Immune and Inflammatory Proteins in Cord Blood as Predictive Biomarkers of Retinopathy of Prematurity in Preterm Infants

Young Joo Park,[1,2] Se Joon Woo,[1] Yu Mi Kim,[3] Subeen Hong,[3] Young Eun Lee,[3] and Kyo Hoon Park[3]

[1]Department of Ophthalmology, Seoul National University College of Medicine, Seoul National University Bundang Hospital, Seongnam, Korea
[2]Department of Ophthalmology, SMG-SNU Boramae Medical Center, Seoul, Korea
[3]Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul National University Bundang Hospital, Seongnam, Korea

Correspondence: Kyo Hoon Park, Department of Obstetrics and Gynecology, Seoul National University Bundang Hospital 82, Gumi-ro 173 Beon-gil, Seongnamsi, Kyeonggido 463-707, Korea; pkh0419@snubh.org.

YJP and SJW contributed equally to this work and should therefore be regarded as equivalent authors.

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Purpose. To determine whether elevated levels of immune/inflammatory proteins in cord blood, alone or in combination with conventional clinical parameters, can predict the occurrence and progression of retinopathy of prematurity (ROP) in preterm infants.

Methods. This was a retrospective cohort study of 110 premature singleton infants who were born at ≤32.0 weeks. Cord plasma at birth was assayed for interleukin-6, C3a, C5a, matrix metalloproteinase-2 (MMP-2), MMP-9, tissue inhibitor of metalloproteinase-1, macrophage colony-stimulating factor, endostatin, a proliferation-inducing ligand, insulin-like growth factor-binding protein-1 (IGFBP-1), IGFBP-2, and calcium-binding protein A8/A9 complex levels. The primary outcome measures were the occurrence of any stage ROP, severe ROP (>stage 3), and vision-threatening type 1 ROP requiring laser treatment.

Results. ROP was diagnosed in 30 of 110 infants (27.3%), including 14 (12.7%) with severe ROP. Laser treatment was performed on 7 infants (6.4%). Multiple logistic regression analyses indicated that elevated levels of cord plasma IL-6 were significantly associated with severe ROP, whereas elevated levels of cord plasma C5a were significantly associated with ROP laser treatments. However, none of the proteins measured in the cord plasma were associated with ROP occurrence. Using a stepwise regression procedure, we developed a combined prediction model, which included high cord plasma IL-6 levels and low birth weight for severe ROP (area under the curve [AUC], 0.840), and high cord plasma C5a levels and low birth weight for laser treatment (AUC, 0.884).

Conclusions. Elevated levels of cord plasma IL-6 and C5a could be used as independent markers to predict severe ROP and laser treatment, respectively, with combined models predicting ROP progression with good accuracy.

Keywords: cord blood, preterm, proteins, retinopathy of prematurity

Retinopathy of prematurity (ROP), which is caused by the insults that disrupt neurovascular growth in immature retinas of preterm neonates, is a leading cause of childhood blindness worldwide.[1] Furthermore, it is considered a complex disease with multiple etiologies, having an overall incidence rate of 30% for very preterm infants (<32 weeks).[1,2] Importantly, significant evidence exists to indicate that earlier detection and treatment of ROP through screening for high-risk neonates dramatically decreased the incidence of irreversible vision loss and significantly reduced unfavorable outcomes.[3,4] Therefore, it is of great importance, for salvaging vision in preterm neonates with ROP, to identify biomarkers for early and more accurate prediction of ROP in high-risk neonates (especially during the prenatal period), thus leading to improved screening, prevention, and timely therapeutic interventions.

A number of significant perinatal and postnatal risk factors associated with ROP have been identified, such as low gestational age, low birth weight, postnatal weight gain, and high oxygen supplementation.[1,5,6] However, little is currently known concerning prenatal risk factors related to ROP. In this respect, the evaluation of biomarkers in preterm newborns through the analysis of umbilical cord blood can be useful and predictive for the risk of ROP linked to events occurring in utero because cord blood at birth can directly reflect the effect of the intrauterine milieu on the fetus (i.e., hypoxia, stress, injury, and infection/inflammation). In fact, in the setting of neurologic, pulmonary, and auditory injuries induced by prenatal insults in preterm infants, several studies have reported a number of cord blood biomarkers that can be useful in identifying neonates at high risk for these diseases.[7–9] However, only very few investigations were conducted regarding the relationship between protein levels in cord blood at birth and ROP[10–12] and were limited by a small number of participants, a lack of controls for known ROP risk factors, a small number of target proteins (e.g., cytokines and growth factors), and restricting the outcome to only ROP occurrences. The purpose of this study was to

determine whether elevated levels of various immune and inflammatory proteins in cord blood are associated with an increased risk of the occurrence and progression of ROP in preterm infants and to develop the best combined model for predicting severe ROP by using these biomarkers in combination with conventional clinical parameters.

Methods

Study Design

This single-center, retrospective cohort study included singleton preterm infants at Seoul National University Bundang Hospital (Seongnam, Korea) between June 2004 and September 2016. The inclusion criteria were (1) 24.0 to 32.0 weeks gestation at birth, (2) survival until 36 weeks postmenstrual age, (3) underwent ROP screening examinations, (4) absence from major structural or chromosomal abnormalities, and (5) availability of cord plasma sample (1 aliquot) for analysis. Gestational age was determined based on the last menstrual period and confirmed by ultrasound in the first or second trimester (≤20 weeks). The Institutional Review Board of Seoul National University Bundang Hospital approved this study (no. B-1006/103-102); the parents gave written informed consent for the collection and use of cord blood samples and clinical information for research purposes. This study was performed in accordance with the tenets of the Declaration of Helsinki.

ROP Screening examination

We followed the ROP screening guidelines that were proposed by the American Academy of Pediatrics and Ophthalmology and Pediatrics, and the Association for Pediatric Ophthalmology and Strabismus.[13,14] The timing of the initial examination was either 4 weeks after birth or 31 weeks of postmenstrual age, whichever was later. The follow-up schedules and the treatment decision adhered to the indications proposed through the Early Treatment for Retinopathy of Prematurity study results.[4,15] Intravitreal antivascular endothelial growth factor (VEGF) treatment, such as bevacizumab, was not considered for the initial treatment. The stage of ROP was graded as the highest stage observed on fundus examination during the entire follow-up periods. Severe ROP included the ROP stages 3, 4, and 5, whereas mild ROP included stages 1 and 2. The outcome parameters were the occurrence of any stage ROP, severe ROP, and clinically significant ROP requiring laser treatment (type 1 ROP).

Clinical Data and Definitions of Various Factors

Data on maternal, perinatal, and neonatal parameters were extracted from the obstetric and neonatal database, as described previously.[10] Acute histologic chorioamnionitis was defined as the presence in any tissue sample (chorionic plate, amnion, umbilical cord, or chorion-decidua) of acute inflammatory change by using previously published criteria.[16] Funisitis was defined as the presence of neutrophil infiltration into the umbilical vessel walls or Wharton's jelly. Clinical chorioamnionitis, intraventricular hemorrhage (IVH), bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), periventricular leukomalacia (PVL), respiratory distress syndrome (RDS), and proven sepsis were diagnosed in accordance with previously proposed criteria.[17]

Analysis of Various Proteins in the Cord Plasma

At birth, cord blood was collected from the umbilical vein into ethylenediaminetetraacetic acid tubes. The samples were centrifuged at 1500*g* at 4°C for 10 minutes, and the plasma was aliquoted for storage at −70°C until assayed. The concentration of interleukin-6 (IL-6) (R&D Systems, Minneapolis, MN, USA), matrix metalloproteinase-2 (MMP-2), MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1), macrophage colony-stimulating factor (M-CSF), endostatin, a proliferation-inducing ligand (APRIL), insulin-like growth factor-binding protein-1 (IGFBP-1), IGFBP-2, calcium-binding protein A8/A9 complex (S100 A8/A9) (DuoSet enzyme-linked immunosorbent assay [ELISA]; R&D Systems), and complements C3a and C5a (BD Biosciences, San Diego, CA, USA) in the stored cord plasma samples were determined using ELISA kits in accordance with the manufacturers' instructions. These proteins were selected for the study based on the fact that they are crucial regulators of inflammation, infection, immune response, oxidative stress, and hypoxia-inducible mechanisms (https://www.uniprot.org/, in the public domain), which may be important for the pathogenesis of ROP.[1,18] The ranges of the protein standard curves and their dilution ratio are described in detail in the Supplementary Materials. The intra-assay and interassay coefficients of variation were <15% for all analyzed proteins.

Statistical Analysis

Continuous data were analyzed using the Student's *t*-test or the Mann-Whitney *U* test, whereas categorical data were compared using the χ^2 test or Fisher's exact test, as appropriate. The normality of the data was tested using the Shapiro-Wilk test. A multivariate logistic regression model was further conducted to assess the independent relationship of the level of each protein in cord plasma with severe ROP and laser treatment, after controlling for baseline risk factors, with a $P < 0.05$ in univariate analysis. In the logistic regression model, continuous data were transformed into dichotomous data to decrease the problem of multicollinearity, especially between the gestational age at birth and cord plasma IL-6 (r = −0.357) or for prediction and/or decision-making purposes. Receiver operating characteristic (ROC) curves were generated for each protein in the cord plasma and clinical risk factors for predicting severe ROP and laser treatment and were used for identifying the best cutoff values (maximizing the sum of sensitivity and specificity) for dichotomization. Additionally, to determine the best detection model for severe ROP and laser treatment, a forward stepwise variable selection procedure was performed in which all predictive variables with a $P < 0.05$ from the univariate analysis were introduced as dichotomous variables. Areas under the ROC curves (AUCs) were computed for each protein in the cord plasma, clinical risk factors, and the best predictive model and compared using the method of DeLong et al.[19] The correlation among the nonnormally distributed continuous variables was analyzed with Spearman's rank correlation test. All data were analyzed using SPSS version 22.0 for Windows (IBM SPSS Statistics, Chicago, IL, USA). All reported *P* values were two-sided, with a significance level of 0.05.

Results

During the study period, a total of 110 preterm neonates, who met the inclusion/selection criteria, were included in the final analysis. The mean gestational age at birth of the cohort was 29.3 weeks (SD, 1.9 weeks; range, 24.5–32.0), and the mean birth weight was 1351 g (SD, 348 g; range, 600–2,090). ROP was diagnosed in 30 of 110 infants (27.3%) from our cohort, including 16 with mild ROP (stage 1, $n = 10$; stage 2, $n = 6$) and 14 with severe ROP (stage 3, $n = 14$). There were no neonates

Table 1. Neonatal Characteristics and Morbidities of Infants in Relation to the Occurrence and Progression of ROP*

	ROP Occurrence (Any Stage)			Severe ROP (Stage 3)			Laser Treatment		
Variables	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**
No. of infants	80	30		96	14		103	7	
Gestational age at birth (weeks)	29.7 ± 1.7	28.1 ± 2.0	**<0.001**	29.6 ± 1.7	27.4 ± 2.1	**<0.001**	29.5 ± 1.8	26.2 ± 1.7	**<0.001**
Birth weight (kg)	1.424 ± 0.330	1.157 ± 0.321	**<0.001**	1.404 ± 0.330	1.024 ± 0.270	**<0.001**	1.387 ± 0.332	0.895 ± 0.203	**0.001**
Male sex	46 (58%)	16 (53%)	0.695	55 (57%)	7 (50%)	0.607	59 (57%)	3 (43%)	0.697
Apgar score <7									
1 min	54 (68%)	26 (87%)	0.055	67 (70%)	13 (93%)	0.107	73 (71%)	7 (100%)	0.186
5 min	21 (26%)	16 (53%)	**0.007**	29 (30%)	8 (57%)	0.068	33 (32%)	4 (57%)	0.222
Umbilical artery pH	7.301 ± 0.056	7.292 ± 0.050	0.237	7.301 ± 0.054	7.289 ± 0.061	0.482	7.304 ± 0.056	7.313 ± 0.039	0.632
Histologic chorioamnionitis	49 (61%)	23 (77%)	0.130	60 (63%)	12 (86%)	0.088	66 (64%)	6 (86%)	0.418
Funisitis	22 (28%)	8 (27%)	0.930	26 (27%)	4 (29%)	1.000	29 (28%)	1 (14%)	0.671
Continuous positive airway pressure	66 (83%)	24 (80%)	0.762	79 (82%)	11 (79%)	0.717	83 (81%)	7 (100%)	0.346
Mechanical ventilation	37 (46%)	21 (70%)	**0.026**	47 (49%)	11 (79%)	**0.038**	51 (50%)	7 (100%)	**0.014**
Use of surfactant	31 (39%)	21 (70%)	**0.003**	41 (43%)	11 (79%)	**0.012**	47 (46%)	5 (71%)	0.252
Proven sepsis	4 (5%)	2 (7%)	0.669	5 (5%)	1 (7%)	0.575	5 (5%)	1 (14%)	0.338
RDS	37 (46%)	21 (70%)	0.026	48 (50%)	10 (71%)	0.134	52 (51%)	6 (86%)	0.117
BPD	25 (31%)	18 (60%)	**0.006**	32 (33%)	11 (79%)	**0.001**	36 (35%)	7 (100%)	**0.001**
IVH, grade 2 or more	5 (6%)	2 (7%)	1.000	6 (6%)	1 (7%)	1.000	6 (6%)	1 (14%)	0.377
PVL	8 (10%)	4 (13%)	0.732	10 (10%)	2 (14%)	0.649	10 (10%)	2 (29%)	0.168
NEC	6 (8%)	2 (7%)	1.000	7 (7%)	1 (7%)	1.000	8 (8%)	0 (0%)	1.000

Values are given as the mean ± SD or *n* (%).
* Significant findings ($P < 0.05$) are presented in bold.

with stage 4 or 5 ROP. Laser treatment was performed on 7 infants (6.4% [7/110]).

The maternal and obstetric characteristics of the study population in relation to the occurrence and progression of ROP are described in Supplementary Table S1. None of the maternal and obstetric variables were associated with the occurrence or progression of ROP.

Table 1 displays neonatal characteristics and morbidities in relation to the occurrence and progression of ROP among the 110 preterm neonates. Based on the univariate analyses, low gestational age, low birth weight, use of mechanical ventilation, and BPD were significantly related with the occurrence and progression of ROP, as well as ROP laser treatment. Apgar scores of <7 at 5 minutes and RDS had a statistically significant association with ROP occurrence, while the use of a surfactant had an association with both ROP occurrence and severe ROP.

Table 2 shows cord plasma levels of immune and inflammatory proteins in relation to the occurrence and progression of ROP. The univariate analysis with regard to the presence or absence of severe ROP showed significantly higher median cord plasma IL-6 and IGFBP-1 levels in preterm infants who developed severe ROP than in those who did not. Moreover, infants with vision-threatening ROP requiring laser treatment had significantly higher median cord plasma IL-6, C5a, and IGFBP-1 levels than infants that did not develop vision-threatening ROP. However, on the basis of univariate analysis, none of the proteins measured in the cord plasma were associated with ROP occurrence. Positive significant correlations between IL-6, IGFBP-1, and C5a levels in cord plasma were identified (all variables, $r = 0.335$–0.472, $P < 0.001$).

A multivariate logistic regression model was conducted to determine the independent association of the different proteins levels in cord plasma with severe ROP and vision-threatening ROP requiring laser treatment after adjusting for the effects of baseline variables. Before performing logistic regression analysis, we checked for multicollinearity among the variables by using the Spearman's rank correlation test. A highly significant correlation was found between gestational age at birth and birth weight ($r = 0.845$); therefore, gestational age alone was included in the analysis instead of both gestational age and birth weight (Table 3). In this model, the variables were selected based on a $P < 0.05$ in univariate analyses, and all continuous parameters were dichotomized using the cutoff values derived from the ROC curves. With respect to the prediction of severe ROP, the optimal cutoff values for cord plasma IL-6 and IGFBP-1 levels, birth weight, and gestational age were ≥6.5 pg/mL, ≥291.6 ng/mL, ≥1.1225 kg, and ≥28.2 weeks, respectively. A multivariate logistic regression model indicated that only high cord plasma levels of IL-6 (≥6.5 pg/mL), and not IGFBP-1 (≥291.6 ng/mL), were still significantly associated with severe ROP when adjusted for low gestational age (≥28.2 weeks) (or low birth weight [≥1.1225 kg] [data not shown]), mechanical ventilation, the use of surfactant, and BPD (Table 3). Similarly, when vision-threatening ROP requiring laser treatment was used as an outcome measure, the following dichotomized variables were used for the logistic regression analysis: high level of cord plasma IL-6 (≥7.5 pg/mL), high level of cord plasma C5a (≥28.0 ng/mL), high level of cord plasma IGFBP-1 (≥291.6 ng/mL), low birth weight (≤1.1225 kg), and low gestational age (≤28.2 weeks). Logistic regression showed that only a high cord plasma C5a level (≥28.0 ng/mL) was still significantly associated with laser treatment, even after adjustment for low gestational age (≤28.2 weeks) (or low birth weight [≤1.1225 kg] [data not shown]), mechanical ventilation, and BPD (Table 3).

To determine the best combination model for the prediction of severe ROP and ROP laser treatment, a multivariate analysis, with a forward selection, was performed on seven variables found to be significant by univariate analysis (<0.05) (Supplementary Table S2a). In this model, all continuous predictors were entered as dichotomous variables using the cutoff values derived from the ROC curves, as described in Table 4. In the severe ROP model, only high cord plasma levels

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TABLE 2. Cord Plasma Levels of Immune and Inflammatory Proteins in Relation to the Occurrence and Progression of ROP*

Variables	ROP Occurrence (Any Stage)			Severe ROP (Stage 3)			Laser Treatment		
	Absent ($n = 80$)	Present ($n = 30$)	*P* Value	Absent ($n = 96$)	Present ($n = 14$)	*P* Value	Absent ($n = 103$)	Present ($n = 7$)	*P* Value
IL-6 (pg/mL)	12.0 ± 15.5	15.7 ± 18.1	0.251	11.8 ± 15.5	21.0 ± 19.1	**0.042**	12.4 ± 16.0	21.5 ± 18.7	**0.039**
C3a (μg/mL)	10.3 ± 5.1	10.1 ± 5.0	0.946	10.1 ± 5.0	11.4 ± 5.7	.286	10.1 ± 5.0	12.2 ± 6.1	0.214
C5a (ng/mL)	26.4 ± 11.4	27.3 ± 14.8	1.000	26.0 ± 11.6	32.6 ± 16.9	.154	26.0 ± 11.8	39.4 ± 15.9	**0.016**
MMP-2 (ng/mL)	180.7 ± 78.1	191.1 ± 73.8	0.501	183.2 ± 765.7	186.2 ± 809.4	.855	183.8 ± 77.2	180.6 ± 76.5	0.945
MMP-9 (ng/mL)	104.4 ± 83.2	111.5 ± 70.1	0.425	104.2 ± 801.6	120.9 ± 769.1	0.459	104.9 ± 79.6	127.3 ± 83.1	0.507
TIMP-1 (ng/mL)	179.1 ± 70.3	204.8 ± 127.0	0.643	179.6 ± 72.8	230.6 ± 161.1	0.247	184.3 ± 90.3	212.8 ± 77.0	0.223
M-CSF (pg/mL)	780.5 ± 382.4	771.3 ± 422.5	0.828	774.2 ± 399.9	803.4 ± 344.1	0.625	769.0 ± 389.7	909.3 ± 431.3	0.408
Endostatin (ng/mL)	83.9 ± 16.5	83.1 ± 16.9	0.727	83.2 ± 16.7	87.2 ± 15.6	0.404	83.8 ± 16.9	81.9 ± 11.2	0.821
APRIL (ng/mL)	257.2 ± 469.7	270.9 ± 325.6	0.430	248.7 ± 432.9	355.5 ± 457.5	0.150	260.6 ± 443.8	265.6 ± 195.2	0.383
IGFBP-1 (ng/mL)	314.6 ± 330.8	418.5 ± 360.5	0.082	315.5 ± 326.5	531.1 ± 387.2	**0.035**	326.9 ± 337.1	581.6 ± 327.3	**0.042**
IGFBP-2 (ng/mL)	331.6 ± 188.6	340.5 ± 240.9	0.752	329.8 ± 192.6	362.8 ± 272.0	0.978	337.8 ± 209.1	281.4 ± 732.0	0.847
S100 A8/A9 (ng/mL)	590.4 ± 1622.7	1271.1 ± 3122.2	0.791	582.3 ± 1550.2	2104.1 ± 4332.3	0.538	668.5 ± 1776.2	237.0 ± 5173.0	0.354
Time interval between cord blood collection and measurements (months)	**92.0 ± 46.2**	**85.5 ± 46.1**	**0.537**	**91.9 ± 46.9**	**79.0 ± 39.6**	**0.342**	**89.4 ± 46.6**	**102.1 ± 37.7**	**0.448**

Values are given as mean ± SD or *n* (%).

* Significant findings ($P < 0.05$) are presented in bold.

of IL-6 (≥6.5 pg/mL) and low birth weight (≤1.1225 kg) were identified as the best combination (Supplementary Table S2a). The AUC of this combination model was 0.840 (95% confidence interval [CI], 0.728–0.953), and the Hosmer-Lemeshow test showed a *P* value of 0.932, indicating an adequate model fit. Likewise, in the laser treatment model, only high levels of C5a (≥28.0 ng/mL) in cord plasma and low birth weight (≤1.1225 kg) were identified as the best combination (Supplementary Table S2b). The AUC of this combination model was 0.884 (95% CI, 0.780–0.988), and the Hosmer-Lemeshow test showed a *P* value of 0.905, indicating an adequate model fit.

Table 4 shows the AUC and best cutoff values, sensitivity, and specificity for various proteins in the cord plasma, clinical factors, and the combined prediction model in relation to severe ROP and ROP laser treatment. In predicting severe ROP, the AUC for the combined prediction model was significantly greater than those for cord plasma IL-6 and IGFBP-1 levels ($P < 0.05$ for each) but not significantly higher than those for birth weight ($P = 0.503$) and gestational age ($P = 0.348$) (Table 4) (Fig. 1). Likewise, for the prediction of laser treatment for ROP, the AUC for the combined prediction model was significantly greater than that for the cord plasma IL-6 level ($P = 0.038$) but not significantly higher than those for cord plasma C5a ($P = 0.398$) and IGFBP-1 ($P = 0.156$) levels, birth weight ($P = 0.774$), and gestational age ($P = 0.557$) (Table 4; Fig. 2).

DISCUSSION

The principal findings of this study are as follows: (1) in preterm neonates, an elevated level of cord plasma IL-6 was independently associated with an increased risk for the progression of ROP, whereas elevated levels of cord plasma C5a were significantly and independently associated with vision-threatening ROP requiring laser treatment; (2) the best combined models including cord plasma IL-6, cord plasma C5a, and birth weight can predict ROP progression with good accuracy; and (3) the development of ROP, especially the mild form, was not associated with cord plasma levels of several immune-inflammatory proteins. These findings are consistent with data reported by Lynch et al.,[2] showing that type 1 or type 2 ROP is an ocular outcome linked with events in the intrauterine environment that trigger a preterm birth. Taken together, these observations strongly suggest that factors that were present prior to birth in preterm infants may influence the development of severe ROP, independent of low birth weight and gestational age.

Previous literature indicates that high IL-6 levels of cord blood at birth play an important role in the development of severe neonatal morbidities, including hearing impairment, BPD, NEC, PVL, neonatal sepsis, and neonatal systemic inflammatory response syndrome, suggesting a deleterious role for IL-6 in a fetal inflammatory condition and its associated diseases.[9,20–22] Also, in retinal vasculature injuries in utero, we found that elevated IL-6 levels in cord plasma at birth were independently associated with the development of severe ROP in preterm infants. Considering that IL-6 is one of the most important proinflammatory cytokines involved in inflammation and infection responses[23] previously identified as important risk factors for ROP,[1,18] this finding is not surprising and supports the hypothesis that a systemic fetal inflammatory response (defined by increased levels of cord plasma IL-6) may cause detrimental effects on the development of the fetal retinal vasculature in preterm infants. In contrast to the current findings, our previous study using ROP occurrence (especially the mild form) as an outcome measure did not detect any association between cord blood IL-6 changes at birth and ROP

Table 3. Multivariate Logistic Regression of Potential Biomarkers in Cord Plasma of Severe ROP and Vision-Threatening ROP Requiring Laser Treatment

Predictors	Adjusted for Low Gestational Age at Birth (≤28.2 weeks)		Adjusted For All Variables Showing Significant Association in the Univariate Model*‡	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
For severe ROP†				
High cord plasma IL-6 level (≥6.5 pg/mL)	6.134 (1.226–30.682)	**0.027**	5.325 (1.047–27.078)	**0.044**
High cord plasma IGFBP-1 level (≥291.6 ng/mL)	1.843 (0.520–6.539)	0.344	1.655 (0.454–6.030)	0.445
For vision-threatening ROP requiring laser treatment§				
High cord plasma IL-6 level (≥7.5 pg/mL)	5.403 (0.582–50.164)	0.138	4.953 (0.505–48.609)	0.170
High cord plasma C5a level (≥28.0 ng/mL)	10.311 (1.118–95.126)	**0.040**	10.210 (1.025–101.726)	**0.048**
High cord plasma IGFBP-1 level (≥291.6 ng/mL)	5.643 (0.609–52.291)	0.128	4.376 (0.441–43.417)	0.207

Significant findings ($P < 0.05$) are presented in bold letters. OR, odds ratio.

* Severe ROP: adjustment for low gestational age, mechanical ventilation, use of surfactant, and BPD.

† All continuous predictors were entered as dichotomous variables using the cut-off values derived from the ROC curves to predict severe ROP.

‡ ROP requiring laser treatment: adjustment for low gestational age, mechanical ventilation, and BPD.

§ All continuous predictors were entered as dichotomous variables using the cutoff values derived from the ROC curves to predict vision-threatening ROP requiring laser treatment.

occurrence.[10] This discrepancy may be derived from the differences in outcome measures (any ROP versus severe ROP) and suggests that IL-6 levels in cord blood samples at birth may reflect disease severity in ROP. This speculation is supported by previous reports that the cord serum level of IL-6 is a significant predictor of the severity of the hypoxic-ischemic encephalopathy and IVH in preterm neonates.[24,25]

An important finding of this study is that elevated C5a cord plasma levels at birth were independently associated with vision-threatening ROP requiring laser treatment after adjusting for important clinical factors, including gestational age at birth and birth weight. To our knowledge, this is the first study that describes changes in cord plasma levels of complement activation fragments at birth in relation to the postnatal development of ROP. Similar to the findings in our study, a recent study using vitreous samples, conducted by Rathi et al.,[26] demonstrated that C3 and CFH proteins were significantly elevated and activated in the vitreous humors of preterm infants with ROP, suggesting a possible involvement of the alternative complement pathway in ROP. Indeed, these observations are natural because the complement system plays an important role in the inflammatory response, innate immunity, and pathologic retinal angiogenesis,[27,28] which were already known to be major risk factors for ROP.[1,18] The findings of our group and Rathi et al.[26] highlight the important role of the complement pathway in ROP pathogenesis, possibly even in utero, and might provide a new selective and specific target for the early prediction for ROP progression and, thus, a more effective ROP treatment. In support of our view, Langer et al.[28] have reported that complement and the C5a-C5aR axes in particular are potent inhibitors of the pathologic retinal angiogenesis in mice.

Similar to our previous report regarding the expression of inflammatory cytokines and growth factors in cord blood at birth related to the occurrence of ROP,[10] we found that levels of various immune and inflammatory proteins in cord blood at

Table 4. Diagnostic Indices of Cord Plasma IL-6, IGFBP-1, and C5a Levels and Clinical Factors to Predict Severe ROP and Vision-Threatening Laser Treatment Among Preterm Infants

Variables	AUC (±SE)	Cutoff Value*	Sensitivity† (95% CI)	Specificity† (95% CI)	PPV	NPV
Severe ROP (stage 3)						
Cord plasma IL-6 (pg/mL)	0.669 ± 0.083‡	≥6.52	85.7 (57.2–98.2)	61.5 (51.0–71.2)	24.5	96.7
Cord plasma IGFBP-1 (ng/mL)	0.675 ± 0.073§	≥291.63	64.3 (35.1–87.2)	62.8 (52.2–72.5)	20.5	92.2
Birth weight (kg)	0.810 ± 0.057	≤1.1225	78.6 (49.2–95.3)	78.1 (68.5–85.9)	34.4	96.2
GA at birth (weeks)	0.789 ± 0.064	≤28.25	71.4 (41.9–91.6)	76.0 (66.3–84.2)	30.3	94.8
Combined model A||	0.840 ± 0.057	≥0.283	71.4 (41.9–91.6)	87.5 (79.2–93.4)	45.5	95.5
Laser treatment						
Cord plasma IL-6 (pg/mL)	0.734 ± 0.059¶	≥7.53	85.7 (42.1–99.6)	62.1 (52.0–71.5)	13.3	98.5
Cord plasma C5a (ng/mL)	0.778 ± 0.120	≥28.03	85.7 (42.1–99.6)	62.1 (52.0–71.5)	13.3	98.5
Cord plasma IGFBP-1 (ng/mL)	0.730 ± 0.078	≥291.63	85.7 (42.1–99.6)	62.4 (52.2–71.8)	13.6	98.4
Birth weight (kg)	0.888 ± 0.052	≤1.1225	85.7 (42.1–99.6)	74.8 (65.2–82.8)	18.8	98.7
GA at birth (weeks)	0.903 ± 0.058	≤28.25	85.7 (42.1–99.6)	73.8 (64.2–82.0)	18.2	98.7
Combined model B#	0.884 ± 0.053	≥0.197	71.4 (29.0–96.3)	90.3 (82.9–95.3)	33.3	97.9

PPV, positive predictive value; NPV, negative predictive.

* Cutoff values corresponding to the highest sum of sensitivity and specificity.

† Values are given as % (95% CI).

‡ $P < 0.01$ compared to combined model A by the method of DeLong et al.[19]

§ $P < 0.01$ compared to combined model A by the method of DeLong et al.[19]

|| Combined model A consists of cord plasma IL-6 ≥6.52 pg/mL and birth weight ≤1.1225 kg.

¶ $P < 0.01$ compared to combined model B by the method of DeLong et al.[19]

Combined model B consists of cord plasma C5a ≥28.03 ng/mL and birth weight ≤1.1225 kg.

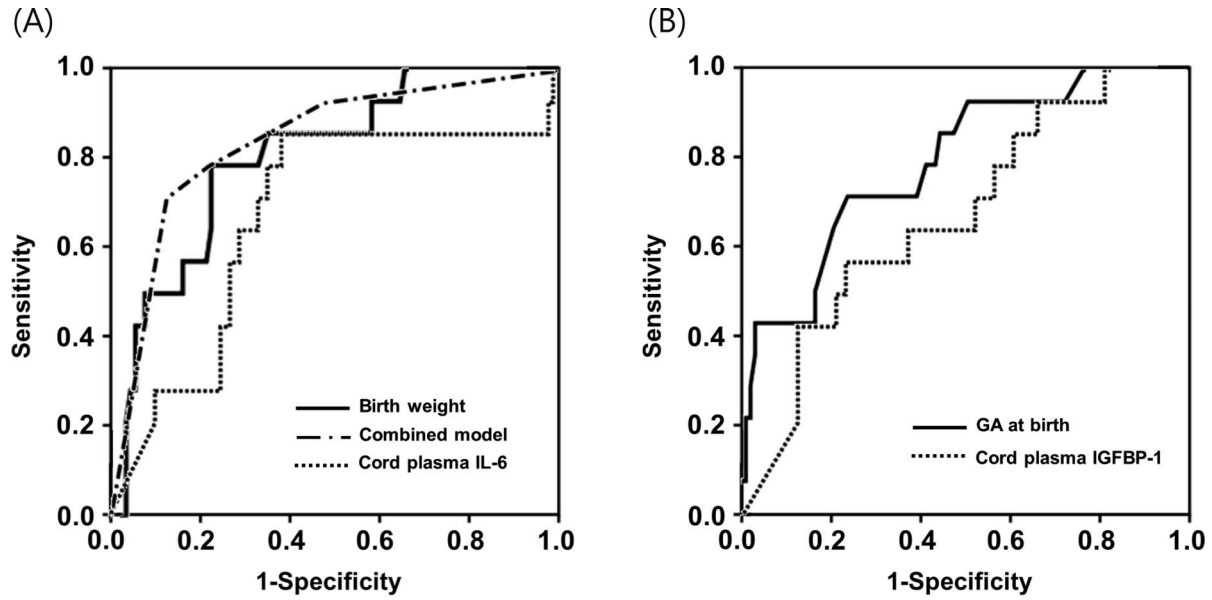

FIGURE 1. (A) ROC curves for the best combined model (including high cord plasma IL-6 and low birth weight) in predicting severe ROP. The AUC for the combined prediction model was 0.840. (B) ROC curves for cord plasma IGFBP-1 and gestational age (GA) at birth in predicting severe ROP (cord plasma IGFBP-1: AUC = 0.675, SE = 0.073; gestational age: AUC = 0.789, SE = 0.064).

birth were not associated with the risk of ROP occurrence, especially the mild form. On the contrary, neonatal blood biomarkers of inflammatory cytokines or growth hormones, as measured in the first few days after birth, were consistently reported to be associated with ROP development, even mild ROP.[29–31] The discrepancy between the null result of the present study using cord blood at birth and other studies using postnatal blood and finding an ROP association[29–31] suggests that environmental and care practices for infants in the postnatal period might be more important in the development and progression of ROP than those in utero. Therefore, we believe that the prediction of ROP risk based on the blood obtained later in postnatal life may be more accurate, although our biomarkers in cord blood at birth may be used to understand the mechanisms of ROP progression and implement novel targets for mechanism-based treatments. Similar observations have been reported in a BPD study.[32]

Our combination models, consisting of high levels of cord plasma IL-6 (or C5a) and low birth weight, were not better than gestational age or birth weight alone in predicting severe ROP. This finding differs from that of Löfqvist et al.,[33] who proposed a WINROP algorithm (a sensitivity of 100% and specificity of 83.6%) by using longitudinal postnatal serum insulin-like growth factor 1 (IGF-1) levels and weight. This

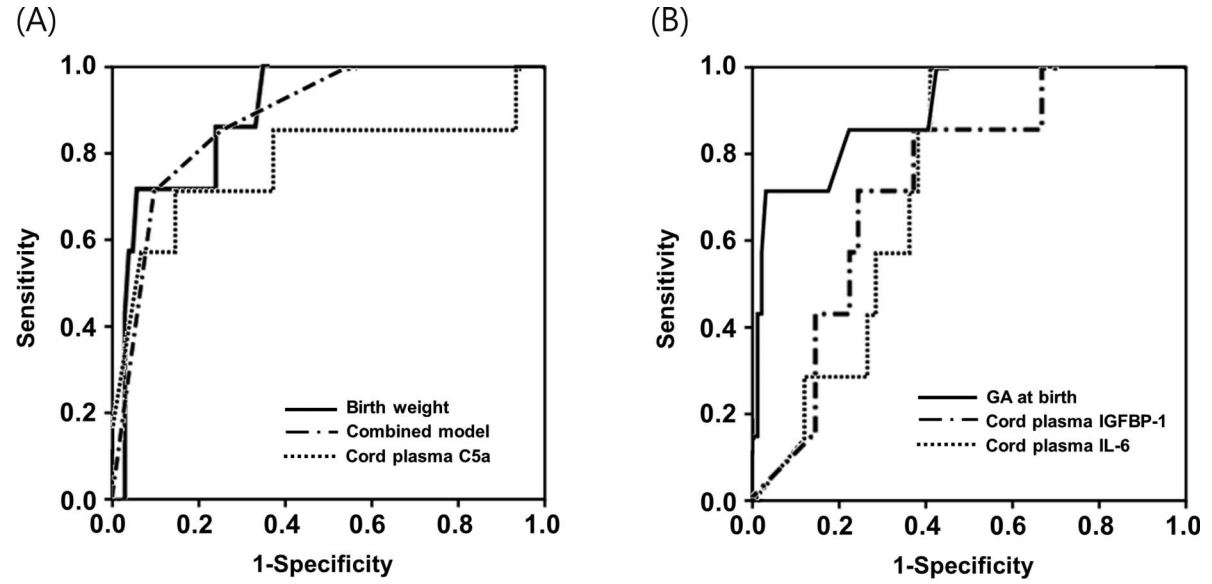

FIGURE 2. (A) ROC curves for the best combined model (including high cord plasma C5a and low birth weight) in predicting vision-threatening ROP requiring laser treatment. The AUC for the combined prediction model was 0.884. (B) ROC curves for cord plasma IGFBP-1, IL-6, and GA at birth in predicting vision-threatening ROP requiring laser treatment (cord plasma IGFBP-1: AUC = 0.730, SE = 0.078; cord plasma IL-6: AUC = 0.734, SE = 0.059; gestational age: AUC = 0.903, SE = 0.058).

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discrepancy may imply the limited clinical usefulness of prenatal data to build a predictive model for ROP risk.

Several limitations of this study should be acknowledged. First, the study is limited by its retrospective nature, single center design, and relatively small sample size in the laser treatment group and the predictive models were not validated in other cohorts, all of which can provide preliminary information on the cord blood biomarkers associated with ROP. Therefore, our findings need to be confirmed in large, prospective cohort studies in other populations. Second, we lacked data for other important markers (such as VEGF, IGF-1, and postnatal weight gain),[1,33,34] which may be used to improve the predictability of our combined prediction model, although we included 12 proteins representing the level of immune-inflammatory responses. Third, this study included umbilical cord blood sampling at birth and did not include serially collected neonatal blood samples, which did not provide the best time point to predict subsequent ROP. Fourth, the current study is limited by the lack of a control group (i.e., gestational age-matched normal neonates), and the results may be affected by unknown confounding factors, which cannot be taken into account during analysis. Fifth, the present study used frozen stored plasma samples to measure protein concentrations. This may lead to potential bias to interpret findings from our study, although the time intervals between cord blood collection and measurements were not associated with any of ROP outcomes (Table 2).

In conclusion, we demonstrated that in preterm infants, an elevated level of cord plasma IL-6 was independently associated with an increased risk of ROP progression, whereas elevated levels of cord plasma C5a were significantly and independently associated with vision-threatening ROP requiring laser treatment; however, the development of ROP, especially the mild form, was not associated with cord plasma levels of several immune-inflammatory proteins. Our results indicate that the immune-inflammatory environment of preterm infants in the prenatal period, as well as in the postnatal period, may be important for the progression to severe ROP. Further studies are needed to confirm our findings and to elucidate the underlying mechanisms by which in utero IL-6 and complement proteins may contribute to the development of severe ROP.

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