasone or hydrocortisone (yes/no)	0.085	0.638	-0.041	0.829	-0.184	0.589
Use of indomethacin	0.119	0.496	-0.056	0.681	-0.125	0.598
BPD over time (days)	-0.010	0.022*	-0.003	0.416	-0.023	0.0002**

Use of either dexamethasone or hydrocortisone, use of indomethacin, BPD and its linear interaction with days of life were included jointly in a mixed effects models with adjustment for days of life using splines; *p<0.05; **p<0.01.