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Perinatal Systemic Inflammatory Response Syndrome and Retinopathy of Prematurity

BEENA G. SOOD, ASHIMA MADAN, SHAMPA SAHA, DIANA SCHENDEL, POUL THORSEN, KRISTIN SKOGSTRAND, DAVID HOUGAARD, SEETHA SHANKARAN, AND WALLY CARLO; ON BEHALF OF THE NICHD NEONATAL RESEARCH NETWORK

Department of Pediatrics [B.G.S., S.S.], Wayne State University, Detroit, Michigan 48201; Department of Pediatrics [A.M.], Stanford University School of Medicine, Palo Alto, California 94305; Statistics and Epidemiology Unit [S.S.], RTI International, Research Triangle Park, North California 27709; Centers for Disease Control and Prevention [D.S.], Atlanta, Georgia 30329; Department of Epidemiology and Social Medicine [P.T.], University of Aarhus, Aarhus, Denmark; Rollins School of Public Health [P.T.], Emory University, Atlanta, Georgia 30322; Department of Clinical Biochemistry and Immunology [K.S., D.H.], Statens Serum Institut DK-2300, Copenhagen, Denmark; Department of Pediatrics [W.C.], University of Alabama at Birmingham, Birmingham, Alabama 35233

ABSTRACT: Fetal and neonatal inflammation is associated with several morbidities of prematurity. Its relationship to retinopathy of prematurity (ROP) has not been investigated. Our objective was to determine the relationship between cytokine levels and ROP in the first 3 postnatal wks. Data for this study were derived from the NICHD Cytokine Study. Dried blood spots (DBS) were obtained from infants <1000 g on days 0–1, 3 ± 1, 7 ± 2, 14 ± 3, and 21 ± 3. Infants were classified into three groups—no, mild, and severe ROP. Multiplex Luminex assay was used to quantify 20 cytokines. Temporal profiles of cytokines were evaluated using mixed-effects models after controlling for covariates. Of 1074 infants enrolled, 890 were examined for ROP and 877 included in the analysis. ROP was associated with several clinical characteristics on unadjusted analyses. Eight cytokines remained significantly different across ROP groups in adjusted analyses. IL-6 and IL-17 showed significant effects in early time periods (D0–3); TGF-β, brain-derived neurotrophic factor (BDNF), and regulated on activation, normal T cell expressed and secreted (RANTES) in later time periods (D7–21) and IL-18, C-reactive protein (CRP), and neurotrophin-4 (NT-4) in both early and later time periods. We conclude that perinatal inflammation may be involved in the pathogenesis of ROP. **(*Pediatr Res* 67: 394–400, 2010)**

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Correspondence: Beena Gaind Sood, M.D., Children's Hospital of Michigan, 3901 Beaubien Boulevard, 4H42, Detroit, MI 48201; e-mail: bsood@med.wayne.edu

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Retinopathy of prematurity (ROP), a vasoproliferative disorder of the developing retina, is a major cause of blindness in infancy. ROP is a biphasic disease consisting of an initial phase of blunted vascular growth followed by a second phase of vasoproliferation that is recognized on ophthalmoscopy 4 to 6 wks after birth. Angiogenesis, the fundamental process involved in retinal vascular development, is tightly regulated by a complex network of cytokines, extracellular matrix components, and growth factors the action of which varies in a time-dependent fashion. Although inflammatory cytokines have the ability to modulate angiogenesis, their role in triggering the dysregulated angiogenesis in ROP has not been investigated (1).

Abbreviations: BDNF, brain-derived neurotrophic factor; **CRP,** C-reactive protein; **FIRS,** fetal inflammatory response syndrome; **GM-CSF,** granulocyte-macrophage colony-stimulating factor; **IVH,** intraventricular hemorrhage; **MCP-1,** monocyte chemoattractant protein-1; **MIP-1α,** macrophage inflammatory protein-1α; **MMP-9,** matrix metalloproteinase-9; **NEC,** necrotizing enterocolitis; **NT-4,** neurotrophin-4; **PVL,** periventricular leukomalacia; **RANTES,** regulated upon activation, normal T cell expressed and secreted; **ROP,** retinopathy of prematurity; **sIL-6R,** soluble IL-6 receptor

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Antenatal intrauterine infection with the resulting fetal inflammatory response syndrome (FIRS) is important in the pathogenesis of preterm birth and its associated morbidities including sepsis, periventricular leukomalacia (PVL), intraventricular hemorrhage (IVH), necrotizing enterocolitis (NEC), and bronchopulmonary dysplasia (2,3). It is not known whether FIRS also predisposes to the development of ROP. Recently, it has been reported that chorioamnionitis may be a risk factor for ROP occurrence and progression; however, this association was not significant on adjusted analyses in this small retrospective study (4). Similarly, although several reports suggest that postnatal severity of illness and *Candida* infection contribute to ROP frequency and/or severity, the role of the associated systemic inflammatory response syndrome (SIRS) as evaluated by cytokine levels in the pathogenesis of ROP has not been investigated (5). Neonatal noninfectious inflammation might further increase the inflammatory burden (6). In this study, we present for the first time, epidemiologic data from a large cohort of preterm babies to support the role of perinatal inflammation in the pathogenesis of ROP.

We hypothesized that inflammatory mediators released during SIRS in the pre-/perinatal period lead to disruption of angiogenesis resulting in ROP. The aim of this study was to investigate the relationship between concentrations of fetal/neonatal inflammatory markers and growth factors in dried blood spots (DBS) from preterm infants collected over the first 3 weeks after birth and ROP.

METHODS

We conducted a secondary analysis using clinical and biologic data collected as part of the multicenter NICHD Cytokine study, a prospective study assessing biomarkers and risk factors for adverse neurodevelopmental outcomes in preterm infants. This study was approved by the institutional review boards at all 17 participating centers, and written informed consent was obtained from the parent(s).

Preterm neonates with birth weight 401-1000 g who were enrolled in the NICHD Cytokine Study and had available DBS and results of ROP diagnostic examinations were eligible for inclusion in the study. Information from ROP diagnostic examinations that was available in this dataset included highest stage and lowest zone of ROP, presence of plus disease, diagnosis of threshold disease, and need for retinal ablative therapy. In addition, the presence of regressing ROP was recorded at 120-d postnatal age, discharge, or death, whichever came first. Infants who died before eye examination for ROP or 36-wk postmenstrual age (PMA) were excluded.

Whole blood was collected on filter paper and frozen at five-time periods (days): 0–1 (D0), 3 ± 1 (D3), 7 ± 2 (D7), 14 ± 3 (D14), and 21 ± 3 (D21). The treatment of blood samples was standardized. After the filter paper blood sample had dried, it was wrapped in stock paper cover and placed in a plastic bag that was stored in a −20°C freezer as soon as possible after the specimen was dry but no later than 24 h. Clinical data were collected by trained research coordinators, and all analyses were performed at a central data coordinating center. The outcome variable was ROP that was classified into three categories—no ROP, mild ROP (ROP other than severe), and severe ROP. Severe ROP included type 1 and type 2 ROP as defined by the Early Treatment for Retinopathy of Prematurity trial (ETROP) (7). Confounding variables included center, gestational age, birth weight, race, sex, premature prolonged rupture of membranes, sepsis (early and late), antenatal steroids, postnatal steroids, days in O_2, IVH (grades 3 and 4), cystic PVL, surgically treated patent ductus arteriosus, and NEC (Bells' stage ≥2).

The stored blood spots were analyzed using a multiplex Luminex assay (Luminex Corp., Austin, TX) as described previously (8,9). This assay has low intra- (<10%) and interassay (7–23%) variation. It has been shown that cytokine measurements on DBS stored at −24°C for >20 y are stable over time. The first part of our study included a hypothesis-driven investigation of 11 cytokines and growth factors reported to be important in the pathogenesis of inflammation, preterm birth, angiogenesis, and or proliferative retinopathies (IL-1β, IL-6, IL-8, sIL-6R, TNF-α, IFN-γ, MIP-1α, monocyte chemoattractant protein-1 [MCP-1], MMP-9, TGF-β, regulated on activation, normal T cell expressed and secreted [RANTES]); subsequently, we broadened our investigation to include nine other markers that were available in the subjects of the primary study [IL-2, IL-4, IL-10, IL-17, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF), C-reactive protein (CRP), brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT-4)] based on exploratory analysis.

Statistical analyses. Statistical analyses were performed using SAS System 9.1 (SAS, Cary, NC). Unadjusted analysis was performed using χ^2 tests for categorical outcomes and analysis of variance test (F test) for continuous outcomes to study the association of several maternal and neonatal variables with ROP (no, mild, and severe ROP). Medians and interquartile ranges were computed for all cytokines at each of the five-time points and the Kruskal-Wallis nonparametric analysis of variance used to assess differences across ROP groups. For each cytokine, levels were plotted against time for each ROP group to assess their temporal profiles during the first 3 postnatal wks. Differences in the temporal profiles across the three ROP groups were further evaluated using mixed-effect models for each cytokine independently to account for the multicenter design, repeated measurement of cytokine levels, significant clinical covariates, and interaction of postnatal age and ROP group. A p value of <0.05 was considered statistically significant.

RESULTS

Among 1074 patients enrolled, 890 had a screening eye examination for ROP. Thirteen infants who died before 36-wk PMA were excluded from the analysis as it has been shown that the median (5, 95%) age at the time of development prethreshold ROP is 36.1 (32.1, 42.1)-wk PMA (10). Of these 13 infants, 10 had no ROP, two had mild ROP, and one infant had severe ROP (type 2 ROP). None of these infants had type 1 or aggressive posterior ROP. Of the remaining 877 infants, 610 (69.6%) were diagnosed with ROP. The numbers of infants who developed each stage of ROP are reported in Table 1. Fifteen infants died before 42-wk PMA and had potentially incomplete ROP outcome data. Of these infants, five had severe ROP [type 1 ROP treated with laser photocoagulation ($n = 2$), type 2 ROP ($n = 3$)], five had mild ROP, and five had no ROP. DBS were available for 560 infants on D0, 709 infants on D3, 811 infants on D7, 789 infants on D14, and 765 infants on D21 (Fig. 1).

Maternal and neonatal characteristics. The mean ± SD birth weight of infants included in this study was 773 ± 129 g. Infants with any ROP were less likely to have received antenatal steroids ($p < 0.05$), and had a lower gestational age and birth weight ($p < 0.0001$; Table 2). There was a statistically significant association between race and development of ROP ($p = 0.04$). Infants who developed ROP were more likely to have received postnatal steroids, longer duration of assisted ventilation and oxygen therapy, late-onset sepsis, grade III-IV IVH, surgically treated patent ductus arteriosus, and bronchopulmonary dysplasia ($p < 0.0001$). Although infants with ROP had a higher incidence of early sepsis, the difference was not statistically significant ($p = 0.09$). Similarly, although mothers of infants with ROP were more likely

Table 1. *Stage of ROP by ROP group*

ROP group	NO ROP ($n = 267$)	Mild ROP ($n = 389$)	Severe ROP ($n = 221$)
Stage 1 no plus disease	0	175 (45)	3 (1)
Stage 2 no plus disease	0	202 (52)	6 (3)
Stage 3 no plus disease	0	9 (2)	67 (30)
Plus disease	0	3 (1)	145 (66)

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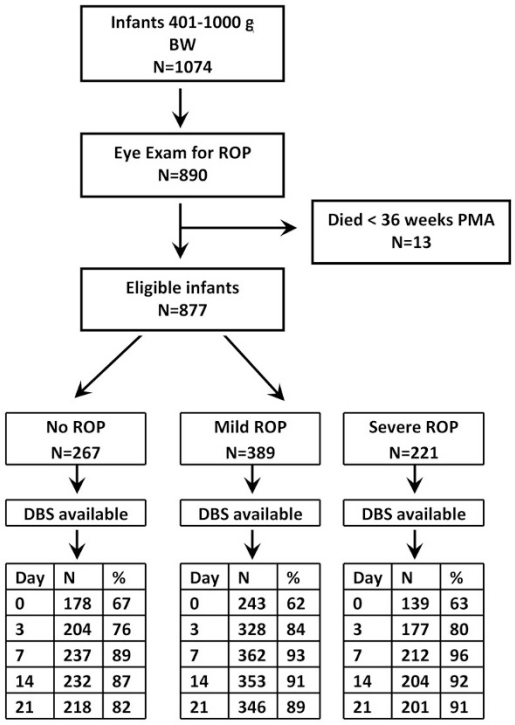

Day	N	%
0	178	67
3	204	76
7	237	89
14	232	87
21	218	82

Day	N	%
0	243	62
3	328	84
7	362	93
14	353	91
21	346	89

Day	N	%
0	139	63
3	177	80
7	212	96
14	204	92
21	201	91

Figure 1. Flow diagram of preterm neonates with available DBS and results of ROP diagnostic examinations. DBS, dried blood spots.

to have received tocolytics, this difference was not statistically significant ($p > 0.1$). There were no differences in sex, incidence of NEC, cystic PVL, PPROM, and mortality between infants who developed ROP compared with those who did not.

Comparison of cytokines by ROP group at each time point. Kruskal-Wallis analysis indicated no difference in median cytokine levels between ROP groups for IL-4, IL-6, TNF-α, and MIP-1α at any of the five-time points. Significant association of several other cytokines with ROP group was observed on D0 (IL-1β, IL-8, IL-10, IL-17, IL-18, sIL-6R, GM-CSF, CRP, TGF-β, and RANTES), D3 (IL-8, IL-10, IL-17, sIL-6R, IFN-γ, GM-CSF, NT-4, MMP-9, and CRP), D7 (IL-1β, IL-2, IL-8, IL-10, IL-17, IL-18, sIL-6R, BDNF, GM-CSF, NT-4, MMP-9, and CRP), D14 (IL-1β, IL-2, IL-8, IL-17, IL-18, BDNF, GM-CSF, NT-4, MCP-1, MMP-9, CRP, and RANTES), and D21 (IL-8, IL-10, IL-17, IL-18, sIL-6R, BDNF, GM-CSF, NT-4, MCP-1, MMP-9, CRP, TGF-β, and RANTES). Mixed-effects model analysis after controlling for important covariates further indicated significant differences across ROP groups for IL-6, IL-17, IL-18, sIL-6R, TGF-β, BDNF, NT-4, CRP, and RANTES (main effect of ROP group on longitudinal data analysis, $p < 0.05$ for all) (Figs. 2 and 3).

Time trends of various cytokines. Mixed-effect model analysis by ROP group indicated that the majority of the cytokines showed significant time trends ($p < 0.05$ for all) during the first 3 weeks of life after controlling for important covariates including center, gestational age, sex, late sepsis, antenatal steroids, postnatal steroids, days in oxygen, race, NEC, severe IVH, and PDA treated with surgery. Cytokines that showed a decreasing trend over time included IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, TNF-B, GM-CSF, NT-4. Cytokines that showed an increasing trend over time included IL-18, BDNF, MMP-9, TGF-β, MIP-1α, RANTES, and CRP.

Interaction of ROP group and cytokines over time. Six cytokines showed significant main effects for ROP group and time with a significant interaction of ROP group with time on mixed-effect model analysis after controlling for important covariates ($p < 0.05$) (IL-6, IL-17, IL-18, TGF-β, BDNF, and RANTES; Fig. 2). Two cytokines showed significant main effects for ROP group without a significant interaction of ROP group with time (CRP, NT-4; Fig. 3). Although sIL-6R had a significant association with ROP ($p = 0.0425$), the association was significant only on D14 and the direction of change of sIL-6R levels on univariate and adjusted analyses was contradictory. Therefore, this has not been described further.

The least-squares means, group comparisons, and significant covariates for cytokines with and without significant ROP-time interaction on adjusted analyses are presented in Tables 3 and 4, respectively. Table 5 summarizes the direction of change in cytokine levels showing significant association with various ROP groups at different time periods on adjusted analyses.

Two cytokines (IL-6 and IL-17) showed significant association with ROP in the early time periods (D0 and D3; Table 5) and three cytokines (TGF-β, BDNF, and RANTES) showed significant association in the later time periods (D7, D14, and D21). IL-18, CRP, and NT-4 showed significant association in both the early and later time periods (D0, D3, D7, D14, and D21). IL-18 was unique in showing biphasic time trends with lower levels in infants with ROP on D0 and higher levels on D7, D14, and D21 compared with infants without ROP.

DISCUSSION

This is the first population study of preterm infants evaluating the association of perinatal inflammation as evaluated by cytokine levels and ROP. In preterm infants, development of ROP was associated with elevated levels of IL-6 and lower levels of IL-17 and IL-18 on D0 suggesting a relationship between FIRS and ROP. IL-17 continued to be significantly lower in infants with mild ROP on D3. Association between ROP group and elevated levels of CRP and lower levels of NT-4 was observed as early as D3; NT-4 continued to be low on D7 and CRP continued to be higher in infants with ROP on D14 and D21. ROP was associated with higher levels of IL-18 and lower levels of TGF-β, BDNF, and RANTES on days 7 to 21 suggesting that postnatal systemic inflammatory response syndrome is also an important factor in the pathogenesis of ROP.

Cytokines have pleiotropic actions including recruitment and activation of immune cells, regulation of angiogenesis, cell proliferation, and apoptosis. Cytokines can have both anti-inflammatory and proinflammatory and anti- and proangiogenic action depending on the target tissue, dose of cytokine, its timing, and duration. In addition to being the site of angiogenesis, the retina is also a neural tissue in which local autocrine loops between neurotrophic, angiogenic and inflammatory mediators play an important role in normal development; disruption of these may result in pathologic states. Cytokine levels at birth are reflective of perinatal events,

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Table 2. *Maternal and neonatal characteristics*

Characteristic	*N*	ROP			*p*
		Absent	Mild	Severe	
Tocolytics (%)	862	38.6	40.4	47.2	0.1384
PPROM (%)	862	22.7	26.2	22.7	0.498
Antenatal steroids (%)	876	83.52	77.38	74	0.0355
Gestational age (wk)	877	27.2 ± 1.8	25.9 ± 1.7	24.8 ± 1.4	<0.0001
Birth weight (g)	877	830.9 ± 121.4	768.3 ± 135.6	712.2 ± 124.1	<0.0001
Male (%)	877	42.0	49.0	49.8	0.1491
Race (%)	877				
African American		51.7	48.1	43.9	
White		34.5	30.1	32.1	0.0412
Others		13.9	21.9	24.0	
Postnatal steroids (%)	877	10.1	27.8	48.0	<0.0001
Ventilator days	877	11.7 ± 18.0	28.8 ± 26.8	49.8 ± 27.1	<0.0001
Days in oxygen	876	36.4 ± 30.0	67.5 ± 35.3	90.0 ± 27.4	<0.0001
Early sepsis (%)	877	0.4	2.3	1.4	0.0871
Late sepsis (%)	877	32.6	42.7	54.3	<0.0001
Grade III or IV IVH (%)	874	6.7	10.4	21.3	<0.0001
Cystic PVL (%)	865	3	4.5	5	0.4957
BPD (%)	877	25.1	53.7	69.2	<0.0001
NEC (proven) (%)	877	9.0	8.2	8.6	0.9425
Deaths (%)	872	2.3	3.9	5.1	0.2573
PDA treated with surgery	877	4.1	15.4	34.8	<0.0001

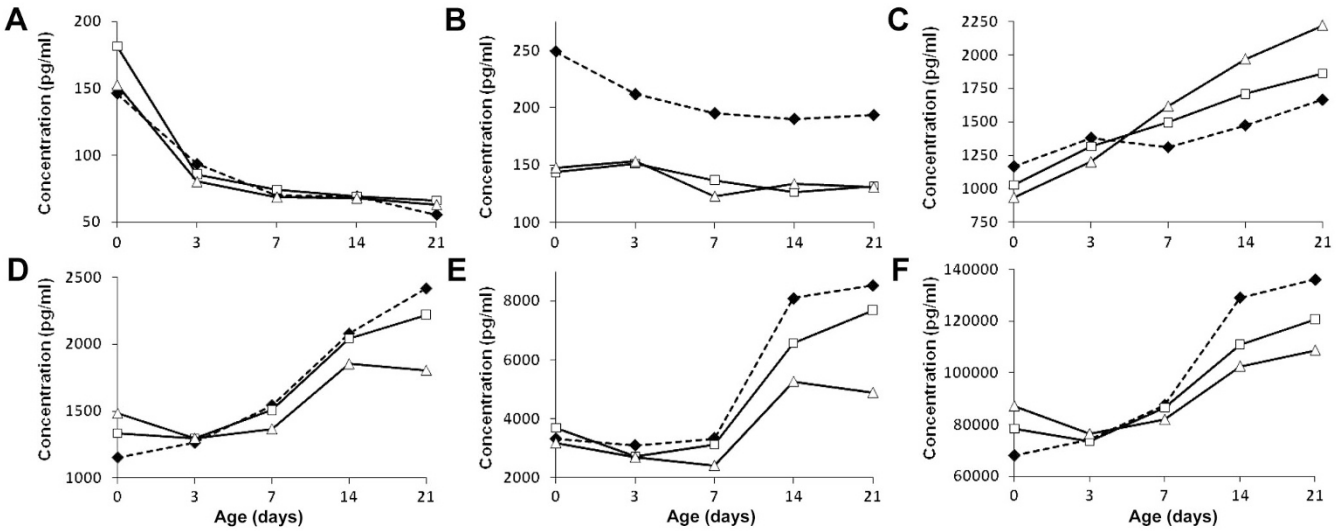

Figure 2. Median concentrations (pg/mL) of cytokines with significant ROP group time interaction. *A*, IL-6; *B*, IL-17; *C*, IL-18; *D*, TGB-β; *E*, BDNF; *F*, RANTES. ◆ no ROP; □: mild ROP; △ severe ROP.

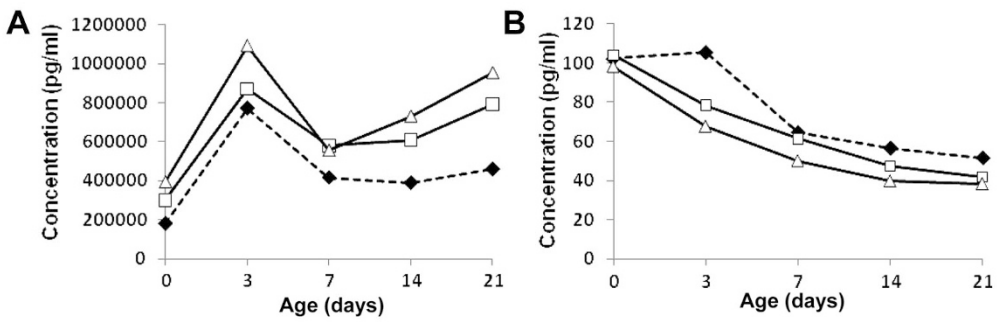

Figure 3. Median concentrations (pg/mL) of cytokines without significant ROP group time interaction. *A*, CRP; *B*, NT-4. ◆ no ROP; □ mild ROP; △ severe ROP.

whereas subsequent levels are reflective of postnatal experiences of the infant (11). Principal proinflammatory cytokines are TNF-α, IL-1, IL-6, IL-8, IL-12, IL-17, IL-18, soluble IL-6 receptor-α (sIL-6rα), IFN- γ, GM-CSF, MCP-1, MIP 1 α, and RANTES. Key anti-inflammatory cytokines include IL-2, IL-4, IL-10, IL-13, IL-11, and TGF-β. BDNF and NT-4 are

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Table 3. *Mixed model analysis for cytokines showing significant ROP group and time interaction after controlling for significant covariates*

Cytokine	Effect—*F* value (Pr > F) ROP	Postnatal age	ROP* age	Time period	Adjusted means by ROP group (pg/mL) No	Mild	Severe	ROP group comparisons (*p*) NO *vs* mild	No *vs* severe	Mild *vs* severe	Significant covariates (*p*)
IL-6	3.57 (0.0284)	23.32 (<0.0001)	2.62 (0.0073)	D0	377	844	499	<.0001	NS	0.0010	Severe IVH (0.0444)
				D3	149	139	103	NS	NS	NS	
IL-17	3.09 (0.0458)	24.28 (<0.0001)	2.31 (0.0182)	D0	286	200	211	<.0001	0.0024	NS	Center (<0.0001)
				D3	228	184	187	0.0331	NS	NS	Severe VH (0.0307)
IL-18	3.45 (0.0323)	87.08 (<0.0001)	6.68 (<0.0001)	D0	722	1,678	1,453	NS	0.0183	0.0324	Center (<0.0001)
				D3	1,847	1,874	1,787	NS	NS	NS	
				D7	1,908	2,047	2,152	NS	0.0187	NS	
				D14	2,047	2,361	2,521	0.0006	<.0001	NS	
				D21	2,163	2,534	2,712	<.0001	<.0001	NS	
TGF-β	3.08 (0.0466)	108.58 (<0.0001)	4.17 (<0.0001)	D7	1,654	1,613	1,407	NS	0.0419	NS	Center (<0.0001)
				D14	2,356	2,141	1,937	0.0449	0.0007	NS	NEC (0.0039)
				D21	2,590	2,436	2,002	NS	<.0001	0.0001	
BDNF	3.84 (0.0219)	55.91 (<.0001)	3.52 (0.0005)	D7	6,694	5,569	4,380	NS	0.0259	NS	Center (<0.0001)
				D14	12,530	10,052	8,108	0.0065	<.0001	0.0401	Male gender (<0.0001)
				D21	12,789	12,505	8,530	NS	0.0001	<.0001	PDA (0.0056)
RANTES	4.04 (0.0179)	58.14 (<.0001)	4.20 (<.0001)	D7	130	120	108	NS	0.0253	NS	Center (<.0001)
				D14	192	152	144	<.0001	<.0001	NS	Late-onset sepsis (0.0010)
				D21	181	166	147	NS	0.0011	0.0373	

* Indicates interaction of ROP group and age.

Table 4. *Mixed model analysis for cytokines without significant ROP group and time interaction after controlling for significant covariates*

Cytokine	Effect—*F* value (Pr > F) ROP	Age	ROP* age	Time period	Adjusted means by ROP group (pg/mL) No	Mild	Severe	ROP group comparisons (*p*) No *vs* mild	No *vs* severe	Mild *vs* severe	Significant covariates (*p*)
NT-4	4.64 (0.0099)	177.58 (<0.0001)	1.89 (0.057)	D3	116.49	99.81	90.48	0.0014	<0.0001	NS	Center (<0.0001)
				D7	85.18	75.76	66.69	NS	0.0017	NS	PN steroids (<0.0055)
CRP	6.07 (0.0024)	32.30 (<0.0001)	0.77 (0.6333)	D0	0.6112	0.8652	0.8416	NS	NS	NS	Center (<0.0001)
				D3	1.2603	1.5552	1.5306	0.016	NS	NS	
				D7	0.8270	0.9629	0.9455	NS	NS	NS	Male gender (0.0023) y
				D14	0.8904	1.1121	1.1748	NS	0.0341	NS	AN steroids (0.0374)
				D21	0.9256	1.3600	1.3190	0.0003	0.0039	NS	Black race (0.0079)

* Indicates interaction of ROP group and age.

Table 5. *Summary of cytokines significantly different between ROP groups by time period after controlling for significant covariates*

ROP group comparisons	D0	D3	D7	D14	D21
No *vs* mild	IL-6 ↑	IL-17 ↓		IL-18 ↑	IL-18 ↑
		NT-4 ↓		TGF-β ↓	
	IL-17 ↓	CRP ↑		BDNF ↓	CRP ↑
				RANTES ↓	
No *vs* severe	IL-17 ↓	NT-4 ↓	IL-18 ↑	IL-18 ↑	IL-18 ↑
			TGF-β ↓	TGF-β ↓	TGF-β ↓
			BDNF ↓	BDNF ↓	BDNF ↓
			RANTES ↓	RANTES ↓	RANTES ↓
	IL-18 ↓		NT-4 ↓	CRP ↑	CRP ↑
Mild *vs* severe	IL-6 ↑			BDNF ↓	TGF-β ↓
					BDNF ↓
	IL-18 ↓				RANTES ↓

Arrows indicate direction of change in marker in the more abnormal ROP group.

neurotrophic factors that also play a role in the regulation of intraocular inflammation.

In this study, we found a panel of six inflammatory markers and two growth factors to be significantly associated with ROP in the first 3 wks of life. In contrast to reports of elevated plasma IL-1β, TNF-α, MMP-9 IL-2, MCP-1 in FIRS and proliferative retinopathies, we did not find these cytokines significantly associated with ROP severity in our large population-based study (12–14). An important factor that may explain this difference is that these studies measured one or a few cytokines in smaller groups of patients.

IL-6 is involved in the regulation of the immune system, in acute phase reaction and inflammation. IL-6 has been shown to play an important role in the pathogenesis of diabetic retinopathy, FIRS, and neonatal sepsis (2,13,15). In this study, significantly higher IL-6 levels were noted on D0 in infants with mild ROP compared with those with severe or no ROP.

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However, because of the lack of increase seen in infants with severe ROP, it is difficult to conclude that IL-6 has an association with the development of ROP.

CRP, a biochemical marker of inflammation, was elevated in infants with ROP both in early and late time periods in this study. Elevated levels of CRP are associated with systemic vascular disorders, diabetic retinopathy, age-related macular degeneration, amniotic fluid infection, congenital neonatal sepsis, funisitis, and late neonatal sepsis (1,16,17). The association of elevated CRP levels and ROP in our study persisted even after controlling for clinical covariates suggesting that a coexistent systemic inflammatory response syndrome may account for the association of elevated levels of CRP and ROP.

Infants with severe ROP tended to have lower levels of cytokines IL-17 and IL-18 on D0 and TGF-β and RANTES on D7-21. These results can be explained by varying proinflammatory and anti-inflammatory actions of the same cytokine in a time-, dose-, and tissue-dependent manner. Another possible explanation for these results could be immune tolerance after chronic fetal exposure to inflammation as has been described previously (18,19). IL-17 and IL-18 are cytokines that have been described recently and whose role in FIRS has not yet been investigated. IL-17 is a proinflammatory cytokine that promotes angiogenesis. It is implicated in intraocular inflammation in uveitis (20). IL-17 has an important role in induction of protective immune response against extracellular bacterial or fungal pathogens such as *Klebsiella pneumonia* and *Candida albicans* (21). Based on the known biologic actions of IL-17, the lower IL-17 levels in infants with ROP in early time points may be responsible for arrest of angiogenesis in early ROP and also predispose these infants to late sepsis that was strongly associated with ROP group.

IL-18 is a pleiotropic proinflammatory cytokine that has immunoregulatory activity. It can act as either an angiogenic or an angiostatic factor (22). High levels of IL-18 have been reported in patients with type 1 diabetes mellitus; however, their relationship to microvascular complications in diabetes is controversial (23). In contrast, in our study, the biphasic and robust association of IL-18 with ROP may be consistent with its role as an immunoregulator and modulator of angiogenesis with time-sensitive expression.

TGF-β is a multifunctional protein that regulates cell growth, differentiation, migration, and extracellular matrix production and plays an important role in embryonic development, wound healing, immune responses, and vascular development (24). TGF-β has been described as a cytokine with bipolarity that can both trigger and inhibit the immune system and angiogenesis (25). Either overproduction or underproduction of TGF-β has been shown to cause ocular abnormalities. In this study, infants with severe ROP had lower levels of TGF-β in the later time periods in this study. These findings are consistent with the role of TGF-β as a regulator of angiogenesis.

The chemokine RANTES plays a significant role in innate immunity, which is particularly important in the neonatal period (26). RANTES cord blood concentrations have been reported to be lower in healthy preterm than in term neonates (27). The increasing levels of RANTES over the first 3 wks in the participants of this study are consistent with the trends described for term infants (26). RANTES has been implicated in the pathogenesis of diabetic retinopathy; its role in ROP has not been investigated to date (14). However, in contrast to elevated levels associated with diabetic retinopathy, RANTES levels were lower with increasing ROP severity in our study.

Clinicians are most familiar with the vascular changes associated with ROP as these are easily distinguishable on ophthalmoscopy (13). Nevertheless, the retina is not only a vascular tissue but also a neural tissue with glial, microglial, and neuronal cells. Direct retinal injury that may not be detectable ophthalmoscopically has been demonstrated early in diabetic retinopathy and in animal models of ROP (28). Astrocytes are involved in the formation of the glia limitans of the retinal vessels and are more sensitive to hypoxia than retinal neurons. Astrocytes subsequently recolonize the retina after a delay that matches the period of leakiness of the proliferative vasculature. BDNF and NT-4 are neurotrophins that have been demonstrated to promote retinal ganglion cell survival after injury (29). Although neurons are the major cellular source of BDNF, BDNF can also be secreted by vascular endothelial cells and immune cells, thus representing a crucial link between the nervous and immune system (30,31). Proinflammatory cytokines regulate the secretion of BDNF and NT-4, which in turn support neuronal survival and further influence the secretion of these same cytokines setting up positive feedback autocrine loops. Serum levels of BDNF have been reported to be higher in adults compared with newborns, and in full-term neonates compared with those delivered preterm suggesting a developmental appearance of neurotrophins in humans. The increase in BDNF and decrease in NT4 levels with age in our cohort are consistent with the trends described in literature for term neonates (32,33). NT-4 levels were lower in infants with ROP compared with controls in our study with significant differences on D3 and D7. BDNF levels were significantly lower in infants with severe ROP at the later time points. A similar trend of BDNF levels has been reported recently in a small study of preterm infants (33). We speculate that lower serum concentrations of BDNF and NT-4 in infants with ROP compared with infants without ROP in our study may reflect glial and neuronal loss associated with ROP.

A majority of clinical characteristics that were strongly associated with ROP in our study have been previously reported in literature (34,35). Center difference was a significant covariate in most of the analyses. This could be related to differing ethnic populations, inborn/outborn infant proportions, clinical practices, or other unidentified factors. Importantly, even after controlling for these covariates, the association between ROP and eight cytokines remained significant suggesting their role in the pathogenesis of ROP.

Strengths of our study include a large sample size; prospectively collected data permitting a thorough investigation of the association of perinatal inflammation and ROP, standardized collection of repeated, timed blood samples for 3 wks starting from birth; assay of multiple cytokines from small volumes of

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blood using the Luminex assay; and multivariate adjustment for control of clinical variables.

Despite the important findings of this study, there are limitations. First, this study is a secondary analysis of the NICHD Cytokine study. Although detailed information regarding serial eye examinations was not available, all infants underwent retinal examinations using an international classification with documentation of highest stage and lowest zone of ROP, presence of plus disease, and need for treatment. Fifteen infants died before 42-wk PMA and had potentially incomplete ROP data. Second, it is difficult to distinguish between primary and secondary mediators in the cytokines described. Systemic levels evaluated in our study may not be representative of ocular levels and it is not possible to ascertain whether systemic levels are secondary to ocular levels or induce ocular changes. However, the fact that changes in cytokine and growth factor concentrations preceded the clinical diagnosis of ROP and that positive association persisted after rigorous adjustment for multiple clinically relevant covariates make the case for causal association plausible. Third, although we included 20 biomarkers representing inflammation and angiogenesis, other markers (VEGF and IGF-I) that have been shown to be important in retinal neovascularization were not included. Finally, we did not correct for multiple comparisons because the post hoc subgroup analysis in this study is exploratory in nature and hypothesis generating. Therefore, reliance should be placed on the observed effect size rather than on statistical significance testing as a basis for decision making (36).

In summary, we have shown a coordinated pattern of increased and decreased levels of eight cytokines in the first 3 wks of life in infants who were diagnosed with ROP. Additional rigorously designed prospective studies are needed to further investigate the preliminary evidence presented in this study. Cytokine dysregulation in ROP may offer a window of opportunity to diagnose and treat ROP medically earlier than the current standards of ROP screening examinations and retinal ablative therapy allow.

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