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Cord blood erythropoietin and interleukin-6 for prediction of intraventricular hemorrhage in the preterm neonate

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Abstract

Objective—To evaluate cord blood erythropoietin (EPO) and interleukin-6 (IL-6) levels to predict preterm infants at risk of developing intraventricular hemorrhage (IVH).

Methods—Levels of umbilical cord EPO, acid–base status and IL-6 were analyzed in 116 consecutive, preterm newborns (GA at delivery: 29 [23–34] weeks) born to mothers who had a clinically indicated amniocentesis to rule out infection. Early-onset neonatal sepsis (EONS) was diagnosed using symptoms, hematological criteria and blood cultures.

Results—IVH was diagnosed by cranial ultrasounds. The prevalence of IVH in our population was 25% (29/116). There was a direct relationship between cord blood EPO and cord blood IL-6 concentration ($r = 0.225$, $p = 0.014$), independent of GA at birth. Elevated cord blood EPO levels ($r = 0.182$, $p = 0.016$) and GA birth at birth ($r = –0.236$, $p = 0.004$) remained significant independent factors associated with the risk of IVH, when evaluated with stepwise logistic regression analyses. Cord blood IL-6, pH, and EONS were not associated with IVH. These relationships remained following correction for GA at birth ($p = 0.027$).

Conclusions—Our results suggest that elevation in cord blood EPO may predict newborns at risk for IVH, independent of fetal inflammatory status. Further studies are warranted to confirm this association.

Keywords

Erythropoietin; IL-6; premature; newborn; IVH

Introduction

In the premature neonate, intraventricular hemorrhage (IVH) is a major cause of morbidity and mortality. The relevance of IVH as a major clinical problem is emphasized by the observation that in the USA, 20–25% of all very low birth weight (VLBW) infants are

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diagnosed with IVH [1]. Among these infants, 10–15% have severe grades (grades 3–4) of IVH, and 75% of this sub-group develop mental retardation or cerebral palsy [1]. Annually, >3600 new cases of mental retardation are attributable to IVH, with lifetime care costs exceeding $3.6 billion [1].

The pathogenesis of IVH is multifactorial with a variety of genetic and environmental factors being implicated [1–3]. Among the latter, the antenatal factors include early gestational age (GA), absence of antenatal steroid exposure, maternal chorioamnionitis, and early-onset neonatal sepsis (EONS). Postnatal factors include respiratory distress syndrome (RDS), and hypotension requiring pressors [1–3].

An effective preventive approach to IVH could have significant clinical implications. Among antenatal interventions, use of steroids has proved to be beneficial [4–6]. A variety of postnatal preventive clinical and pharmacologic interventions have been tried [1]. Among them, indomethacin has been found to be most effective in decreasing the rate of IVH [7,8]. However, indomethacin appears to mostly impact on the more severe grades of IVH [7,9]. In addition, indomethacin does not appear to consistently improve long-term neurodevelopmental outcomes [9,10].

In a majority of cases, IVH occurs within the first 24 h of birth, and may progress over the next 48 h [1]. Biomarkers with the potential to identify the preterm neonate at high-risk for IVH, could allow for early and more effective targeted therapy to prevent this serious morbidity. For clinical relevance, such a biomarker would need to be detected at or soon after birth.

Maternal and/or intra-uterine infection has been suggested to possibly be associated with IVH, mostly in relation to histologic chorioamnionitis [11]. Among proinflammatory cytokines, cord blood interleukin-6 (IL-6) has not been consistently shown to be associated with IVH [12–14].

Erythropoietin (EPO) has been previously proposed as a potential marker for central nervous system (CNS) injury in neonates with asphyxia, meningitis, and IVH [15]. In this study, mean cerebrospinal fluid (CSF) EPO levels were higher in neonates with IVH, compared to controls [15]. Interestingly, in 11 patients with IVH, CSF EPO concentrations obtained closer to birth (and presumably to the time of hemorrhage) tended to be higher, as compared to those obtained later [15]. Plasma EPO levels of these patients with IVH were not significantly different from controls [15]. These results may have been affected by the small sample size, and variability in the timing of collection of samples that were obtained from neonates who had an IVH within 1 week before the spinal tap [15]. Several premature babies with culture-proven or clinically presumed meningitis were also studied in the aforementioned study; however, it was not reported whether these neonates were delivered in the setting of intra-amniotic infection/inflammation [15]. This distinction is important because as shown in humans and animal models of sepsis, intra-amniotic inflammation may sensitize the fetus to postpartum stressors or initiate early brain hemorrhage or IVH via cytokine effects on cardiovascular instability [16,17].

The above observation led us to hypothesize that cord blood IL-6 and/or EPO concentrations in preterm neonates exposed to intra-amniotic inflammation/infection are higher in infants who develop IVH. The objective of the present study was to evaluate, in a prospective fashion, the cord blood IL-6 and EPO concentrations of babies delivered in the setting of intra-amniotic inflammation-related preterm birth, and its' relationship to IVH.

Material and methods

Study population and research design

We prospectively studied 116 consecutive preterm neonates born to mothers who had amniocenteses to rule out intra-amniotic infection. Women were recruited following admission to the Labor and Birth or to the Maternal Special Care antepartum units at Yale New Haven Hospital (YNHH). The decision to perform amniocenteses was made by clinicians responsible for the care of the patient, independent of our study protocol. The Human Investigation Committee of Yale University approved our study.

Amniotic fluid (AF) was collected by ultrasound-guided amniocentesis for all the participants and each woman was followed prospectively up to the point of delivery. Inclusion criteria included: GA ≥ 23.1 weeks, symptoms of preterm labor, preterm premature rupture of membranes (PPROM), advanced cervical dilatation (≥3 cm), and/or uterine contractions intractable to tocolysis. Women with anhydramnios, human immunodeficiency or hepatitis viral infections were not enrolled. GA was determined based on last menstrual period and ultrasound information obtained prior to 20 weeks of gestation [18]. Preterm labor was defined as the presence of regular uterine contractions and documented cervical effacement and/or dilatation in patients <37 weeks GA. The diagnosis of PPROM was confirmed by vaginal AF “pooling”, “nitrazine”, “ferning” or by an amniocentesis-dye positive test. No digital exams were permitted, and in the absence of infection, PPROM was managed expectantly. Patients received corticosteroids for fetal lung maturity and antibiotic therapy in accordance to the American Congress of Obstetrics and Gynecology (ACOG) recommendations [19]. Delivery was performed for routine obstetrical indications and the decision to deliver was made by the clinical team responsible for the care of the patient. The neonatology resuscitation team was present at the time of delivery for all cases.

AF was cultured for aerobic and anaerobic bacteria, *Ureaplasma urealyticum* and *Mycoplasma hominis*. Additional clinical laboratory tests performed for the purpose of diagnosing infection/inflammation included: glucose, lactate dehydrogenase (LDH), Gram stain, and white blood cell (WBC) count. For clinical management, an AF glucose cut-off of ≤15 mg/dl and LDH levels ≥419 U/l were considered suggestive of intra-amniotic infection [20,21]. AF not used for clinical purposes was spun at 3000*g* at 4°C for 20 min, aliquoted and immediately stored at –80°C until IL-6 levels were measured by a specific and sensitive immunoassay.

Umbilical cord blood

Umbilical cord blood was obtained by aseptic puncture of the clamped umbilical vein at the time of delivery. Acid–base status was determined within 10 min of delivery. Immediately following collection, the cord blood was centrifuged at 1000*g* for 15 min. Serum was aliquoted in sterile polypropylene tubes and stored at –80°C until IL-6 and EPO levels were examined.

Immunoassays for IL-6 and EPO

Enzyme-linked immunosorbent assays for human IL-6 (Pierce-Endogen, Rockford, IL) and EPO (R&D Systems, Minneapolis, MN) were performed in duplicate according to manufacturers’ instructions by investigators unaware of the AF and umbilical cord blood sample origin. The minimal detectable concentration for the umbilical cord and AF IL-6 was 1 pg/ml. The minimal detectable concentration for EPO was 0.6 mIU/ml. The inter- and intra-assay coefficients of variation were <10%.

Evaluation of early-onset neonatal sepsis

Neonatal hematological indices and sepsis categorization were assessed from blood specimens and cultures obtained within 2 h from the time of birth by an investigator (VB) unaware of the results of the umbilical cord IL-6 levels. All 116 neonates were admitted to the Yale Newborn Special Care Unit (NBSCU). EONS was defined as the presence of confirmed or suspected sepsis at ≤72 h after birth. A diagnosis of EONS was based on clinical symptoms corroborated with hematological laboratory results [22,23]. Any two or more of the following hematological criteria were used as indicators of EONS [23]: (1) absolute neutrophil count of <7500 or >14,500 cells /mm^3; (2) absolute band count >1500 cells/mm^3; (3) immature/total neutrophil (I:T) ratio >0.16; (4) platelet count <150,000 cells/mm^3. EONS was dichotomized into present (when sepsis was either confirmed or suspected) or absent. All neonates with confirmed or suspected sepsis received antibiotic therapy, per institutional protocol.

Evaluation of IVH

This was done using cranial ultrasounds, as per nursery protocol, and read by experienced pediatric radiologists. Routinely, the first scans are done by day of life (DOL) 3, and repeated at DOL 7–10 and DOL 30. Additional scans are done if clinically indicated. We used the worst grade of IVH for our analyses.

Statistical analysis

Statistical analyses were performed with Sigma Stat, version 2.03 (SPSS Inc, Chicago, IL) and MedCalc (Broekstraat, Belgium) statistical software. Normality testing was performed using the Kolmogorov–Smirnov test. Data were compared with one-way ANOVA followed by Dunnet's tests (parametric) or Mann–Whitney Rank Sum Test, or Kruskal–Wallis on ranks followed by Dunn's tests (non-parametric), to adjust for multiple comparisons as appropriate. The IL-6 concentrations were presented as arithmetic means with inter-quartile range. Statistical analysis was completed before (Kruskal–Wallis ANOVA) or after (one-way ANOVA) logarithmic transformation of data. Pearson correlations were used to measure co-linearity between the selected independent variables as well as other relevant relationships between dependent and independent variables. Comparisons between proportions were done with Chi-square tests. Stepwise multivariable regression analysis was used to determine concurrent relationships between variables and to correct for possible influences of GA and BW. A p value of <0.05 was considered significant throughout the analysis.

Results

The demographics and clinical characteristics of the maternal and neonatal study population are presented in Table I. The prevalence of IVH in our population was 25% (29/116). Mothers of infants who developed IVH were more likely to have a lower GA at enrollment ($p = 0.006$) and evidence of intra-amniotic infection (positive AF cultures) ($p = 0.04$) and inflammation as reflected by the AF IL-6 ($p = 0.0007$). Infants without IVH were more likely to be delivered via cesearean section, have significantly higher GA, BW, and Apgar scores at 1 and 5 min (all $p \leq 0.02$).

Table II shows the results of umbilical vein IL-6 and EPO levels. While there were no differences in the EPO levels, the cord blood IL-6 values were significantly elevated in infants with IVH ($p = 0.004$). Since intra-amniotic infection/inflammation increases the risk of a fetal inflammatory status [24], we analyzed the hematological indices from the infants' blood, done as part of their sepsis evaluation. Infants with IVH had significantly lower hematocrits ($p = 0.003$), but elevated absolute band count ($p = 0.035$) and I:T ratios ($p =$

0.007). EPO levels were not different based on the route of delivery (cesarean section versus vaginal delivery, $p = 0.454$), and did not significantly correlate with Apgars at 1 ($r = –0.101$, $p = 0.282$) or 5 min ($r = –0.049$, $p = 0.607$).

The cord blood gases were available for 85 neonates (Table III). Our analysis demonstrated that the pO_2 and O_2 saturation values were significantly higher, while pCO_2 and HCO_3 values significantly lower, in the umbilical vein of infants who developed IVH. At birth, none of the infants had either acidemia or hypoxia as defined by the ACOG criteria [25]. IL-6 levels were significantly higher in the infants who developed IVH, with no difference in EPO values, in this subset, similar to the entire study cohort.

When examining the relationships with GA at birth we found that cord blood IL-6 levels were dependent and inversely correlated ($r = –0.298$, $p = 0.001$) with GA at birth. In contrast, cord blood EPO concentration appeared to vary independently of GA at birth ($r = 0.131$, $p = 0.162$).

Relationship between severity of IVH and IL-6 and EPO values

The distribution of the severity of IVH in our study population, and the IL-6 as well as EPO levels in the respective categories have been shown in Table IV. There was a significant difference between no IVH (Grade 0) and severe (Grades 3–4) IVH, for both analytes (Table IV).

Relationships between IVH, EPO, EONS, and IL-6

There was a direct relationship between cord blood EPO and cord blood IL-6 concentration ($r = 0.225$, $p = 0.014$) (Figure 1), independent of GA. However, in multivariate regression analysis, elevated cord blood EPO levels, but not cord blood IL-6, pH and EONS status, was the strongest predictor of IVH. These relationships remained following correction for GA ($p = 0.027$). Table V shows the results of stepwise logistic regression analysis. The model included IVH (dichotomized) as dependent variable and GA at delivery along with BW, IL-6, and EPO as independent variables. When our outcome data were evaluated by using stepwise logistic regression analyses, elevated cord blood EPO levels ($r = 0.182$, $p = 0.016$) and GA at birth ($r = –0.236$, $p = 0.004$) remained significant independent risk factors associated with the risk of IVH. Cord blood IL-6 and BW were excluded from the model ($p > 0.1$ for both).

Discussion

The prevalence of IVH in our population mirrors what has been reported nationally [1]. While a variety of factors [1–3,26,27] have been associated with the risk of developing IVH, a majority of them (VLBW, RDS, severity of illness) are related to the GA at delivery. In our study, however, the association of IVH with cord blood EPO levels remained significant after correcting for GA. Cesarean delivery has not been shown to impact on IVH [28], and our study noted a higher incidence of Cesarean-section delivery in infants without IVH. As noted in Table I, we also did not find differences in antenatal steroid exposure or gender [29] in the infants with or without IVH in our study cohort.

The cord blood EPO levels reported in our study in the infants with IVH ranged from 1.6 to 184.7 mIU/ml, similar to the plasma levels (4.7 to 164.2 mU/ml) reported in the earlier study [15]. In that study, no differences were found in patients with IVH versus controls; however, the sample size was small and the timing of collection of samples was quite variable [15]. Interestingly, we did find that severe IVH (Grades 3–4) had higher levels of cord blood IL-6 and EPO, compared to infants with no IVH (Table IV).

While we did not have detailed information about hypotension [30] or postnatal blood gases collected in our study cohort, these and other commonly cited clinically-relevant factors were not found to be significant risk factors for severe IVH [3]. It is also important to point out that our NBSCU utilized the indomethacin protocol for prophylaxis against IVH [7,8] during the study period.

With regards to IL-6 levels and IVH, elevated umbilical vein levels (median: 87; 25–75 centile: 30–310 pg/ml) in VLBW infants ($n = 69$) with IVH, versus those without (median: 0; 25–75 centile: 0–4 pg/ml) have been reported [31]. Increased post-natal serum IL-6 values have also been suggested as a marker for IVH [32]. Our results are in accord with these studies. However, following correction for GA we found that IL-6, pH and EONS were not associated with IVH.

While one study has shown an association of IVH with severe acidemia (pH < 7) at birth [33], the majority of studies have reported that cord blood gas analyses are not helpful in predicting IVH in VLBW infants [3,16,34,35]. Our blood gas data results support that latter contention.

Hypoxia could be implicated as a potential mechanism, since EPO is well known to increase in the CNS following hypoxia [15]. Elevated circulating nucleated erythrocytes (NRBCs) in the newborn period can be a marker of chronic fetal hypoxia and levels measured in the first 6 days of postnatal life have been associated with IVH [36]. In the absence of hypoxia, our earlier work has noted that elevations in NRBCs in the early neonatal period may be a direct response of exposure to inflammatory mediators *in utero* [37]. In our cohort, however, there was no difference in NRBC counts between newborns with and without IVH (Table II). In addition, the data on cord blood gases also do not support an acute hypoxic perinatal event in our study cohort.

The lack of association of EONS with IVH in our study are in accord with a recent study in 125 premature newborns, where funisitis (OR 1.6, $p = 0.06$) and fetal inflammation (OR 2.6, $p = 0.06$) were not associated with early hemorrhage [12]. In that study, fetal inflammation was defined as cord blood IL-6 concentrations >7.6 pg/ml [12]. The data on the association of histological chorioamnionitis with IVH is controversial [38–40].

In VLBW infants, most of the literature regarding the CNS has focused on the use of recombinant EPO as a neuroprotective strategy, specifically, against hypoxic-ischemic encephaolopathy [41] or IVH and periventricular leukomalacia [42]. We are unaware of any previous study evaluating cord blood EPO levels in relationship to IVH in VLBW infants. Our data, however, are supported by the earlier report of the levels and timing of collection in CSF in infants with IVH [15]. Most importantly, our results remained true even after adjusting for GA.

In conclusion, our results suggest that the level of cord blood EPO, in association with GA at birth, may predict newborns at risk for IVH, independent of fetal inflamma-tory status. Further studies are warranted to confirm this association.

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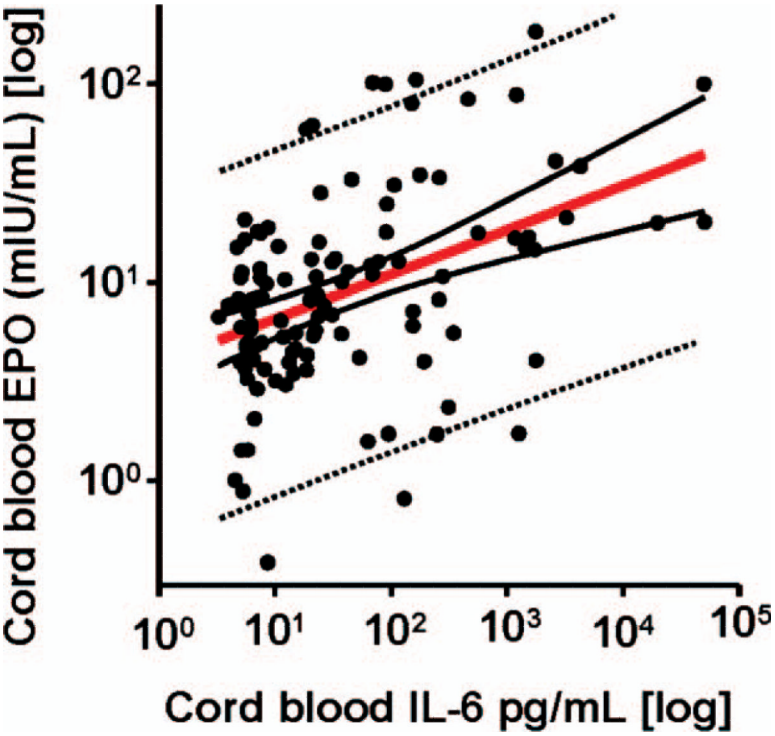

Figure 1.
Relationship between the umbilical cord blood interleukin-6 (IL-6) and erythropoietin (EPO) levels. Distribution of the cord blood IL-6 (*y*-axis) in relationship to the EPO on the *x*-axis. Data presented in logarithmic format. (pg, picograms; mIU, milli international units; ml, milliliters). The regression line (solid red) and 95% confidence (solid black lines) and prediction (dotted black lines) intervals are also shown ($n = 116$).

Table I

Demographic and clinical characteristics of women and their newborns.

	IVH		
Variable	**No, *n* = 87**	**Yes, *n* = 29**	***p*-value**
Characteristics at enrollment			
Maternal age, years★	29 [23–35]	26 [20–32]	0.182
Maternal parity★	1 [0–2]	0 [0–1]	0.063
Non-Caucasian race, *n* (%)†	48 (55)	22 (76)	0.080
Product of twin gestation, *n* (%)†	12 (14)	6 (21)	0.554
Gestational age at enrollment, weeks★	28 [25–30]	26 [24–28]	0.006
PPROM, *n* (%)†	52 (60)	13 (45)	0.235
Clinical chorioamnionitis, *n* (%)†	6 (7)	7 (24)	0.027
Steroid exposure during pregnancy, *n* (%)†	79 (91)	29 (100)	0.204
Intra-amniotic infection (positive cultures), *n* (%)†	34 (39)	18 (62)	0.044
Intra-amniotic inflammation IL-6, ng/ml★	6.8 [1.4–23.7]	24.6 [6.5–98.8]	0.007
Outcome characteristics			
Gestational age at delivery, weeks★	29 [26–30]	26 [25–29]	0.011
Amniocentesis-to-delivery, days★	0.5 [0.2–2.2]	0.4 [0.2–0.8]	0.747
Birth weight, grams★	1,190 [849–1,503]	955 [730–1,225]	0.018
Cesarean delivery, *n* (%)†	51 (59)	9 (31)	0.018
Apgar score 1 min,★	7 [5–8]	4 [2–6]	0.001
Apgar score 5 min,★	8 [8–9]	6 [6–8]	< 0.001
Male sex, *n* (%)†	43 (50)	16 (55)	0.748
Histological chorioamnionitis stages II & III, *n* (%)†	49 (56)	21 (72)	0.189
Histological funisitis grades 1–4, *n* (%)†	37 (43)	16 (55)	0.333
Early-onset neonatal sepsis (EONS), *n* (%)†	21 (24)	13 (45)	0.060
EONS confirmed by blood cultures, *n* (%)†	3 (3)	3 (10)	0.333

PPROM, preterm premature rupture of membranes; AF, amniotic fluid.

★Data presented as median [interquartile range – IQR] and analyzed by Mann–Whitney tests.

†Data presented as *n* (%) and analyzed by Chi-square tests.

Table II

Cord blood IL-6 and EPO levels with hematological indices for evaluation of sepsis in infants.

	IVH		
Variable	**No, *n* = 87**	**Yes, *n* = 29**	***p*-value**
Umbilical vein research analytes			
Interleukin-6, pg/ml★	12.4 [6.6–69.2]	89.3 [14.9–724.5]	0.004
Erythropoietin, mIU/ml★	8.0 [4.0–15.1]	8.2 [5.2–21.2]	0.287
Hematological indices			
Hematocrit, %★	46 [43–50]	41 [39–48]	0.003
Hemoglobin, g/dl★	15 [14–16]	13 [12–15]	0.002
WBC, cells × 1000/mm^3★	10 [8–14]	8 [6–17]	0.386
Platelets, cells × 1000/mm^3★	242 [212–308]	233 [198–288]	0.241
Segmented, %★	33 [24–43]	28 [21–34]	0.048
Lymphocytes, %★	41 [29–57]	42 [26–56]	0.962
ANC, cells/mm^3★	3,529 [2,091–5,875]	2,323 [1,206–5,600]	0.154
ABC, cells/mm^3★	336 [146–1,177]	910 [409–2,455]	0.035
I:T ratio, %★	4 [1–12]	12 [4–20]	0.007
NRBC, cells/mm^3★	18 [13–33]	20 [13–33]	0.382

ANC, absolute neutrophil count; ABC, absolute band count; I:T, immature/total neutrophil count; NRBC, nucleated red blood cells.

★Data presented as median [interquartile range – IQR] and analyzed by Mann–Whitney tests.

Table III

Umbilical cord blood analysis.

	IVH		
Variable	**No, *n* = 69**	**Yes, *n* = 16**	***p*-value**
Umbilical artery clinical gas analysis			
pH★	7.3 [7.2–7.4]	7.3 [7.3–7.3]	0.926
pO_2, mmols/l★	22 [18–33]	28 [25–35]	0.048
O_2 sat, %★	47 [27–63]	59 [405–755]	0.133
pCO_2, mmols/l★	42 [37–48]	44 [40–48]	0.854
HCO_3, mmols/l★	21 [19–22]	22 [20–23]	0.344
Base deficit, mmols/l★	4.9 [6.2–3.6]	4.7 [5.3–3.7]	0.493
Umbilical vein clinical gas analysis			
pH★	7.4 [7.3–7.4]	7.4 [7.3–7.4]	0.971
pO_2, mmols/l★	31 [24–40]	39 [33–54]	0.004
O_2 sat, %★	65 [40–80]	83 [65–93]	0.013
pCO_2, mmols/l★	38 [36–41]	33 [29–38]	0.030
HCO_3, mmols/l★	21 [19–22]	19 [17–21]	0.005
Base deficit, mmols/l★	4.0 [4.9–2.0]	4.3 [6.7–3.7]	0.051
Umbilical vein research analytes			
Interleukin-6, pg/ml★	12.4 [6.8–56.7]	201.2 [25.3–1,651]	< 0.001
Erythropoietin, mIU/ml★	7.2 [4.1–15.6]	12.3 [5.7–39.9]	0.139

★Data presented as median [interquartile range – IQR] and analyzed by Mann–Whitney tests.

Table IV

IL-6 and EPO levels and severity of IVH.

IVH groups	*n*	IL-6, pg/ml‡	EPO, mIU/ml‡
Grade 0	87	12.4 [6.6–69.2]	8.0 [4.1–15.2]
Grade 1	7	21.4 [8.6–30.0]	6.0 [4.3–7.5]
Grade 2	9	89.3 [13.8–109.4]	8.6 [4.3–43.8)
Grades 3–4	13	563.0 [53.2–3026.6]★	17.0 [7.6–39.3)★★

IVH, intraventricular hemorrhage; EPO, erythropoietin; IL-6, interleukin-6.

‡Data presented as median [interquartile range – IQR] and analyzed by Mann–Whitney tests.

★$p < 0.001$, vs. Grade 0

★★$p = 0.037$, vs. Grade 0.

Table V

Logistic regression analyses for predicting IVH.

Variables	*R*	*p* value
GA at delivery	–0.236	0.004
CB EPO	0.182	0.016
CB IL-6	0.135	>0.1
Birth weight	–0.219	>0.1

GA, gestation age; CB, cord blood; EPO, erythropoietin; IL-6, interleukin-6.