Inflammatory and Angiogenic Mediators in Amniotic Fluid Are Associated With the Development of Retinopathy of Prematurity in Preterm Infants

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Received: January 22, 2020
Accepted: April 13, 2020
Published: May 23, 2020

Citation: Woo SJ, Park JY, Hong S, et al. Inflammatory and angiogenic mediators in amniotic fluid are associated with the development of retinopathy of prematurity in preterm infants. *Invest Ophthalmol Vis Sci.* 2020;61(5):42. https://doi.org/10.1167/iovs.61.5.42

Purpose. To investigate whether elevated levels of inflammatory/angiogenic and growth mediators in amniotic fluid (AF) and the presence of intra-amniotic infection are associated with the occurrence and progression of retinopathy of prematurity (ROP) in preterm infants.

Methods. This retrospective cohort study included 175 premature singleton infants who were born between 23+0 and 32+0 weeks. AF obtained via amniocentesis was cultured, and endoglin, endostatin, insulin-like growth factor-binding protein (IGFBP)-2, IGFBP-3, IGFBP-4, IL-6, IL-8, matrix metalloproteinase-8, matrix metalloproteinase-9, and vascular endothelial growth factor receptor-1 levels were assayed by ELISA. The primary outcome measures included the occurrence of any stage ROP, severe ROP (stage ≥3), and vision-threatening type 1 ROP requiring treatment.

Results. Multiple logistic regression analyses revealed that there are significant associations between elevated AF endoglin levels and ROP occurrence; between elevated AF endoglin, endostatin, and IGFBP-2 levels and severe ROP; and between high AF endoglin, IL-6, and IL-8 levels and vision-threatening ROP requiring treatment, after adjusting for potential postnatal confounders. Using stepwise regression analyses, antenatal prediction models based on these AF biomarkers and prenatal factors were developed for the ROP outcomes, which had good discriminatory power (area under the curves, 0.731–0.863). However, we found that intra-amniotic infection is not associated with ROP occurrence and progression.

Conclusions. Elevated levels of inflammatory (IL-6 and IL-8) and angiogenic (endoglin and IGFBP-2) mediators in the AF, but not the presence of intra-amniotic infection, are independently associated with the occurrence and progression of ROP in preterm infants. These findings suggest that the pathophysiologic events that predispose preterm neonates to ROP may begin before delivery.

Keywords: amniotic fluid, antenatal prediction model, intra-amniotic infection, mediators, retinopathy of prematurity

Retinopathy of prematurity (ROP), a vasoproliferative retinal disease, affects very preterm infants and is a leading cause of potentially avoidable childhood blindness, worldwide.[1] The prevalence of ROP was estimated to be approximately 30% in very preterm neonates (<32 weeks). Moreover, approximately 20% of infants with any stage of ROP developed treatment-requiring ROP, and about 27% of infants with treatment-requiring ROP became blind or had severe visual impairment.[2,3] Despite the high prevalence and clinical importance of ROP in preterm neonates, as well as the preventable nature of ROP early in its pathogenesis, little is known about the risk factors and the possible preventive interventions for ROP, especially in the prenatal period.

Important risk factors of ROP include early gestational age, low birth weight, postnatal weight gain, and high or fluctuating levels of oxygen in the postnatal period.[4,5] The literature suggests that elevated levels of inflammatory factors and growth factors (insulin-like growth factor [IGF]-1, matrix metalloproteinase [MMP], placenta growth factor, and angiopoietins) in blood obtained later in postnatal life are associated with ROP development.[6–12] Furthermore, using cord blood sampled at birth, we have recently shown that elevated cord plasma levels of IL-6 and C5a were independently associated with severe ROP and laser treatment,[13] thereby suggesting that elevated levels of inflammatory and angiogenic proteins in the postnatal blood of

infants with ROP may reflect ongoing prenatal or perinatal inflammatory response and its associated mediators, leading to subsequent unfavorable visual outcomes for ROP. In fact, in the context of severe neonatal morbidities, such as bronchopulmonary dysplasia (BPD), periventricular leukomalacia (PVL), cerebral palsy, and hearing impairment, significant associations of elevated cytokine levels in the postnatal and cord bloods, as well as in amniotic fluid (AF), with each of these outcomes have been already reported in very preterm infants.[14–17] However, to date, no rigorous clinical investigations have addressed the association between ROP development, altered protein levels, and the presence of microbes in AF, both of which reflect events that occur in the intrauterine environment. To improve ROP prevention and treatment, it is vital that we increase our understanding of its pathogenesis and that we identify the causative agents of ROP, especially in the antenatal period. Thus, the aim of this work was to investigate whether elevated levels of inflammatory/angiogenic and growth mediators in AF, and the presence of intra-amniotic infection (IAI) are independently associated with the occurrence and progression of ROP in preterm infants, and to develop antenatal prediction models for ROP using these biomarkers in combination with prenatal factors.

Methods

Study Design and Population

The study was approved by the local ethics committee at Seoul National University Bundang Hospital (IRB no. B-1105/128-102). Written informed consent was obtained from all parents for amniocentesis and for the collection and use of AF samples and clinical information for research purposes. In this retrospective single-center cohort study, we included all consecutive women with preterm delivery between 23+0 and 32+0 weeks of gestation, and their neonates admitted to a neonatal intensive care unit between June 2004 and July 2018. Infants were included if they met the following criteria: (i) singleton gestation, (ii) neonates whose mothers underwent transabdominal amniocentesis for microbiological or fetal lung maturation, (iii) availability of AF samples for analysis, (iv) survival until 36 weeks postmenstrual age, and (v) infants who underwent ROP screening examinations. We excluded infants with multiple gestations and any major congenital anomalies, and infants who were transferred to another hospital after amniocentesis. We used both the last menstrual period and a first or second trimester (≤20 weeks) ultrasound examination to determine gestational age.

ROP Screening Examination

For ROP screening, we adhered to the guidelines proposed by the American Academy of Pediatrics and Ophthalmology and Pediatrics, and the Association for Pediatric Ophthalmology and Strabismus.[18,19] The initial examination was conducted either 4 weeks after birth or 31 weeks of postmenstrual age, whichever occurred later. The follow-up schedules and the treatment decision were identical to the indications proposed by the Early Treatment for Retinopathy of Prematurity study.[20,21] Either intravitreal anti-VEGF treatment (e.g., bevacizumab or ranibizumab) or laser treatment were considered as the initial treatment of type 1 ROP. The stage of ROP was graded as the highest stage observed on fundus examination during the entire follow-up period. Severe ROP included stages 3, 4, and 5, and mild ROP included stages 1 and 2. The outcome parameters were the occurrence of any stage of ROP, severe ROP, and vision-threatening ROP requiring treatment (type 1 ROP).

Clinical Data and Definitions

Data on maternal and obstetric factors, and newborn parameters were abstracted from the obstetric and neonatal database as previously described.[22] IAI was diagnosed after positive AF microbial cultures. Diagnostic criteria for acute histologic chorioamnionitis, funisitis, and clinical chorioamnionitis have previously been described in detail,[23,24] and supplementary descriptions are provided in the Supplementary Materials. The definitions of respiratory distress syndrome (RDS), BPD, necrotizing enterocolitis (NEC), intraventricular hemorrhage, periventricular leukomalacia, and early-onset neonatal sepsis (EOS) have been described in previous publications.[23,25,26]

Diagnostic criteria and management of preterm labor, preterm premature rupture of membranes, and preeclampsia have previously been described in detail.[26–28] Decisions regarding the use and type of prophylactic antibiotics were left to the discretion of the attending obstetrician and treatments with antibiotics, corticosteroids, and tocolytics were started after amniocentesis.

AF Collection and Determination of Various Proteins in the AF Samples

To test the AF for infection and inflammation or fetal lung, a transabdominal amniocentesis was performed under ultrasound guidance with aseptic conditions. Following previously described methods, the samples of AF were cultured to identify the presence of microorganisms (e.g., genital mycoplasmas (*Mycoplasma hominis* and *Ureaplasma urealyticum*) and aerobic and anaerobic bacteria).[26] The remaining AF was centrifuged at 1500 *g* at 4°C for 10 minutes, and the supernatant was aliquoted and stored at −70°C until further use. Managing physicians had access to the AF culture results.

The concentrations of endoglin, endostatin, IGF-binding protein (IGFBP)-2, IGFBP-3, IGFBP-4, IL-6, IL-8, MMP-8, MMP-9, and VEGF receptor-1 in the stored AF samples were determined using ELISA kits (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions. These factors were chosen for the study because they have been previously shown to be important regulators of biologic action of IGFs, angiogenesis, infection, inflammation, oxidative stress, and immune response (www.uniprot.org/), which may be the main pathogenetic mechanisms underlying ROP development.[2,29] The ranges of the protein standard curves and dilution factors are described in detail in the Supplementary Materials. The intra-assay and interassay coefficients of variation were less than 15% for the analyzed proteins, with the exception of IGFBP-3 and MMP-8; for which the interassay coefficients of variation were 16.2% and 15.5%, respectively.

Statistical Analysis

Statistical analyses were performed using IBM SPSS 25.0 (IBM SPSS Inc., Chicago, IL). The Student *t*-test or the Mann–

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Whitney *U* test was used to analyze continuous data, and the χ^2-test or Fisher's exact test was used to compare categorical data. A multivariable logistic regression analysis was used to evaluate the independent association between the concentrations of each protein in the AF and occurrence and progression of ROP, after adjusting for baseline risk factors, with a *P* value of less than 0.1 in univariable analysis. In the logistic regression analysis, continuous data were converted to binary data to reduce the issue of multicollinearity (especially between the gestational age at birth and AF endoglin, endostatin, IL-6 and IL-8 levels [r = −0.442 to −0.313]) or to be used for risk prediction and decision making. All proteins in the AF and clinical risk factors were dichotomized at the highest quartile and compared against the lower three quartiles. To evaluate the independent association between inflammatory factors (IL-6 and IL-8 levels) in the AF and the occurrence and progression of ROP, gestational age at sampling (rather than the gestational age at delivery) was adjusted for in multivariable analyses. Gestational age at delivery forms part of the causal pathway (intermediate variable) between infection and inflammation and ROP and thus is not a confounding variable.[30] Additionally, to develop the antenatal prediction model for the occurrence and progression of ROP, a stepwise forward regression analysis was performed in which all predictive variables with a *P* value of less than 0.1 from the univariable analysis were introduced as dichotomous variables. Prenatal factors only (i.e., AF proteins and gestational age at sampling) associated with the risk of ROP were entered into this model. To compare the discriminatory power of each protein in the AF, clinical risk factors, and the antenatal prediction model, the areas under the curve (AUCs) for different tests were compared as previously described.[31] The correlation between continuous parameters with non-normal distribution was assessed by the Spearman's rank correlation test. Two-sided *P* values of less than 0.05 were considered to be statistically significant.

Results

Of the 175 preterm neonates with a gestational age of 32.0 weeks or less included in the final analysis, 50 developed ROP (28.6%, 50/175; stage 1, n = 17; stage 2, n = 6), 27 developed severe ROP (stage 3, n = 27), and 19 (10.9%; 19/175) were treated with laser retinal ablation. Positive AF culture results were obtained for 79 women (45.1%; 79/175), including 46 women with preterm premature rupture of membranes and 33 women with preterm labor. The types of microorganisms isolated from the AF samples are shown in the Supplementary Table S1.

Maternal and Neonatal Characteristics

Table 1 presents the maternal demographic and clinical characteristics of the study population in relation to the occurrence and progression of ROP. Based on the univariable analyses, only low gestational age at amniocentesis was significantly associated with the occurrence and progression of ROP, as well as vision-threatening ROP requiring treatment.

Table 2 shows the neonatal characteristics in relation to the occurrence and progression of ROP. In univariable analyses, low gestational age at birth, low birth weight, use of mechanical ventilation, EOS, RDS, BPD, and NEC had statistically significant associations with the occurrence and progression of ROP, as well as vision-threatening ROP requiring treatment. A low 5-minute Apgar score (<7) was statistically significantly associated with ROP occurrence, whereas administration of surfactant was significantly related to both ROP occurrence and severe ROP.

Various Proteins in AF in Relation to ROP

Table 3 shows the concentrations of various AF proteins in relation to the occurrence and progression of ROP. Based on the univariable analyses, elevated AF levels of endoglin, endostatin, and IL-6 were significantly associated with occurrence and progression of ROP, as well as vision-threatening ROP requiring treatment. Moreover, elevated AF IL-8 levels had a statistically significant association with both severe ROP and vision-threatening ROP requiring treatment, and elevated AF IGFBP-2 was linked with both ROP occurrence and severe ROP. However, elevated AF levels of IGFBP-3, IGFBP-4, MMP-8, MMP-9, and VEGF receptor-1 and IAI were associated with neither the occurrence nor progression of ROP.

The correlations between AF levels of endoglin, endostatin, IL-6, IGFBP-2, and IL-8 are described in the Supplementary Table S2. From these five proteins, endoglin, endostatin, IL-6, and IL-8 levels, and not IGFBP-2, were negatively correlated with gestational age at birth (r = −0.442 to −0.313, $P < 0.001$), whereas endoglin, endostatin, and IGFBP-2 levels, and not IL-6, and IL-8, were negatively correlated with gestational age at the time of amniocentesis (r = −0.485 to −0.165; $P < 0.05$).

Multivariable Analysis

Multivariable logistic regression analyses were performed to further evaluate the independent association of the various proteins in AF (i.e., endoglin, endostatin, IGFBP-2, IL-6, and IL-8) with the occurrence and progression of ROP, with adjustments for baseline variables. Before the regression analyses, multicollinearity was checked among the parameters using the Spearman's rank correlation test. Gestational age at amniocentesis and at birth, and birth weight were significantly correlated with each other (r = 0.683–0.865) and thus were summarized in the analysis; instead of including the three variables simultaneously, gestational age at birth alone was included in the analysis (Table 4). However, gestational age at sampling, rather than at delivery, was adjusted for in the multivariable analyses of infections/inflammations (i.e., AF IL-6 and Il-8) and ROPs, as described in the Methods section (Statistical Analysis). The highest quartile values that were selected as the cutoff points for dichotomization are as follows: 8.73 ng/mL for AF endoglin, 75.34 ng/mL for AF endostatin, 41.41 ng/mL for AF IL-6, 11.75 ng/mL for AF IL-8, 1.56 µg/mL for AF IGFBP-2, 27.0 weeks for gestational age at birth, and 26.3 weeks for gestational age at sampling.

In the multivariable analysis regarding prediction of ROP occurrence, only high AF levels of endoglin (≥8.73 ng/mL) was still significantly and independently associated with ROP occurrence when adjusted for low gestational age at birth (≤27.0 weeks), use of tocolytics, low 5-minute Apgar score (< 7), mechanical ventilation, the use of surfactant, EOS, RDS, and BPD (Table 4). Likewise, with respect to the prediction of severe ROP, logistic regression indicated that high levels of AF endoglin (≥8.73 ng/mL), endostatin (≥75.34 ng/mL), and IGFBP-2 (≥1.56 µg/mL) were still significantly associated with severe ROP when we adjusted

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Table 1. Maternal and Obstetric Factors in Relation to the Occurrence and Progression of ROP*

	ROP Occurrence (Any Stage)			Severe ROP (Stage 3)			Vision-Threatening ROP Requiring Treatment (Type 1)		
Variables	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**
No. of mothers	125	50		148	27		156	19	
Maternal age (y)	32.0 ± 4.0	31.7 ± 3.8	0.703	31.8 ± 4.0	32.2 ± 3.9	0.624	31.8 ± 3.9	32.8 ± 4.0	0.259
Nulliparity	54 (43.2)	27 (54.0)	0.196	68 (45.9)	13 (48.1)	0.833	74 (47.4)	7 (36.8)	0.382
Cause of preterm delivery			0.264			0.575			0.768
Preterm labor	60 (48.0)	28 (56.0)		72 (48.6)	16 (59.3)		77 (49.4)	11 (57.9)	
PPROM	63 (50.4)	20 (40.0)		72 (48.6)	11 (40.7)		75 (48.1)	8 (42.1)	
Preeclampsia	2 (1.6)	2 (4.0)		4 (2.7)	0 (0.0)		4 (2.6)	0 (0)	
Cesarean delivery	66 (52.8)	24 (48.0)	0.566	78 (52.7)	12 (44.4)	0.430	82 (52.6)	8 (42.1)	0.389
Antenatal corticosteroids	118 (94.4)	46 (92.0)	0.512	140 (94.6)	24 (88.9)	0.379	148 (94.9)	16 (84.2)	0.102
Antenatal antibiotics	97 (77.6)	40 (80.0)	0.728	114 (77.0)	23 (85.2)	0.344	121 (77.6)	16 (84.2)	0.768
Use of tocolytics	107 (85.6)	37 (74.0)	0.069	123 (83.1)	21 (77.8)	0.505	131 (84.0)	13 (68.4)	0.112
Gestational age at sampling (weeks)	28.4 ± 2.2	27.1 ± 2.3	**<0.001**	28.4 ± 2.2	26.6 ± 2.3	**0.001**	28.3 ± 2.2	25.6 ± 1.9	**<0.001**
Histologic chorioamnionitis	88 (70.4)	40 (80.0)	0.196	105 (70.9)	23 (85.2)	0.125	111 (71.2)	17 (89.5)	0.089
Funisitis	43 (34.4)	13 (26.0)	0.282	48 (32.4)	8 (29.6)	0.774	50 (32.1)	6 (31.6)	0.967
Clinical chorioamnionitis	11 (8.8)	8 (16.0)	0.167	16 (10.8)	3 (11.1)	1.000	16 (10.3)	3 (15.8)	0.439

* Significant findings ($P < 0.05$) are presented in bold.
Values are mean ± standard deviation or number (%). PPROM, preterm premature rupture of membranes.

Table 2. Neonatal Characteristics and Morbidities of Infants in Relation to the Occurrence and Progression of ROP*

	ROP Occurrence (Any Stage)			Severe ROP (Stage 3)			Vision-threatening ROP Requiring Treatment (Type 1)		
Variables	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**
No. of infants	125	50		148	27		156	19	
GA at birth (weeks)	29.5 ± 1.7	27.7 ± 2.0	**<0.001**	29.4 ± 1.8	27.0 ± 2.1	**<0.001**	29.4 ± 1.7	26.1 ± 1.7	**<0.001**
Birth weight (kg)	1.430 ± 0.324	1.118 ± 0.301	**<0.001**	1.403 ± 0.323	1.004 ± 0.277	**<0.001**	1.393 ± 0.321	0.913 ± 0.240	**<0.001**
Male	68 (54.4)	24 (48.0)	0.444	81 (54.7)	11 (40.7)	0.181	85 (54.5)	7 (36.8)	0.146
Apgar score <7									
1 minute	90 (72.0)	40 (80.0)	0.274	107 (72.3)	23 (85.2)	0.159	113 (72.4)	17 (89.5)	0.163
5 minutes	36 (28.8)	25 (50.0)	**0.008**	48 (32.4)	13 (48.1)	0.115	51 (32.7)	10 (52.6)	0.085
Continuous positive airway pressure	102 (81.6)	42 (84.0)	0.707	122 (82.4)	22 (81.5)	1.000	127 (81.4)	17 (89.5)	0.533
Mechanical ventilation	59 (47.2)	37 (74.0)	**0.001**	75 (50.7)	21 (77.8)	**0.009**	79 (50.6)	17 (89.5)	**0.001**
Use of surfactant	39 (31.2)	29 (58.0)	**0.001**	52 (35.1)	16 (59.3)	**0.018**	57 (36.5)	11 (57.9)	0.071
EOS	13 (10.4)	13 (26.0)	**0.009**	18 (12.2)	8 (29.6)	**0.034**	19 (12.2)	7 (36.8)	**0.010**
RDS	51 (40.8)	36 (72.0)	**<0.001**	68 (45.9)	19 (70.4)	**0.020**	73 (46.8)	14 (73.7)	**0.027**
BPD	31 (24.8)	29 (58.0)	**<0.001**	41 (27.7)	19 (70.4)	**<0.001**	43 (27.6)	17 (89.5)	**<0.001**
IVH, grade ≥2	7 (5.6)	4 (8.0)	0.515	8 (5.4)	3 (11.1)	0.380	8 (5.2)	3 (15.8)	0.104
PVL	11 (8.9)	5 (10.0)	0.779	15 (10.2)	1 (3.7)	0.472	15 (9.7)	1 (5.3)	1.000
NEC	8 (6.4)	8 (16.0)	**0.047**	10 (6.8)	6 (22.2)	**0.010**	11 (7.1)	5 (26.3)	**0.018**

* Significant findings ($P < 0.05$) are presented in bold.
Values are mean ± standard deviation or number (%). GA, gestational age; IVH, intraventricular hemorrhage; PVL, periventricular leukomalacia.

Table 3. AF Levels of Immune and Inflammatory Proteins and Results of AF Cultures in Relation to the Occurrence and Progression of ROP*

	ROP Occurrence (Any Stage)			Severe ROP (Stage 3)			Vision-Threatening ROP Requiring Treatment (Type 1)		
Variables	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**	**Absent**	**Present**	***P* Value**
No. of infants	125	50		148	27		156	19	
AF endoglin (ng/mL)	6.4 ± 2.9	8.8 ± 3.2	**<0.001**	6.7 ± 3.1	9.0 ± 2.9	**<0.001**	6.8 ± 3.1	9.4 ± 3.0	**0.001**
AF endostatin (ng/mL)	60.6 ± 25.4	75.6 ± 31.8	**0.004**	62.7 ± 28.0	76.6 ± 26.2	**0.006**	63.6 ± 28.3	75.3 ± 24.3	**0.026**
AF IGFBP-2 (μg/mL)	1.1 ± 0.5	1.3 ± 0.5	**0.036**	1.1 ± 0.5	1.4 ± 0.5	**0.031**	1.1 ± 0.5	1.4 ± 0.6	0.117
AF IGFBP-3 (ng/mL)	427.9 ± 317.8	456.0 ± 252.3	0.267	437.2 ± 317.1	429.2 ± 185.1	0.614	431.9 ± 312.1	469.0 ± 175.3	0.223
AF IGFBP-4 (μg/mL)	1.0 ± 0.8	1.2 ± 0.9	0.312	1.0 ± 0.8	1.3 ± 0.9	0.197	1.0 ± 0.8	1.4 ± 1.0	0.186
AF IL-6 (ng/mL)	19.5 ± 21.2	28.2 ± 24.0	**0.048**	19.8 ± 21.6	34.0 ± 22.8	**0.002**	19.8 ± 21.3	40.1 ± 23.0	**<0.001**
AF IL-8 (ng/mL)	6.5 ± 6.3	8.2 ± 6.7	0.160	6.6 ± 6.4	9.4 ± 6.2	**0.021**	6.5 ± 6.3	11.1 ± 5.8	**0.003**
AF MMP-8 (ng/mL)	332.9 ± 555.2	417.0 ± 608.0	0.430	336.1 ± 561.9	471.0 ± 612.7	0.192	340.8 ± 569.2	489.5 ± 577.2	0.051
AF MMP-9 (ng/mL)	10.3 ± 8.6	10.5 ± 8.5	0.950	10.1 ± 8.6	12.3 ± 8.0	0.288	10.0 ± 8.6	13.9 ± 7.5	0.102
AF VEGFR-1 (ng/mL)	500.8 ± 466.5	607.1 ± 471.4	0.111	524.2 ± 471.1	569.4 ± 464.1	0.620	516.8 ± 469.1	649.1 ± 463.1	0.128
Positive AF cultures	56 (44.8)	23 (46.0)	0.885	66 (44.6)	13 (48.1)	0.733	69 (44.2)	10 (52.6)	0.487

* Significant findings ($P < 0.05$) are presented in bold letters.
Values are mean ± standard deviation or number (%).
VEGFR-1, VEGF receptor 1.

Table 4. Multivariable Logistic Regression of Potential Biomarkers in AF in Relation to the Occurrence and Progression of ROP

Predictors*	Adjusted for Low Gestational Age at Birth (Quartile 4, ≤27.0 weeks)†		Adjusted for All Variables Showing Significant Association in the Univariate Model†	
	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
For ROP‡				
AF endoglin level (quartile 4, ≥8.73 ng/mL)	4.0 (1.8–8.8)	**0.001**	3.2 (1.4–7.6)	**0.007**
AF endostatin level (quartile 4, ≥75.34 ng/mL)	2.3 (1.1–4.8)	**0.036**	2.3 (1.0–5.2)	0.052
AF IGFBP-2 level (quartile 4, ≥1.56 µg/mL)	1.9 (0.9–4.1)	0.091	1.8 (0.8–4.0)	0.153
AF IL-6 level (quartile 4, ≥41.41 ng/mL)	2.2 (1.0–4.6)	0.042	1.7 (0.8–4.0)	0.197
For severe ROP§				
AF endoglin level (quartile 4, ≥8.73 ng/mL)	4.1 (1.6–10.4)	**0.003**	2.9 (1.1–7.9)	**0.033**
AF endostatin level (quartile 4, ≥75.34 ng/mL)	2.7 (1.1–6.7)	**0.032**	2.7 (1.0–7.2)	**0.048**
AF IGFBP-2 level (quartile 4, ≥1.56 µg/mL)	2.6 (1.0–6.5)	**0.039**	3.1 (1.2–8.4)	**0.023**
AF IL-6 level (quartile 4, ≥41.41 ng/mL)	2.4 (1.0–6.0)	0.051	1.8 (0.6–4.8)	0.269
AF IL-8 level (quartile 4, ≥11.75 ng/mL)	3.0 (1.2–7.4)	**0.021**	2.2 (0.8–6.0)	0.135
For vision-threatening ROP requiring laser treatment‖				
AF endoglin level (quartile 4, ≥8.73 ng/mL)	4.5 (1.5–14.2)	**0.009**	5.5 (1.3–22.4)	**0.018**
AF endostatin level (quartile 4, ≥75.34 ng/mL)	2.0 (0.7–5.9)	0.209	1.7 (0.5–6.2)	0.406
AF MMP-8 level (quartile 4, ≥472.99 ng/mL)	1.2 (0.4–3.6)	0.771	0.6 (0.1–2.8)	0.537
AF IL-6 level (quartile 4, ≥41.41 ng/mL)	7.0 (2.2–22.5)	**0.001**	9.4 (1.7–52.2)	**0.001**
AF IL-8 level (quartile 4, ≥11.75 ng/mL)	8.4 (2.4–30.1)	**0.001**	8.8 (1.6–48.3)	**0.013**

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

For the ORs shown in the highest quartile (quartile 4), the reference category is the lower three quartiles.

OR, odds ratio; CI, confidence interval; MMP, matrix metallopeptidases.

* All continuous predictors were entered as dichotomous variables using the highest quartile cutoff points.

† Gestational age at sampling (rather than gestational age at birth) was adjusted for in multivariable analyses to evaluate the independent association between IL-6 and IL-8 levels in the AF and ROP. Gestational age at delivery forms part of the causal pathway (intermediate variable) between infection and inflammation and ROP and thus is not a confounding variable.

‡ Adjustment for low gestational age at birth (≤27.0 weeks), use of tocolytics, low 5-minute Apgar score (<7), mechanical ventilation, use of surfactant, early-onset neonatal sepsis, RDS, and BPD.

§ Adjustment for low gestational age at birth (≤27.0 weeks), mechanical ventilation, use of surfactant, early-onset neonatal sepsis, RDS, BPD, and NEC.

‖ Adjustment for low gestational age at birth (≤27.0 weeks), histologic chorioamnionitis, low 5-minute Apgar score (< 7), mechanical ventilation, use of surfactant, early-onset neonatal sepsis, RDS, BPD, and NEC.

for low gestational age at birth (≤27.0 weeks), mechanical ventilation, use of surfactant, EOS, RDS, BPD, and NEC (Table 4). For vision-threatening ROP requiring laser treatment, logistic regression showed that only high AF endoglin (≥8.73 ng/mL), IL-6 (≥41.41 ng/mL), and IL-8 (≥11.75 ng/mL) levels were significantly associated with the risk of this outcome, after adjustment for low gestational age at birth (≤27.0 weeks, gestational age at sampling [≤26.3 weeks] for IL-6 and IL-8), histologic chorioamnionitis, low 5-minute Apgar score (<7), mechanical ventilation, use of surfactant, EOS, RDS, BPD, and NEC (Table 4).

Development of an Antenatal Combined Prediction Model for ROP

To develop the best antenatal prediction model for ROP, AF protein levels and baseline prenatal variables were included in the multivariable analysis with a forward selection. In this model, all continuous predictors with a *P* value of less than 0.1 from the univariate analysis were entered as dichotomous variables using the highest quartile values for a cutoff point. In the ROP occurrence model, only high AF levels of endoglin (≥8.73 ng/mL) and IL-6 (≥41.41 ng/mL) were identified as the best combination (Hosmer-Lemeshow test, $P = 0.905$); in the severe ROP model, only high AF levels of endoglin (≥8.73 ng/mL) and IL-6 (≥41.41 ng/mL) were identified as the best combination (Hosmer-Lemeshow test, $P = 0.400$). Likewise, in the laser treatment model, only high levels of endoglin (≥8.73 ng/mL), IL-8 (≥11.75 ng/mL), and low gestational age at sampling (≤26.3 weeks) were identified as the best combination (Hosmer-Lemeshow test, $P = 0.320$). The AUC for ROP occurrence, severe ROP, and laser treatment models, were 0.731 (95% confidence interval, 0.643–0.818), 0.736 (95% confidence interval, 0.622–0.850), and 0.863 (95% confidence interval, 0.756–0.970), respectively (Tables 5 and 6; Figure).

Discussion

The main findings of this study are as follows: (i) in preterm neonates, elevated levels of AF inflammatory (IL-6 and IL-8) and angiogenic (endoglin, endostatin, and IGFBP-2) mediators are independently associated with an increased risk for the occurrence and progression of ROP; (ii) based on these biomarkers and prenatal factors (gestational age at presentation), the best combined antenatal models can predict the occurrence and progression of ROP with good accuracy; and (iii) the presence of IAI in utero was not associated with the development of ROP. Previous studies, including ours, also noted (i) elevated cord plasma levels of IL-6, C3a, and C5a, and (ii) events in the intrauterine environment that trigger spontaneous preterm delivery are significantly associated with an increased risk of ROP progression.[13,32,33] Taken

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Table 5. Regression Coefficients, ORs, and 95% CI of the Final Prenatal Model for Predicting ROP Occurrence, Severe ROP, and Vision-threatening ROP Requiring Treatment Among Preterm Infants

Predictor	Beta-Coefficient	SE	OR (95% CI)	*P* Value
For ROP[*]				
High AF endoglin level(quartile 4, ≥8.73 ng/mL)	1.920	0.393	6.8 (3.2–14.7)	<0.001
High AF IL-6 level(quartile 4, ≥41.41 ng/mL)	0.999	0.408	2.7 (1.2–6.0)	0.014
Constant	–1.808	0.279	0.164	<0.001
For severe ROP (stage 3)[†]				
High AF endoglin level (quartile 4, ≥8.73 ng/mL)	1.916	0.463	6.8 (2.7–16.8)	<0.001
High AF IL-6 level(quartile 4, ≥41.41 ng/mL)	1.109	0.483	3.0 (1.2–7.8)	0.022
Constant	–2.779	0.389	0.062	<0.001
For vision-threatening ROP requiring laser treatment[‡]				
Low GA at sampling(quartile 4, ≤26.3 weeks)	2.394	0.706	11.0 (2.7–43.7)	0.001
High AF endoglin level(quartile 4, ≥8.73 ng/mL)	1.604	0.654	5.0 (1.4–17.9)	0.014
High AF IL-8 level(quartile 4, ≥11.75 ng/mL)	2.259	0.691	9.6 (2.5–37.1)	0.001
Constant	–4.723	0.741	0.009	<0.001

CI, confidence interval; GA, gestational age; OR, odds ratio; SE, standard error.

[*] Final model resulting from a forward regression analysis including the following predictive parameters: low gestational age at sampling (quartile 4, ≤26.3 weeks), use of tocolytics, high AF endoglin level (quartile 4, ≥8.73 ng/mL), high AF endostatin level (quartile 4, ≥75.34 ng/mL), high AF IGFBP-2 level (quartile 4, ≥1.56 µg/mL), and high AF IL-6 level (quartile 4, ≥41.41 ng/mL).

[†] Final model resulting from a forward regression analysis including the following predictive parameters: low gestational age at sampling (quartile 4, ≤26.3 weeks), high AF endoglin level (quartile 4, ≥8.73 ng/mL), high AF endostatin level (quartile 4, ≥75.34 ng/mL), high AF IGFBP-2 level (quartile 4, ≥1.56 µg/mL), high AF IL-6 level (quartile 4, ≥41.41 ng/mL), and AF IL-8 level (quartile 4, ≥11.75 ng/mL).

[‡] Final model resulting from a forward regression analysis including the following predictive parameters: low gestational age at sampling (quartile 4, ≤26.3 weeks), high AF endoglin level (quartile 4, ≥8.73 ng/mL), high AF endostatin level (quartile 4, ≥75.34 ng/mL), high AF IL-6 level (quartile 4, ≥41.41 ng/mL), high AF IL-8 level (quartile 4, ≥11.75 ng/mL), and high AF MMP-8 level (quartile 4, ≥472.99 ng/mL).

For the ORs shown in the highest quartile (quartile 4), the reference category was the lower three quartiles.

together, these findings suggest that pathophysiologic events that predispose preterm neonates to ROP begin before delivery and that therapeutic strategies to decrease the risk of ROP may need to be implemented during pregnancy (e.g., specific treatment with antibiotics, anti-inflammatory drugs, and/or antiangiogenic drugs). Additionally, our findings strongly support the theory of Lee and Dammann[34] regarding the important etiological role of antenatal factors, especially prenatal and perinatal infection/inflammation, in ROP.

An important observation of the current study is that elevated levels of the inflammatory mediators in AF IL-6 and IL-8 are independently associated with vision-threatening ROP requiring laser treatment, after adjusting for potential postnatal confounders. Similar to the current findings in AF, our recent study using cord blood samples at birth, showed that elevated levels of cord plasma IL-6 were significantly associated with severe ROP.[13] Furthermore, previous studies using postnatal blood have demonstrated that inflammatory proteins are significantly elevated in the peripheral blood obtained in the postnatal period of preterm infants with ROP.[6–9,11,12] Therefore, these findings show that in utero to postnatal systemic inflammation is linked to ROP occurrence and progression, and highlight the importance of inflammation in the pathogenesis of ROP.

We found that novel angiogenic signaling-related molecules in AF (endoglin, endostatin, and IGFBP-2) are associated with the development and progression of ROP. Endoglin is a TGF-β auxiliary co-receptor that modulates TGF-β signaling, and is involved in the recruitment of smooth muscle cells, angiogenesis, neovascularization, and vascular remodeling, therefore, making it an important protein for postocclusion reperfusion, neovascular diseases, tumor growth, and metastasis.[35,36] In the context of oxygen-induced retinopathy, previous research in cell- and animal-based models has shown that decreased endoglin expression inhibits retinal neovascularization, suggesting that endoglin may serve as a useful predictor of incipient neovascular disease.[37,38] The circulating form of endoglin (also known as soluble endoglin [sEng] has an antiangiogenic effect by inhibition of TGF-β.[37,38] The sEng results in the present study are similar to those in previous reports, which showed that patients with proliferative diabetic retinopathy had higher vitreous levels of sEng than patients without diabetes,[39] thereby suggesting that increased sEng levels in AF may result in impaired retinal vascular growth and contribute to the increased risk of neonatal ROP. Similar to sEng, endostatin is an endogenous inhibitor of angiogenesis and may interfere with the proangiogenic action of growth factors, including VEGF.[40] In line with the known biology and function of endostatin, elevated endostatin levels in ocular fluid samples have been linked to neovascular AMD and proliferative diabetic retinopathy.[41,42] These findings are similar to the results of the present study.

IGFBP-2 is a member of a family of IGFBPs that serves as a carrier protein for IGF-1, an important growth hormone involved in promoting the development of retinal vasculature.[2] IGFBP-2 in particular has been shown to have mainly inhibitory effects of IGF actions, and is expressed in human fetal and placental tissues.[43,44] Previous studies have shown that low serum levels of IGF-1 in the early postnatal period are associated with the poor postnatal weight gain and the development of ROP in preterm children.[2,45,46] Given the biological characteristics and site of production of IGFBP-2, our finding that increased AF levels of IGFBP-2 is associated with ROP pathogenesis is quite evident, and the following plausible mechanisms can be proposed to explain this observed relationship. The high AF IGFBP-2 level inhibits the prenatal growth of retinal vascular endothelial cells and

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Table 6. Diagnostic Indices of AF Endoglin, Endostatin, IL-6, IL-8, IGFBP-2, Clinical Factors, and Antenatal Model to Predict Occurrence and Progression of ROP Among Preterm Infants

Variables	Area (±SE) Under the ROC Curve	Cutoff Value*	Sensitivity† (95% CI)	Specificity† (95% CI)	PPV	NPV
ROP occurrence						
AF endoglin (ng/mL)	0.719 ± 0.044	≥7.3	62.0 (47.2–75.4)	71.2 (62.4–79.0)	46.3	82.4
AF endostatin (ng/mL)	0.641 ± 0.047	≥60.86	64.0 (49.2–77.1)	62.4 (53.3–70.9)	40.5	81.3
AF IGFBP-2 (μg/mL)	0.597 ± 0.048‡	≥1.22	58.0 (43.3–71.5)	62.4 (53.3–70.8)	38.2	78.8
AF IL-6 (ng/mL)	0.596 ± 0.050‡	≥10.49	66.0 (51.1–78.4)	54.4 (45.3–63.3)	36.7	80.0
Birth weight (kg)	0.755 ± 0.040	≤1.117	56.0 (41.4–69.7)	83.2 (75.2–89.1)	57.1	82.5
GA at birth (weeks)	0.747 ± 0.040	≤28.25	50.0 (35.7–64.3)	78.4 (70.0–85.1)	48.1	79.7
Combined model A§	0.731 ± 0.045	≥0.22	72.0 (57.5–83.8)	65.6 (56.6–73.9)	45.6	85.4
Severe ROP (stage 3)						
AF endoglin (ng/mL)	0.725 ± 0.054	≥7.3	70.4 (49.8–86.3)	67.6 (59.4–75.0)	28.4	92.6
AF endostatin (ng/mL)	0.666 ± 0.056	≥61.34	70.4 (49.8–86.3)	60.1 (51.8–68.1)	24.4	91.8
AF IGFBP-2 (μg/mL)	0.613 ± 0.062	≥1.60	44.4 (26.0–64.4)	80.4 (73.1–86.5)	29.3	88.8
AF IL-6 (ng/mL)	0.684 ± 0.055	≥10.49	81.5 (61.3–93.0)	53.4 (45.0–61.6)	24.2	94.1
AF IL-8 (ng/mL)	0.640 ± 0.054	≥4.63	77.8 (57.3–90.6)	53.4 (45.0–61.6)	23.3	92.9
Birth weight (kg)	0.825 ± 0.044	≤0.947	55.6 (35.6–74.0)	95.3 (90.1–97.9)	68.2	92.1
GA at birth (weeks)	0.799 ± 0.046	≤27.20	55.6 (35.6–74.0)	87.8 (81.2–92.4)	45.5	91.5
Combined model B‖	0.736 ± 0.058	≥0.11	74.1 (53.7–88.9)	60.1 (51.8–68.1)	25.3	92.7
Vision-threatening ROP requiring treatment¶						
AF endoglin (ng/mL)	0.745 ± 0.063	≥9.1	68.4 (43.5–87.4)	84.0 (77.3–89.4)	34.2	95.6
AF endostatin (ng/mL)	0.657 ± 0.061#	≥72.86	57.9 (33.5–79.8)	75.0 (67.5–81.6)	22.0	93.6
AF IL-6 (ng/mL)	0.747 ± 0.062	≥10.49	89.5 (65.4–87.2)	53.2 (45.1–61.2)	18.9	97.6
AF IL-8 (ng/mL)	0.706 ± 0.059**	≥6.38	84.2 (59.5–95.8)	60.9 (52.7–68.5)	20.8	96.9
Birth weight (kg)	0.885 ± 0.041	≤0.947	73.7 (48.6–89.9)	94.9 (89.8–97.6)	63.6	96.7
GA at birth (weeks)	0.893 ± 0.037	≤27.15	78.9 (53.9–93.0)	88.5 (82.1–92.8)	45.5	97.2
Combined model C††	0.863 ± 0.055	≥0.19	78.9 (54.4–93.9)	89.1 (83.1–95.3)	46.0	97.2

CI, confidence interval; GA, gestational age; NPV, negative predictive; PPV, positive predictive value; ROC, receiver operating characteristics; SE, standard error.

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

† Values are given as percent (95% CI).

‡ $P < 0.05$ compared with the combined model A by the method of DeLong et al.[31]

§ Combined model A consists of high AF levels of endoglin (quartile 4, ≥8.73 ng/mL) and IL-6 (quartile 4, ≥41.41 ng/mL).

‖ Combined model B consists of high AF levels of endoglin (quartile 4, ≥8.73 ng/mL) and IL-6 (quartile 4, ≥41.41 ng/mL).

# $P < 0.001$ compared with the combined model C by the method of DeLong et al.[31]

** $P < 0.05$ compared with the combined model C by the method of DeLong et al.[31]

†† Combined model C consists of high levels of endoglin (quartile 4, ≥8.73 ng/mL), IL-8 (quartile 4, ≥11.75 ng/mL), and low gestational age at sampling (quartile 4, ≤26.3 weeks).

facilitates the initial stage of ROP after preterm birth (arrest of vascular growth).[2]

Traditionally, most of the predictive models for ROP are based primarily on gestational age at birth and birth weight[47]; recently, additional factors, including IGF-1 and postnatal weight gain, were incorporated into these model developments.[47,48] Contrary to previous predictive models using postnatal factors, our prenatal prediction model for

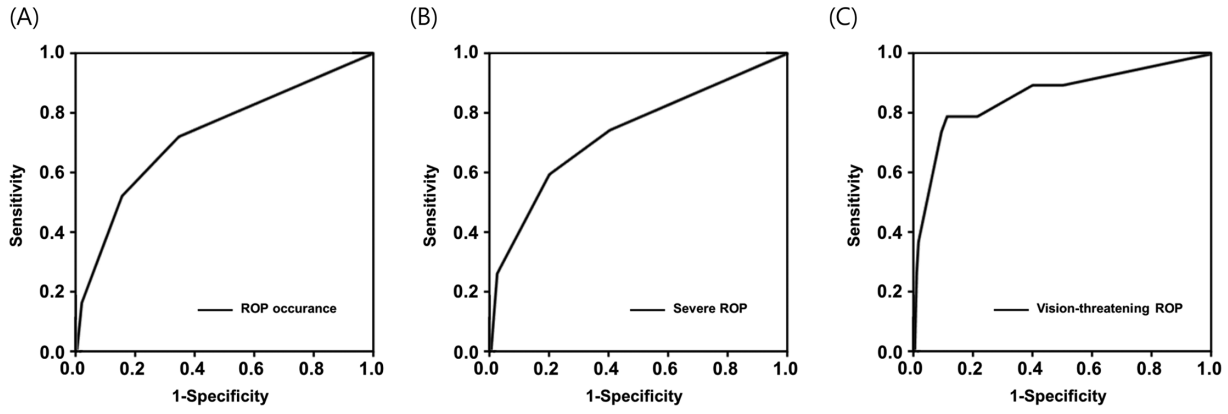

Figure. (**A**) Receiver operating characteristic (ROC) curve for the best antenatal prediction model (including high AF endoglin and high AF IL-6) for ROP occurrence (AUC, 0.731; SE, 0.045). (**B**) ROC curve for the best antenatal prediction model (including high AF endoglin and high AF IL-6) for severe ROP (AUC, 0.736; SE, 0.058). (**C**) ROC curve for the best antenatal prediction model (including high AF endoglin, high AF IL-8, and low gestational age at sampling) for vision-threatening ROP requiring treatment (AUC, 0.863; SE, 0.055).

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ROP was unique in that it combined both AF proteins and prenatal characteristics (gestational age at presentation) as candidate predictive variables. Moreover, the diagnostic performance of our prenatal model is similar to that of gestational age at birth or birth weight alone in predicting the occurrence and progression of ROP. In particular, a prenatal model yielded an AUC of 0.863 and negative predictive value of 97.2% in predicting type 1 ROP, which indicates a good discriminatory ability and negative predictive value, suggesting that this model may be used in the clinic to rule out the possibility that fetuses of patients with impending preterm birth develop type 1 ROP requiring treatment.

There are several limitations to be considered in our study. First, the current study was conducted in a single center and was retrospective in nature, which could have the potential for inherent selection bias. Second, we did not pursue a full characterization of the inflammatory, angiogenic, and growth factors associated with ROP, and thus lacked the information on the AF regarding important biomarkers of ROP in the postnatal blood, such as VEGF and IGF-1.[2,29] Third, ROP risk prediction using AF biomarkers requires invasive sampling of AF obtained via amniocentesis, which may limit clinical usefulness, particularly in low-risk patients. Fourth, the AF culture results were routinely reported to the managing physicians, which may have affected our decisions about the beginning of antibiotic therapy and optimal timing of delivery, although we adjusted AF-related factors (gestational age at birth and use of antibiotics) in multivariable analyses. Fifth, we did not perform a pre hoc sample size calculation before patient recruitment. Thus, the possibility of type II errors cannot be entirely excluded in certain analyses, especially in the current analysis in which the odds ratios of greater than 2.0 did not achieve a statistical significance. Nonetheless, we are the first to report the relationship between changes in AF levels of inflammatory, angiogenic, and growth mediators and the postnatal development of ROP, which may place some infants at risk for blindness.

In conclusion, for the first time to our knowledge, we demonstrate that the increases in endoglin, endostatin, IGFBP-2, IL-6, and IL-8 in AF are independently associated with the subsequent development and progression of ROP in preterm neonates, whereas the presence of IAI is not. Further studies are required to examine the impact of an early maternal perinatal therapy, such as use of antibiotics, anti-inflammatory agents, and antiangiogenic agents, on the development and progression of ROP.

Acknowledgments

The authors thank Soyeon Ahn, PhD, Jaebong Lee, MS, and the Medical Research Collaboration Center (MRCC) of Seoul National University Bundang Hospital for their assistance in statistical analysis.

Supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant number: HI18C0063).

Disclosure: **S.J. Woo,** None; **J.Y. Park,** None; **S. Hong,** None; **Y.M. Kim,** None; **Y.H. Park,** None; **Y.E. Lee,** None; **K.H. Park,** None

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