Increased postnatal concentrations of pro-inflammatory cytokines are associated with reduced IGF-I levels and retinopathy of prematurity

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Abstract

Objective—Retinopathy of prematurity (ROP) is a multifactorial disease linked to low insulin-like growth factor (IGF)-I levels and perhaps to postnatal inflammation. Here, we investigated the longitudinal postnatal serum concentrations of pro-inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α in relation to IGF-I levels and ROP.

Design—The study cohort included 52 infants born before 31 gestational weeks. The infants were screened for ROP and classified as non-ROP ($n = 33$), non-proliferative ROP (stages 1 and 2; $n = 10$), or proliferative ROP (stage 3, all treated for ROP; $n = 9$). Blood samples were collected at birth, 24 h after birth, and then weekly until at least 36 weeks postmenstrual age (PMA) (i.e., up to 13 weeks after birth). Circulating levels of IL-6 and TNF-α were evaluated in relation to circulating IGF-I levels and ROP.

Results—IL-6 levels negatively correlated with IGF-I levels between 5 and 8 weeks after birth, ($p < 0.01$ to $p < 0.05$). At birth, the IL-6 and TNF-α levels were similar independent of later ROP. Twenty-four hours after birth, both IL-6 and TNF-α levels had increased in infants later treated for ROP ($p < 0.05$). Postnatal, infants treated for ROP had higher IL-6 levels than infants without ROP.

Conclusions—The pro-inflammatory response is associated with low IGF-I levels and the development of ROP.

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Keywords

Retinopathy of prematurity; IGF-I; Pro-inflammatory cytokines

1. Introduction

Improved neonatal intensive care has resulted in an increased survival rate among extremely preterm infants, but the morbidity rate is still high in this vulnerable group of newborns. Extremely preterm infants are at high risk of developing morbidities, including retinopathy of prematurity (ROP), a multifactorial disease with a complex pathogenesis that is not yet fully understood. We and others have identified low postnatal levels of IGF-I as a strong risk factor for ROP [1–3]. Other factors beyond low gestational age (GA) at birth and low postnatal levels of IGF-I that have been identified as risk factors for ROP include low birth weight and poor postnatal growth, sub-optimal oxygen therapy, depletion of the maternal supply of growth and nutritional factors, and red blood cell transfusion [1,4–6]. In addition to risk factors specifically associated with preterm birth, perinatal or neonatal infections followed by a systemic inflammatory response with the activation of pro-inflammatory cytokines have been suggested to increase the risk for severe ROP [7–10], but contradictory data have been reported [11–13].

Complex interactions occur between cytokines and growth factors, including IGF-I [14]. Pro-inflammatory cytokines seem to suppress several components of the IGF-I pathway, and many of the cytokines share common signaling components with IGF-I, such as extracellular signal-regulated kinas (Erk)1/2 and mitogen-activated proteinkinase (MAPK). Furthermore, association between elevated levels of the pro-inflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 and suppression of both circulatory levels of and locally produced IGF-I has been described in critically ill patients [15]. Less is known about associations between pro-inflammatory cytokines and IGF-I levels, and ROP in preterm infants.

As stated above, low postnatal IGF-I levels is an established risk factor for ROP. The hypothesis of an inflammatory response as a risk factor for ROP is supported by data showing that infants treated with adenocorticotropic hormone (ACTH), which suppresses inflammation, have a lower incidence of ROP [16,17], and the inflammatory cytokines TNF-α, IL-1, and IL-8 are associated with inflammatory-mediated angiogenesis [18–22]. Inhibition of TNF-α improves physiological angiogenesis and reduces pathological neovascularization in oxygen-induced retinopathy in mice [23,24]. Furthermore, in both experimental and clinical studies inflammation have been identified as a key factor in the development of diabetic retinopathy and macular degeneration, with increased retinal expression of pro-inflammatory cytokines, macrophage infiltration, microglial cell activation, and complement activation [25–27]. Thus, inflammation during the first weeks of life may affect retinal angiogenesis and predispose preterm infants for later development of ROP.

The aim of the present study was to investigate the postnatal pattern of the pro-inflammatory cytokines IL-6 and TNF-α in relation to IGF-I levels, and to ROP outcome in a preterm study population.

2. Methods

2.1. Ethical considerations

The study protocol was approved by the regional ethical review board in Lund, Sweden. The study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents of all patients.

2.2. Study population

This prospective longitudinal study cohort of infants born at Lund University Hospital, Lund, Sweden between January 2005 and May 2007 has been described previously [28]. Inclusion criteria were GA < 31 weeks at birth and the absence of major congenital anomalies. Pregnant women admitted to Lund University Hospital with a risk of delivery before 31 gestational weeks were identified. All pregnancies were dated by ultrasound at 17–18 gestational weeks. Out of 173 infants born < 31 gestational weeks, 64 infants were enrolled before delivery. During the study, nine infants died, and three infants were denied continued participation by their parents, leaving 52 infants for the evaluation of longitudinal circulatory concentrations of IL-6, TNF-α, and IGF-I.

Blood plasma samples from the cord at birth and 24 h after birth were collected. For longitudinal analyses, blood serum sampling was performed before enteral feeding at 72 h and 7 days postnatal age (PNA) and weekly thereafter until a postmenstrual age (PMA) of at least 36 weeks or up to 13 weeks PNA. After centrifugation, plasma and serum samples were stored at −80 °C until assayed.

2.3. Growth measurements

Standardized weight measurements were performed within 24 h after birth. The standard deviation score (SDS) for weight (weight$_{SDS}$) was calculated from an intrauterine growth curve based on ultrasonically estimated fetal weights in Scandinavia [29].

2.4. ROP examinations

ROP screening started at 5–6 weeks of age, but not before 31 weeks PMA. Retinal examinations through dilated pupils were performed biweekly to twice a week depending on the severity of the ROP until the retina was fully vascularized or the condition considered stable. ROP was classified according to international classification [30,31]. Each child was classified according to the most advanced ROP stage observed. For treatment, the recommendations of the Early Treatment for Retinopathy of Prematurity Cooperative Group [32] were followed.

2.5. Quantitative TNF-α and IL-6 analyses

Blood samples were examined in duplicate using a human Luminex xMAP assay including TNF-α and IL-6 (Bio-Rad Laboratories, Hercules, CA, USA) according to the

manufacturer's protocol. All samples were diluted 1:4 before analysis. Early samples (cord and 24 h) and longitudinal samples collected at 72 h and later were analyzed in separate assays. The lowest levels of quantification for TNF-α and IL-6 were 1.3 and 1.7 pg/mL, respectively, in the assay used for early samples (birth and 24 h) and 0.38 and 0.10 pg/mL, respectively, in the assay for longitudinal samples. The inter-assay coefficient of variation (CV) for early samples was 2.71% at 4.80 pg/mL for IL-6. For TNF-α, the pooled serum control was below the detection limit. In the assay for longitudinal samples, the inter-assay CVs were 19.3% and 24.3% at 4.66 pg/mL and 7.94 pg/mL for TNF-α and IL-6, respectively. For early samples, the mean intra-assay CVs between duplicates were 5.2% and 4.2% for TNF-α and IL-6, respectively. For longitudinal samples, the intra-assay CVs were 13.9% and 8.7% at 5.48 pg/mL and 7.34 pg/mL, respectively. Within respective assay, beads and antibodies were from the same batches and all plates were run by the same operator. In addition, all samples from the same infant were run in the same plate.

2.6. Analysis of IGF-I

Samples for measurements of IGF-I concentrations were diluted 1:50 and analyzed by an IGF binding-protein blocked competitive RIA, performed without extraction in the presence of an approximately 250-fold excess of IGF-II. The method has been described previously (Mediagnost GmbH, Tübingen, Germany) [33]. Briefly, after 3 days incubation with primary antibody, a secondary antibody, diluted in PolyEthyleneGlycol was added, and then bound IGF-I was precipitated by centrifugation. Lowest level for detection was 0.064 μg/L. Inter-assay CVs were 29.0%, and 11.3% at 9 μg/L and 33 μg/L, respectively. Intra-assay CVs were 18.5%, and 11.4% at 9 μg/L and 33 μg/L, respectively.

2.7. Statistical analysis

Statistical analyses were performed using the program package IBM SPSS Statistics 20 for Microsoft Windows (IBM, Armonk, NY, USA). The Mann Whitney U test was used for analysis of independent groups. Differences in proportions between groups were tested by Fisher's exact test, and correlation analyses were performed by Spearman correlation analysis. Binary logistic regression was used to calculate the impact on mean IL-6 levels for ROP. Cord blood and 24 h samples were not included in longitudinal data analyses after birth, as these analyses were performed from plasma samples whereas longitudinal analyses were performed from serum samples. Values under detection limit were set to half this value and were then included in following data analyses. The plasma and serum samples were analyzed using different assay setups. $P < 0.05$ was considered significant for all analyses.

3. Results

Clinical data at birth and morbidity data for the study population are shown in Table 1. Nineteen of the 52 infants (36.5%) had culture-verified sepsis during the period from birth to a PNA of 3 weeks, and sepsis was more frequent in infants treated for ROP than in infants without any ROP (7/9, 78% vs. 7/33, 21%; $p < 0.01$).

3.1. Elevated IL-6 levels are associated with decreased levels of IGF-I

To investigate a potential relationship between the inflammatory response and IGF-I levels, correlation analyses were performed between IGF-I and the cytokines IL-6 and TNF-α from 72 h after birth until 13 weeks PNA. Significant negative correlations between IGF-I and IL-6 were found at 5–8, 11, and 13 weeks PNA (Table 2). Mean IGF-I levels during the period from 5 to 8 weeks PNA inversely correlated with mean IL-6 levels during the same period ($r_s = -0.495$, $p < 0.001$; Fig. 1). TNF-α levels did not correlate with circulating IGF-I levels at any time point.

3.2. Early concentrations of IL-6 and TNF-α in relation to ROP

To investigate the role of increased levels of pro-inflammatory cytokines in early life as potential risk factor for ROP, concentrations of IL-6 and TNF-α were measured in plasma at birth and 24 h after birth. At birth, Mann-Whitney U test reviled no difference in IL-6 and TNF-α concentrations in cord blood between infants without any ROP and infants later treated for ROP. However, 24 h after birth, both IL-6 and TNF-α concentrations were higher in infants who were later treated for ROP ($p < 0.05$) (Table 3, Fig. 2). The maximum TNF-α level at 24 h in infants without later ROP originated from one outlier within this group. At both birth and 24 h after birth, a substantial number of TNF-α values were below the detection limit. However, at 24 h the frequency of infants with TNF-α values above the detection limit was higher in the group of infants later treated for ROP than in the group of infants without any ROP (3/10 vs. 1/33, $p < 0.05$).

3.3. Longitudinal pattern of IL-6 and TNF-α concentrations in relation to ROP

Next, we investigated if circulatory levels of examined cytokines during the postnatal period were related to ROP. Infants requiring treatment for ROP had significantly higher median IL-6 serum concentrations 72 h after birth and at 1, 5, 8, 11, and 12 weeks PNA than infants without ROP (Table 4, Fig. 3). Weekly IL-6 serum concentrations in infants without ROP and infants treated for ROP are given in Table 4. Furthermore, mean IL-6 levels for the periods 3–7 days and 5–8 weeks PNA were higher in infants treated for ROP than for infants without ROP; median (min; max) 49.7 (13.3; 201.9) vs 20.0 (6.9; 179.7) pg/mL, $p < 0.01$ for postnatal days 3–7, and 9.0 (4.16; 207.5) vs 5.7 (2.4; 13.8) pg/mL, $p < 0.05$ for postnatal weeks 5–8.

Direct logistic regression was performed to assess the impact of mean IL-6 concentrations 5–8 postnatal weeks on the likelihood to develop severe ROP. The model was significant with Chi^2 = 12.60, $p < 0.001$ and explained between 27.6% (Cox and Snell R square) and 41.8% (Nagelkerke R squared) of the variance of the development of severe ROP. Differences in TNF-α concentrations did not reach the predetermined level of statistical significance between infants requiring treatment for ROP and those without ROP at a PNA > 24 h, though at the PNA of 72 h, 11 weeks, and 12 weeks, slightly higher serum TNF-α concentrations were measured in infants treated for ROP ($p = 0.072$ to $p = 0.086$).

4. Discussion

In this longitudinal study elevated IL-6 levels were frequently associated with lower IGF-I levels during the second and third month of life. Furthermore, we found an association between elevated levels of the pro-inflammatory cytokines IL-6 and TNF-α and severe ROP requiring treatment. Though increased levels of TNF-α were only observed in very early life (24 h after birth), infants treated for ROP had continuously higher postnatal levels of IL-6 than infants without any stage of ROP. In addition, episodes of sepsis were more frequent in infants with ROP.

A negative correlation between IL-6 and IGF-I in plasma from cord blood was previously shown in this study cohort [34]. However, 72 h after birth no such correlation was observed. In the present study, the longitudinal pattern of pro-inflammatory cytokines and IGF-I was analyzed up to 13 weeks after birth, which to our knowledge, has not been reported previously. In line with a negative correlation between IL-6 and IGF-I, an inhibiting effect of IL-6 on IGF-I expression has been shown in mice [35]. Mice overexpressing IL-6 are normal size at birth but suffer from impaired postnatal growth and lower IGF-I levels, despite normal food intake. The administration of antibodies neutralizing the IL-6 receptor improves postnatal growth, and the administration of recombinant IL-6 reduced IGF-I levels, supporting an inhibitory effect of IL-6 on IGF-I.

Similarly, high levels of IL-6 and low levels of IGF-I have been observed in humans suffering from starvation, infection, or trauma, which are all characterized by a catabolic state. Catabolism is also characteristic of very preterm infants. A negative correlation has been observed between IL-6 and IGF-I in relation to severe starvation. Bartz et al. reported high IL-6 and low IGF-I levels in children (mean age 16.3 months) suffering from severe starvation. Two weeks of nutritional intervention resulted in a 3-fold increase in IGF-I and a 6-fold decrease in IL-6 [36]. Similarly, a negative correlation was found between plasma IGF-I levels and inflammation markers, including IL-6, in growth-stunted Zimbabwean children followed from 6 weeks of life [37]. Furthermore, in connection with infectious disease, low levels of IGF-I and elevated levels of inflammation markers have been reported, indicating that recent acute illness is associated with decreased IGF-I levels [37]. Similar to starvation, infection and trauma in extremely preterm birth is associated with catabolism. Catabolism has been associated with muscle wasting, in which the induction of cytokine networks involving TNF-α, IL-1β, and IL-6 has been suggested to be crucial [38]. These cytokines are all associated with reduced circulating levels and locally produced IGF-I [15].

Neonatal sepsis [12,39,40] and elevated levels of pro-inflammatory cytokines [7–10] were previously identified as risk factors for ROP. Silveira et al. found that high IL-6, IL-8, and TNF-α levels within 72 h after birth are associated with a higher incidence of severe ROP [9], and Sood et al. reported that infants treated for ROP have elevated IL-6 levels within 1 day after birth [8]. These reports are in line with our data. However, the higher incidence of inflammation at birth in infants with low GA at birth may serve, at least partly, as a confounder, as low GA is also a risk factor for ROP. In contrast to our results, Sood et al. did not find any difference in IL-6 levels in the postnatal period between infants with and without later ROP. However, Sood et al. followed infants for three weeks after birth, whereas

infants in our study were followed up to 13 weeks PNA. In infants treated for ROP we found higher IL-6 levels early after birth, but also frequently from five weeks PNA. These studies were association studies showing a relationship between markers of inflammation and ROP. The mechanisms underlying this association remain to be elucidated.

In our study, circulating TNF-α levels were elevated early after birth in infants treated for ROP, but later in postnatal life no significant differences were found in TNF-α levels between infants with and without ROP, though TNF-α levels tended to be higher. In this study pro-inflammatory cytokines at birth did not differ between infants without ROP and those later treated for ROP, and severe ROP was not associated with maternal infection.

The strength of this study is the longitudinal design. To the best of our knowledge, this study is the first in very preterm infants presenting weekly data on pro-inflammatory cytokines and IGF-I data collected over several months. The study also has limitations. The study was small with few infants treated for ROP. This is a limiting factor for multivariate analyses taking confounders in consideration. Furthermore, analyses of pro-inflammatory cytokines were limited to IL-6 and TNF-α. The half-life of IL-6 is relatively short, and further implications of a systemic inflammatory response might have been observed if we had been able to measure additional cytokines or IGFBP-1, which is known to increase rapidly with sustained levels in response to endotoxins. In this study, TNF-α levels were commonly under detection limit, both in infants with and without ROP. Although the frequency of samples with detectable TNF-α levels was higher in infants with ROP, the association between increased TNF-α levels and ROP has to be further investigated.

5. Conclusion

In conclusion, data presented in this study suggest that postnatal episodes of inflammation with elevated pro-inflammatory cytokine levels are associated with low serum levels of IGF-I and the development of severe ROP.

Acknowledgments

Abbreviations

ACTH	Adenocorticotropic hormone
CV	Coefficient of variation
Erk	Extracellular signal-regulated kinas
GA	Gestational age

IL	Interleukin
IQR	Interquartile range
MAPK	Mitogen-activated proteinkinase
PMA	Postmenstrual age
PNA	Postnatal age
ROP	Retinopathy of prematurity
SDS	Standard deviation score
TNF	Tumor necrosis factor

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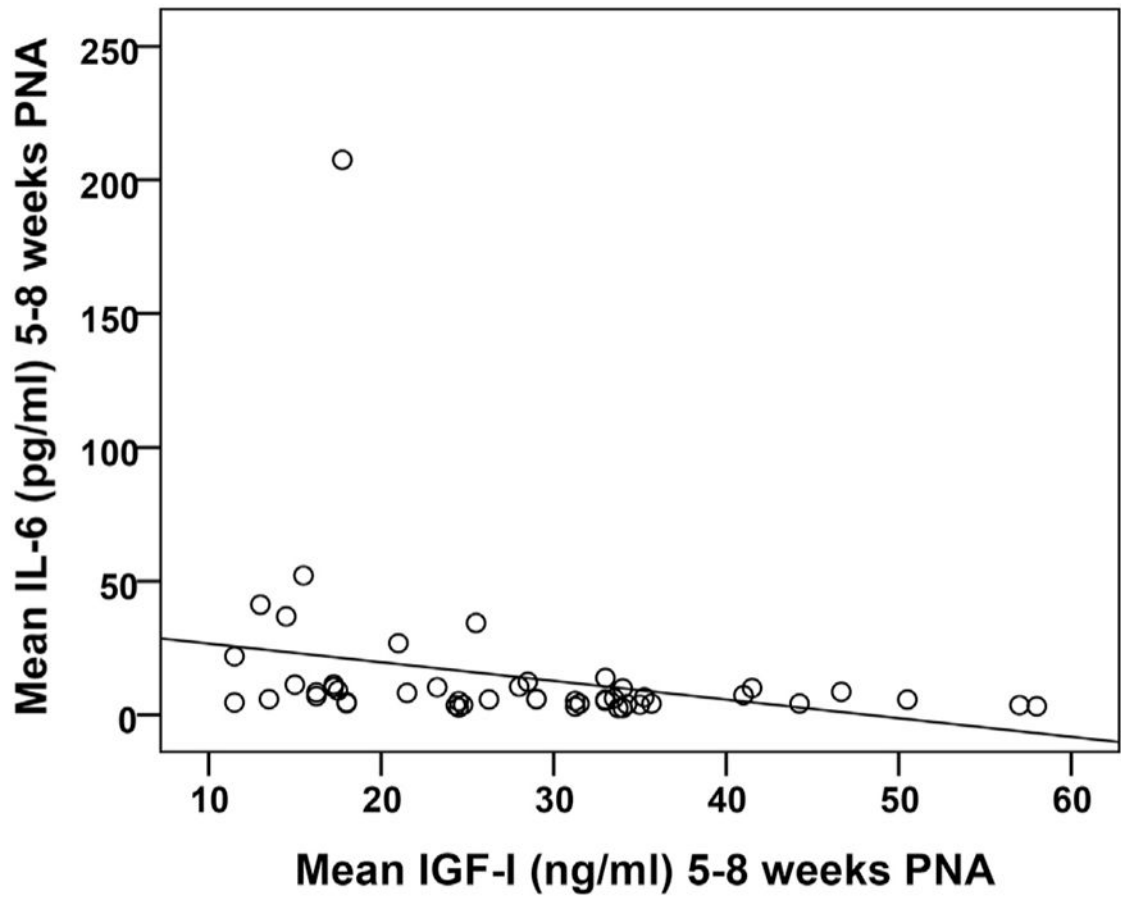
Fig. 1.
Correlation between IGF-I and IL-6.
The correlation between mean IGF-I (ng/mL) and mean IL-6 (pg/mL) from 5 to 8 weeks postnatal age (PNA).
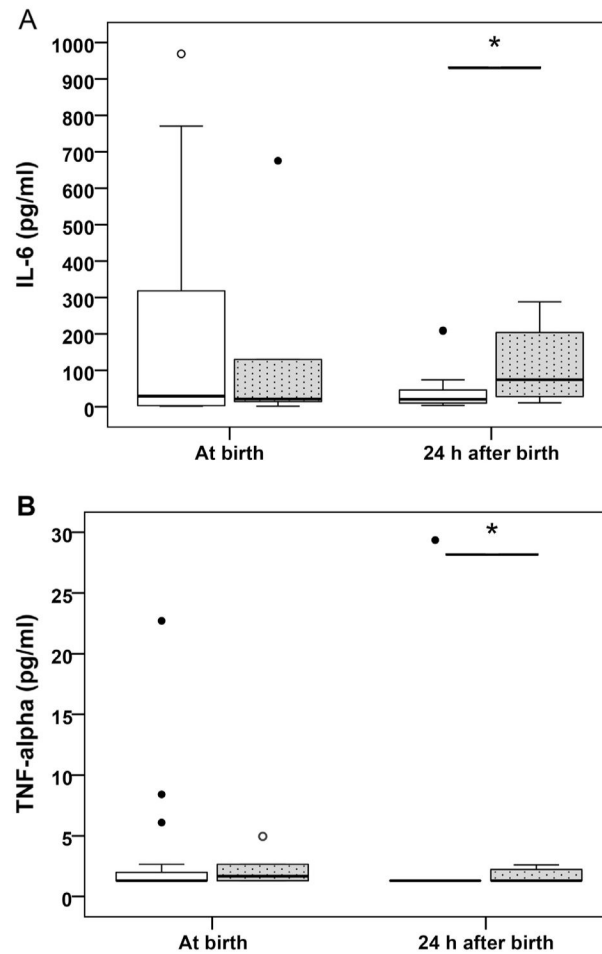
Fig. 2.
Early levels of pro-inflammatory cytokines in relation to ROP.

Plasma concentrations of (A) IL-6 and (B) TNF-α at birth and 24 h after birth in infants without any stage of ROP ($n = 25$) (white boxes) and infants later treated for ROP ($n = 5$) (grey, dashed boxes). Solid dots represent extreme values > 3 interquartile ranges (IQRs) from the end of the box, and open circles represent outliers between 1.5 and 3 IQRs from the end of the box. In Fig. A, four extreme values, two over detection limit and one with IL-6 concentration 4833 pg/mL at birth, and one with the concentration of 9796 pg/mL 24 h after birth, in infants without later ROP are not shown in the figure. In Fig. B, two extreme values with TNF-α concentrations 59 and 446 pg/mL, respectively, at birth in infants without later ROP are not shown in the figure. $*p < 0.05$.

Fig. 3.
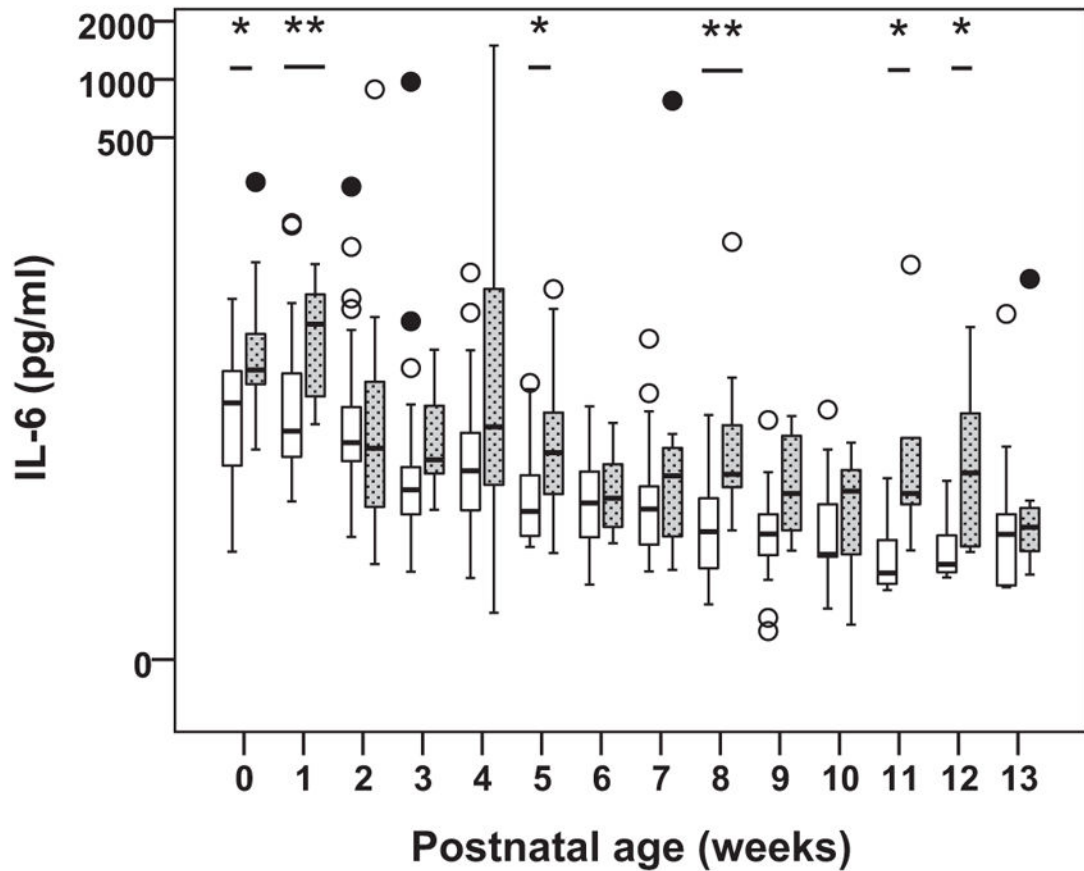Postnatal levels of IL-6 in relation to ROP.

Longitudinal serum concentrations of IL-6 in infants without any stage of ROP (white boxes) and infants treated for ROP (grey, dashed boxes). Solid dots represent extreme values > 3 interquartile ranges (IQRs) from the end of the box, and open circles represent outliers between 1.5 and 3 IQRs from the end of the box. $*p < 0.05$, $**p < 0.01$.

Table 1

Axiological data at birth.

	All	No rop	ROP stages 1 and 2	Treated ROP
Number	52	33	10	9
GA, weeks	26.5 (23.0; 30.6)	27.4 (24.3; 30.6)	25.1 (23.4; 26.4)	24.1 (23.0; 27.1)
Boys/girls	27/25 (52/48)	16/17 (48/52)	7/3 (70/30)	4/5 (44/56)
AGA/SGA	39/13 (75/25)	29/4 (88/12)	4/6 40/60)	6/3 67/33)
Single/twin	27/25 (52/48)	16/17 (48/52)	7/3 (70/30)	4/5 (44/56)
Weight, g	850 (348; 1716)	970 (592; 1716)	625 (348; 796)	638 (460; 854)
Weight$_{SDS}$	−0.78 (−5.00; 0.89)	−0.69 (−4.66; 0.89)	−2.30 (−5.00; 0.60)	−0.72 (−3.16; 0.39)
Sepsis	19 (37)	7 (21)	5 (50.0)	7 (78)
Maternal infection	25 (49)	19 (58)	3 (30)	3 (38)
Preeclampsia	11 (21)	7 (21)	2 (20)	2 (22)

Data are presented as median (min; max) or n (%). AGA, appropriate for gestational age; ROP, retinopathy of prematurity; SDS, SD score; SGA, small for gestational age.

Table 2

Correlations between IL-6 and IGF-I concentrations week by week.

PNA (weeks)	n	r_s	*p*-value
0 (72 h)	49	0.110	0.452
1	44	−0.183	0.235
2	43	−0.281	0.068
3	46	−0.239	0.110
4	43	−0.008	0.957
5	45	−0.322	**0.031**
6	37	−0.472	**0.003**
7	43	−0.390	**0.010**
8	41	−0.447	**0.003**
9	36	−0.228	0.182
10	31	0.052	0.780
11	26	−0.525	**0.006**
12	24	−0.175	0.414
13	24	−0.454	**0.026**

PNA, postnatal age; r_S, Spearman coefficient of correlation. Significant values are bolded.

Table 3

IL-6 and TNF-α plasma levels in cord blood at birth and 24 h after birth.

	At birth		p-value	24 h after birth			p-value
	No ROP (n = 25)	Treated ROP (n = 5)		No ROP (n = 34)	Treated ROP (n = 10)		
IL-6 (pg(mL)	29.2	21.5	0.933	20.4	74.3		**0.012**
	(1.71; 4833)	(1.7; 675)		(3.5; 9796)	(10.9; 288)		
TNF-a (pg/mL)	1.3	1.7	0.439	1.3	1.3		**0.012**
	(1.3; 446)	(1.3; 5.0)		(1.3; 29)	(1.3; 2.6)		

Data are presented as median (min; max). ROP, retinopathy of prematurity. Stattistical analyses were performed by Mann-Whitney U test. Significant values are bolded.

Table 4

Weekly IL-6 serum levels measured in pg/mL.

PNA (weeks)	No ROP (pg/mL)	n	Treated ROP (pg/mL)	n	p-value
0 (72 h)	20.3	30	30.5	9	**0.016**
	(2.6; 72.4)		(11.2; 293)		
1	14.2	26	53	9	**0.005**
	(5.6; 180)		(15.5; 110)		
2	12.2	26	11.4	7	0.509
	(3.3; 278)		(2.1; 889)		
3	6.6	30	9.8	7	0.081
	(1.9; 973)		(5.0; 39.1)		
4	8.5	25	15.0	8	0.179
	(1.6; 99.3)		(0.8; 1500)		
5	4.8	28	10.8	9	**0.037**
	(2.8; 26.0)		(2.6; 81.5)		
6	5.4	23	6.0	6	0.686
	(1.4; 19.4)		(3.0; 15.75)		
7	5.0	27	7.9	8	0.326
	(1.9; 44.6)		(1.9; 776)		
8	3.6	26	8.1	7	**0.002**
	(0.9; 17.4)		(3.7; 143)		
9	3.5	19	6.2	8	0.063
	(0.4; 16.3)		(2.7; 17.2)		
10	2.5	17	6.5	6	0.263
	(0.8; 18.6)		(0.5; 12.2)		
11	1.8	11	6.2	6	**0.021**
	(1.3; 7.7)		(2.7; 109)		
12	2.1	10	9.0	6	**0.013**
	(1.7; 7.4)		(2.6; 51.5)		
13	3.5	12	3.8	7	0.447
	(1.4; 60.4)		(1.8; 92.0)		

Data are presented as median (min; max). PNA, postnatal age; ROP, retinopathy of prematurity. Significant values are bolded.