Serum neutrophil gelatinase-associated lipocalin as a predictor of the development of bronchopulmonary dysplasia in preterm infants

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ABSTRACT

Background: Bronchopulmonary dysplasia (BPD) is a chronic lung disease mostly occurring in preterm infants. The pathogenesis of BPD involves early inflammation and remodeling of the premature lung.

Aim: To search for the novel predictive marker of BPD development, we studied serum levels of neutrophil gelatinase-associated lipocalin (NGAL), an innate immune mediator, in preterm infants.

Methods: Serum NGAL concentrations at birth were measured by enzyme-linked immunosorbent assay. The reference levels were determined in 52 infants having no anomalies or inherited diseases. The levels and clinical variables were assessed in association with BPD.

Results: Geometric means (95%CI) of serum NGAL levels at birth of infants having no underlying diseases were 32.4 (22.1-47.5), 58.6 (47.9-71.8), and 126.2 (99.0-168.7) ng/mL for <31, 31-36 and >36 gestational weeks (GW), respectively ($p<0.001$). These levels positively correlated with neutrophil ($p<0.0001$) or monocyte counts ($p<0.0001$). The median NGAL levels (307.8 ng/mL) and neutrophil counts (4141 /μL) at birth of 16 preterm infants (<31 GW) who developed BPD were higher than those (42.9 ng/mL and 1357 /μL) of 20 infants (<31 GW) who did not ($p<0.0001$ and $p=0.012$), respectively. In multivariable analysis for 36 infants born less than 31 GW, higher NGAL levels ($\geq$82 ng/mL) but not neutrophil counts at birth had a significant association with developing BPD (gestational-age adjusted odds ratio [OR]=37.45 [3.08-455.49], $p<0.01$).

Conclusions: High serum levels of NGAL at birth could be an early sensitive marker for BPD in preterm infants, because their levels were physiologically low.

KEY WORDS

neutrophil gelatinase-associated lipocalin, bronchopulmonary dysplasia, preterm infants

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease commonly occurring in preterm infants. It is a major cause of long-term pulmonary complications, including recurrent respiratory infections, reactive airway disorders, and abnormal pulmonary function in children [1]. The pathogenesis of BPD remains unclear, although lung injuries due to mechanical ventilation, oxygen toxicity, and infection, as well as genetic predispositions are implicated [2]. The development of BPD is associated with pulmonary inflammation, as demonstrated by greater numbers of inflammatory cells and more increased levels of proinflammatory mediators in the premature lung [3]. Early phase inflammatory cells (neutrophils and macrophages) could contribute to the alveolar destruction by releasing proteolytic enzymes such as neutrophil elastases or matrix metalloproteases (MMPs) [4]. Thus, the normal structural complexity of the lung is lost as a result: lack of alveolar septation, fewer alveoli, and abnormal vascular development of the BPD lung [2].

Neutrophil gelatinase-associated lipocalin (NGAL, known as lipocalin-2) is a 25-kDa glycoprotein originally isolated from specific granules of human neutrophils [5]. NGAL is expressed at a low level in the lung, gastrointestinal tract, and kidney [6]. The increased levels were suggested to be a biomarker of acute kidney injury, cancer, rheumatologic diseases and respiratory diseases [6, 7]. High NGAL levels were found in peripheral blood, bronchoalveolar lavage fluid (BALF) or sputum obtained from patients with bronchial asthma, chronic obstructive pulmonary disease, cystic fibrosis and emphysema [6]. NGAL may have a critical role in preterm birth associated with intra-amniotic infection and inflammation, based on the high expression in stimulated trophoblast tissues [8]. Chorioamnionitis has a great impact on the development of BPD

in preterm infants. Serum NGAL levels were increased in children with bacterial rather than viral infection [9]. In murine sepsis models, NGAL production is up-regulated through toll-like receptor 4, and exerts a protective effect against *Escherichia coli (E. coli)* infection [10]. Recently, it has been reported that NGAL is required for the immune defense against pulmonary infection by *E. coli* [11] or *Klebsiella pneumonia* [12]. Circulating neutrophil counts of the newborn infants are the highest during the lifetime. Considering the biological functions of NGAL in the innate immune response, it may be involved in the inflammatory process of premature organs. However, there is little information about the pathophysiological role of NGAL in neonatal diseases.

The identification of early predictors of BPD may warrant the strategy for the protection of premature lung. In this study, to search for the novel marker of BPD development, we studied serum NGAL levels at birth in preterm infants who developed BPD or not. The origin and effects of circulating NGAL in preterm infants were discussed in terms of the pathophysiology of BPD.

METHODS

Subjects

Nine hundred twenty-five infants were consecutively admitted to the tertiary neonatal intensive care unit at Kyushu University Hospital between October 2005 and June 2009. Patients with underlying disorders, including chromosomal aberrations, anomalies, inherited diseases and hematological diseases were excluded. The study enrolled all 86 infants who provided analyzable samples of cord or peripheral bloods at birth. Complete blood counts, serum chemistry tests, blood gas analysis, and flow-cytometric analysis were performed. BPD was defined as oxygen dependency for at least 28 days and assessed at 36 weeks' postmenstrual age [13]. The infection risk at birth was defined as the presence of at least one of premature rupture of membrane (PROM) >72 hours, cloudy amniotic fluid, histological chorioamnionitis, C-reactive protein (CRP) >0.1 mg/dL at birth, or IgM >20 mg/dL at birth. Following events before discharge were defined as the major complications: death, BPD, severe intraventricular hemorrhage (IVH) (grade 3-4), cystic periventricular leukomalacia (cPVL), surgical ligation for patent ductus arteriosus, laser photocoagulation for retinopathy of prematurity and intestinal perforation. No cPVL or severe IVH was found, assessed by the cranial ultrasound or magnetic resonance imaging.

Of 86 infants studied, 36, 35 and 15 infants were born at <31, 31-36 and >36 gestational weeks, respectively (**Figure 1**). Sixteen infants suffered from BPD, and all of them were born prior to 31 gestational weeks. In order to investigate a novel marker of BPD, we compared clinical parameters at birth of 16 infants who developed BPD with those of 20 infants born at <31 gestational weeks who did not, and additionally with those of gestational-age matched 5 infants who did not. We further statistically

assessed the impact of NGAL level at birth on developing BPD in all 36 infants born at <31 gestational weeks (16 BPD and 20 non-BPD infants). To determine the physiological levels of NGAL at birth, 52 infants (10, 28 and 14 infants born at <31, 31-36 and >36 gestational weeks, respectively) were recruited after the exclusion of infants with the any infection risk at birth and the aforementioned complications. Informed consents were obtained from all parents. The Ethics Committee of Kyushu University approved the study.

Measurement of serum NGAL concentrations

Serum was separated from blood samples and stored at −30°C until the analysis. NGAL concentrations were measured by a research enzyme-linked immunosorbent assay (KIT 036, BioPorto Diagnostics, Gentofte Denmark). Duplicate samples were used to determine the concentrations. The detectable range was 5-500 ng/mL.

Statistics

Correlation coefficients (CC) of NGAL levels with gestational age and leukocyte counts were calculated for 52 subjects having no infection risk at birth and major complications before discharge. The log-transformed values were used for NGAL levels, neutrophil and monocyte counts because of the skewed distributions. Mean NGAL values according to the gestational week were calculated. Clinical characteristics and biochemical parameters were examined in relation to the occurrence of BPD among gestational age-matched infants by Wilcoxon rank-sum test for continuous variables and Fisher's exact test for dichotomous variables, respectively. Effect of NGAL level at birth on developing BPD in 36 infants born before 31 gestational weeks was examined by logistic regression analysis in a case-control design treating infants without BPD as control. The NGAL levels, neutrophil counts and CRP levels were dichotomized with an

optimal cut-off point, determined by analysis of receiver operating characteristics (ROC) curve. Statistics were performed using STATA version 10.0 (Stata Corporation, College Station, TX, USA). Statistical significance was defined as *p*-value <0.05.

RESULTS

Serum NGAL levels at birth according to the gestational age

Physiological levels of NGAL at birth were first determined in 52 infants who had neither infection risk at birth nor complications until discharge. Median gestational age and birth weight of these infants were 34.5 (range: 25.9-41.0) weeks and 1813 (552-3350) gram, respectively. Twenty-three (44%) were small-for-gestational age (SGA) infants. Median Apgar score at 5 minute was 9 (3-10). The NGAL levels at birth positively correlated with the gestational age (CC=0.6962, p<0.0001, **Figure 2A**), neutrophil (CC=0.6591, p<0.0001, **Figure 2B**), and monocyte (CC=0.6100, p<0.0001, **Figure 2C**), but not lymphocyte (CC=0.2251, p=0.1280, **Figure 2D**) counts. Geometric means (95%CI) of NGAL were 32.4 (22.1-47.5), 58.6 (47.9-71.8), and 126.2 (99.0-168.7) ng/mL for <31, 31-36 and >36 gestational weeks, respectively (trend p<0.001, **Table 1**). When 29 appropriate-for-gestational age (AGA) infants and 23 SGA infants were separately analyzed, the positive correlation of NGAL was significant with gestational age (AGA; CC=0.7668, p<0.0001, SGA; CC=0.4404, p=0.0354), neutrophil (AGA; CC=0.8065, p<0.0001), and monocyte (AGA; CC=0.652, p=0.0003, SGA; CC=0.5957, p=0.0034) counts, but not with neutrophil counts only in SGA infants (CC=0.4026, p=0.0704). There was no correlation between NGAL and creatinine levels at birth (CC=0.1963 and p=0.1764).

Clinical profiles and parameters at birth of infants who developed BPD

Clinical profiles of 36 infants born before 31 gestational weeks (16 BPD and 20 non-BPD patients) are shown in **Table 2**. Median gestational age in BPD infants (24.8 weeks) was younger than that in non-BPD infants (28.2 weeks) (p<0.0001). Median birth weight did not differ between BPD and non-BPD infants, and SGA infants were

not found in BPD infants (p=0.004). BPD infants had lower Apgar scores (p=0.001), higher frequency of surfactant therapy (p=0.001), infection risk at birth (p=0.044) and complications (p<0.0001) than non-BPD infants. No other parameters differed between BPD and non-BPD infants, including maternal hypertension, antenatal steroids use, early-onset sepsis, PROM>72 hours, IgM>20 mg/dL, death and intestinal perforation.

Biochemical parameters at birth are shown in **Table 3**. Serum NGAL levels and neutrophil counts at birth of BPD infants were higher than those of non-BPD infants (p<0.0001 and p=0.012, respectively). Leukocyte, monocyte and early activation marker (CD69) positive-lymphocyte counts did not differ between BPD and non-BPD infants. There was no difference in any variables among patient's groups according to the severity of BPD. When these parameters of 16 BPD infants were further compared with those of gestational age-matched 5 non-BPD infants (Gestational age; median 26.9 weeks, range 25.9-27.3), the NGAL levels were only higher than those of non-BPD infants (median NGAL; 80.9 ng/mL, p=0.039).

Risk of developing BPD in infants born before 31 gestational weeks

To investigate whether the NGAL levels at birth could be a biomarker of BPD in preterm infants, we conducted a multiple logistic regression analysis in all 36 infants born before 31 gestational weeks (16 infants developed BPD and 20 did not). ROC curve analysis indicated that serum NGAL levels ≥82 ng/mL at birth had an area under the curve of 0.91, a sensitivity of 94 %, and a specificity of 85 % for the prediction of BPD in infants born before 31 weeks of gestation. These corresponding indices for neutrophil counts ≥4141 /μL at birth were 0.83, 57.1%, and 94.1%, respectively. These corresponding indices for CRP levels ≥0.1 mg /dL at birth were 0.69, 37.5%, and 100%, respectively. Higher NGAL levels (≥82 ng/mL) were significantly associated with the

risk of BPD as compared with the lower levels (gestational-age adjusted odds ratio [OR]; 37.45, 95%CI: 3.08-455.49, $p<0.01$) (**Table 4**). Neutrophil count ($\geq$4141 /μL) and infection risk at birth were not associated with the risk of BPD when adjusted for gestational age.

DISCUSSION

The notable finding of the present study was that preterm infants who developed BPD had higher concentration of serum NGAL at birth than those who did not. The NGAL levels of uncomplicated newborns correlated with the gestational age and neutrophil counts. On the other hand, the preterm infants having high NGAL values at birth had a higher risk of BPD than those having other variables studied. These results suggested that circulating NGAL levels of preterm newborns could be an early sensitive marker for developing BPD.

This is the first report that indicates the maturational increase of serum NGAL levels in the newborn infants. NGAL is produced mainly by neutrophils, and the plasma levels are expected to correlate with the total number of neutrophils in the body [9]. Neutrophil counts of the newborn infants physiologically increase as gestational age advanced, as the result of labor, placental G-CSF and catecholamine-stimulated demargination of neutrophils [14, 15]. Serum NGAL levels of uncomplicated full-term and preterm infants (<31 weeks' gestation) in the present study were higher and lower than those of healthy adults (median: 78.4 ng/mL, range: 37.9-190.8) [6], respectively. Non-stimulated neutrophils showed a significant expression of NGAL gene (*data not shown*). Taken together, the close association between serum NGAL levels and neutrophil counts in newborn infants having no underlying disease might indicate that the major source of NGAL in resting condition is circulating neutrophils. La Manna et al [7] reported that urinary NGAL levels at birth might be a biomarker of acute kidney injury in preterm infants, as well as children and adults. However, they revealed that serum NGAL levels collected 24 to 48 hours after birth did not predict the developing renal failure in the first week of life in preterm infants [7]. We found no correlation

between serum NGAL and creatinine levels at birth. These may indicate the different source of NGAL between serum and urine.

There are a few reports on the association between NGAL and BPD. Capoluongo et al [16] first documented an increased NGAL concentration in BALF on 3 days of life in preterm infants who developed BPD compared with those who did not. In premature rat models exposed to prolonged hyperoxia, lipocalin-2 gene expression was up-regulated on day 10 [17]. By contrast, Dik et al [18] reported that NGAL levels in BALF on postnatal day 2 were not different between BPD and non-BPD group, but those on postnatal day 4 were significantly higher in non-BPD group than BPD group. Sveger et al [19, 20] found that NGAL levels in BALF and plasma after 3 days of life were not correlated with the development of BPD. In these previous studies, all NGAL levels were evaluated after the second day of life, although preterm infants (especially <31 weeks' gestation) are generally treated with surfactant, antibiotics, steroids or non-steroid anti-inflammatory drugs and ventilator immediately after birth. NGAL has an elimination rate (t1/2) of 10-20 minutes [9]. Therefore, unless analysis of NGAL is employed immediately after birth as in our study, the results might not reflect the close relationship between the development of BPD and NGAL levels. Moreover, the dilution effects by using BALF samples might affect the discrepancy among the previous results. Peripheral blood neutrophils but not T-cells expressed NGAL gene, and the expression levels increased in response to LPS in a dose-dependent manner (*data not shown*). These results implied that intrauterine inflammation or other triggers in the innate immune response led to the increased production of NGAL from neutrophils of preterm infants. Miharada et al [21] reported that expression of NGAL was observed not only in granulocytes but also in monocyte/macrophage, hematopoietic stem/progenitor cells and

erythroblasts isolated from human cord blood. NGAL could be additionally produced from monocyte/erythroblast or respiratory epithelium [6, 21] in the inflammatory lung of preterm infants. Recent studies suggested that increased activity of lung MMP-9 was associated with the development of BPD in humans [4, 22] and animal models [23]. NGAL modulates MMP-9 activity by protecting it from degradation [24]. It may raise the possibility that sustained high production of NGAL until the delivery, being mainly produced by stimulated neutrophils, contribute to the early inflammation and subsequent disruption of the premature lung.

The limitations of our study were the small number of samples, and the lack of direct evidence for the increased production of NGAL in periphery and in the lung of infants who developed BPD. Further prospective studies are required to determine the pathophysiological role and the clinical impact of NGAL in the development of BPD.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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Figure 1

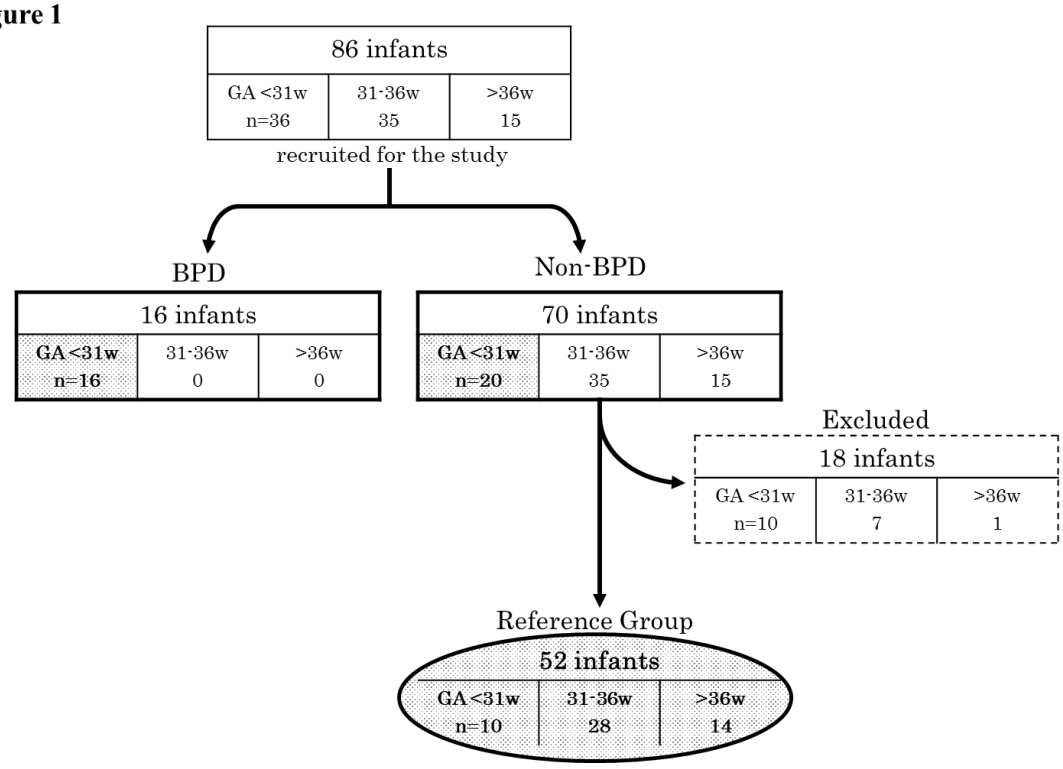

Figure 2

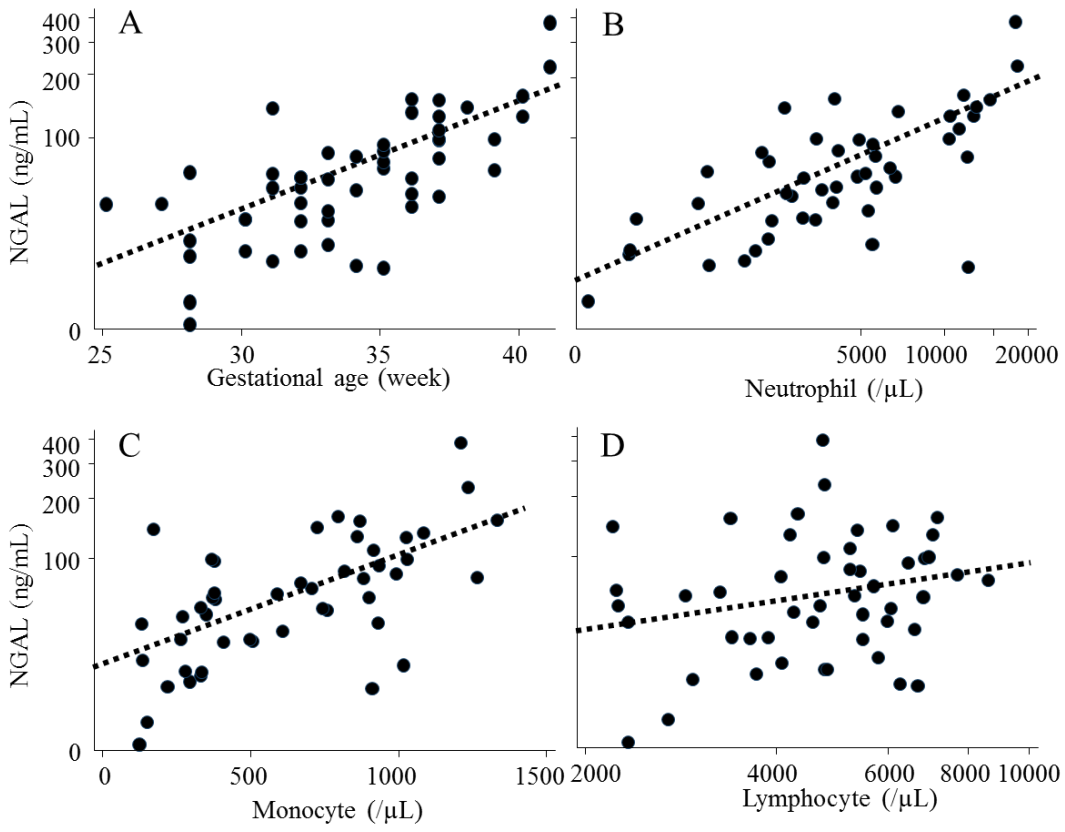

FIGURE LEGENDS

Figure 1. Flowchart of patients in this study. Of 86 infants studied, 36, 35 and 15 infants were born at <31, 31-36 and >36 gestational weeks, respectively. All 16 infants who developed BPD were born at <31 gestational weeks (gray column). Of 70 non-BPD patients, 20 infants were born at <31 gestational weeks (gray column). For the reference group, 52 infants were recruited after the exclusion of infants having the infection risk at birth and the complications (see METHODS). GA, gestational age; w, weeks; BPD, bronchopulmonary dysplasia.

Figure 2. Correlation of NGAL with the gestational age and leukocyte counts in 52 infants having no underlying disorders or complications. Serum NGAL levels at birth significantly correlated with gestational age (*A*, CC=0.6929, p<0.0001), peripheral blood neutrophils (*B*, CC=0.6591, p<0.0001), and monocytes (*C*, CC=0.6100, p<0.0001), but not lymphocytes (*D*, CC=0.2251, p=0.1280).

Table 1. Reference normal values of serum NGAL at birth by gestational weeks

		NGAL (ng/mL)	
Gestational age (weeks)		Mean	95% Confidence interval
<31	(n=10)	32.4	22.1 – 47.5
31 -36	(n=28)	58.6	47.9 – 71.8
>36	(n=14)	126.2	99.0 – 168.7

NGAL, neutrophil gelatinase-associated lipocalin.

Table 2. Demographics of 36 infants born < 31 gestational weeks

	BPD (n=16)		Non-BPD (n=20)		*p*-value
Gestational age (weeks)	24.8 (22.9 - 30.3)		28.2 (25.9 - 30.9)		<0.0001
Birth weight (gm)	707 (504 - 1470)		876 (548 - 1514)		0.171
Small-for-gestational age	0	(0)	8	(40)	0.004
Apgar score 5 min	5	(3 - 8)	8	(3 - 10)	0.001
Maternal hypertension	0	(0)	4	(20)	0.058
Antenatal steroids	9	(56)	15	(75)	0.236
Surfactant therapy	14	(88)	11	(55)	0.035
Early-onset sepsis	1	(6)	1	(5)	0.658
Infection risk at birth	11	(69)	7	(35)	0.044
PROM >72 hrs	3	(19)	6	(30)	0.439
Cloudy amniotic fluid	7	(44)	0	(0)	0.001
Histological CAM	5	(31)	1	(5)	0.036
CRP >0.1 mg/dL	6	(38)	0	(0)	0.003
IgM >20 mg/dL	2	(13)	1	(5)	0.419
Major complications other than BPD	16	(100)	4	(20)	<0.0001
Death before discharge	0	(0)	1	(5)	0.364
PDA ligation	6	(38)	1	(5)	0.039
Photocoagulation for ROP	6	(38)	1	(5)	0.039
Intestinal perforation	1	(6)	1	(5)	0.658

BPD, bronchopulmonary dysplasia; PROM, premature rupture of membrane; CAM, chorioamnionitis; CRP, C-reactive protein; PDA, patent ductus arteriosus; ROP, retinopathy of prematurity. No intraventricular hemorrhage or periventricular leukomalacia was found. All continuous variables expressed as median (range); dichotomous variables expressed as n (%). *p*-values from Wilcoxon rank-sum test (continuous variables) and Fisher's exact test (dichotomous variables).

Table 3. Parameters at birth of 36 infants born < 31 gestational weeks

	BPD (n=16)	Non-BPD (n=20)	*p*-value
NGAL(ng/mL)	307.8 (21.3 - 581.2)	42.9 (11.9 - 370.8)	<0.0001
WBC (/μL)	8290 (4700 - 46090)	5965 (3440 - 17830)	0.075
Neutrophil (/μL)	4141 (964 - 21137)	1357 (422 - 5790)	0.012
Lymphocyte (/μL)	3019 (1076 - 8723)	3753 (2207 - 8516)	0.235
Monocyte (/μL)	422 (109 - 1150)	205 (49 - 652)	0.085
pH	7.287 (7.050 - 7.718)	7.307 (7.044 - 7.555)	0.524
Base exess (mmol/L)	-6.2 (-15.6 - 0.8)	-5.0 (-21.3 - 3.7)	0.260
$CD3^+/HLA\text{-}DR^+$ cell (/μL)	68 (21 - 387)	119 (4 - 535)	0.245
$CD3^+/CD45RO^+$ cell (/μL)	168 (29 - 509)	215 (12 - 545)	0.727
$CD69^+/CD19^+$ cell (/μL)	28 (7 - 134)	19 (2 - 75)	0.161
$CD69^+/CD56^+$ cell (/μL)	45 (0 - 202)	36 (3 - 134)	0.743
$CD69^+/CD3\gamma\delta^+$ cell (/μL)	1 (0 - 6)	1 (0 - 10)	0.747
$CD69^+/CD3\gamma\delta^-$ cell (/μL)	37 (7 - 317)	24 (13 - 54)	0.302

NGAL, neutrophil gelatinase-associated lipocalin; WBC, white blood cell.

All variables expressed as median (range). *p*-values from Wilcoxon rank-sum test.

Table 4. Risk of developing BPD according to NGAL level, neutrophil count, infection risk at birth and gestational age

Variables		N	Crude odds ratio	95% Confidence interval Lower	Upper	*p* -value	Adjusted* odds ratio	95% Confidence interval Lower	Upper	*p* -value
NGAL (ng/mL) **	<82	18	1.00	Reference			1.00	Reference		
	≥82	18	85.00	7.97 -	906.81	<0.01	37.45	3.08 -	455.49	<0.01
Neutrophil (/μL) **	<4141	19	1.00	Reference			1.00	Reference		
	≥4141	5	21.33	1.73 -	263.7	0.017	5.01	0.21 -	119.39	0.319
Infection risk at birth	No	18	1.00	Reference			1.00	Reference		
	Yes	18	4.09	1.01 -	16.58	0.049	4.06	0.63 -	26.20	0.141
Gestational age (wks)	≥27	20	1.00	Reference			-	-	-	-
	<27	16	24.56	4.24 -	142.11	<0.01	-	-	-	-

* Adjusted for gestational age. ** An optimal cut-off point based on receiver operating characteristics curve. *p*-values from logistic regression analysis.