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ORIGINAL

# Cord blood interleukin-8 levels correlate with airway flow limitation at eight years of age in ex-very low birth weight infants

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## ABSTRACT

*Background. Exposure to prenatal inflammation increases the risk for development of bronchopulmonary dysplasia.*

*Aim. To evaluate the correlation between cord blood and gastric aspirate levels of interleukine-6 (IL-6) and interleukine-8 (IL-8) in preterm infants, and lung function at the age of 8 years.*

*Methods. Between 2000-2002 we recruited 129 infants of gestational age < 30 wks. The concentration of IL-6 and IL-8 were measured in gastric aspirate and cord blood. At the age of 8 years, 30 ex-preterm infants, with mean gestational age of 27 wks and mean birth weight of 955 g, returned for pulmonary function measurement. To exclude major bias, a comparison between the study group and non-responder group was done and showed no statistically significant difference with respect to perinatal characteristics, ventilation days, bronchopulmonary dysplasia and cytokine concentration.*

*Results. Pulmonary function test measurments in children born preterm were lower than in their term pairs. However, only the difference in forced mid-expiratory flow (FEF25-75%) was statisticaly significant. The concentration of IL-6 and IL-8 in cord blood and in gastric aspirate inversely correlated to all parameters of lung fuction at the age of 8 years, however only correlations between the concentration of IL-8 in cord blood and forced expired volume in one second/forced vital capacity (FEV1/FVC) ($r = -0.38$, $p = 0.04$) and FEF 25%-75% ($r = -0.44$, $p = 0.02$) were statistically significant.*

*Conclusion. These results show a negative correlation between the concentration of IL-8 in cord blood and FEF25%-75% and FEV1/FVC, which suggests the important role of IL-8 in early airway remodeling.*

**Key words: IL-8, lung function, preterm infant.**

## Introduction

The study by Watterberg et al. (1) in 1996 established that the presence of chorioamnionitis (CA) is associated with a lower incidence of respiratory distress syndrome (RDS) and a higher incidence of bronchopulmonary dysplasia (BPD), indicating that lung injury can start before birth. Later studies confirmed that CA, (2,3) funisitis, (4) and fetal inflammatory response syndrome (5,6) were associated with an increased incidence of BPD; however, results were not reproduced in some other studies. (7,8) It has been suggested that the association between CA and BPD is more complicated. (9) Van Marter et al. (10) introduced the "multiple hit" theory. They established that CA does not increase the incidence of BPD by itself, but only in combination with already known risk factors for BPD, such as mechanical ventilation longer than 7 days and exposure to postnatal infection. In our cohort of infants, with gestational age (GA) < 30 weeks exposure to prenatal inflammation did not increase the risk of BPD. However, low gestational age, male sex, need for resuscitation, mechanical ventilati-

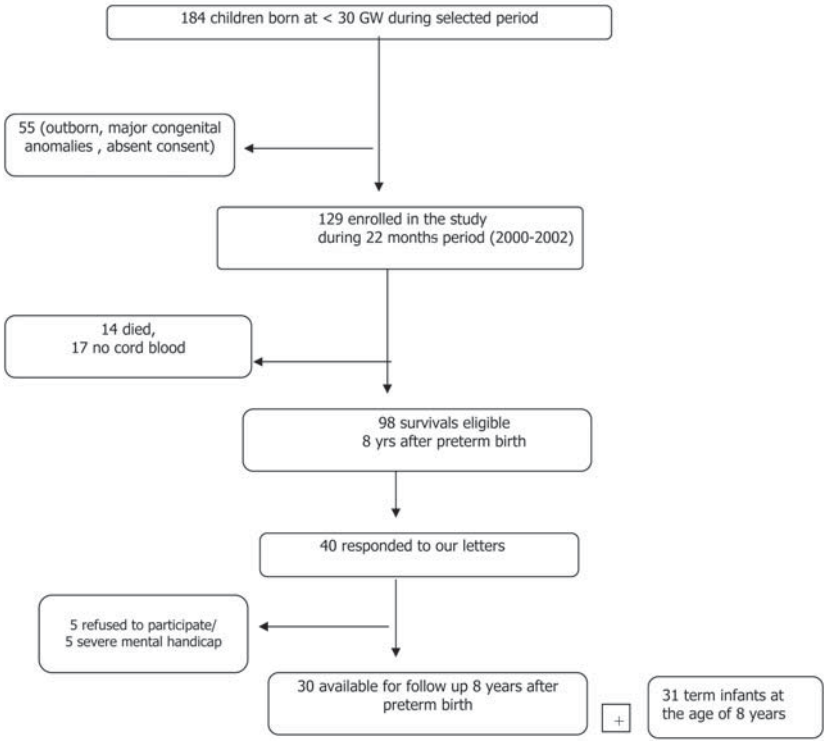


**Figure 1. Flow-chart of the study group from birth to school age.**
**GW; gestational weeks.**

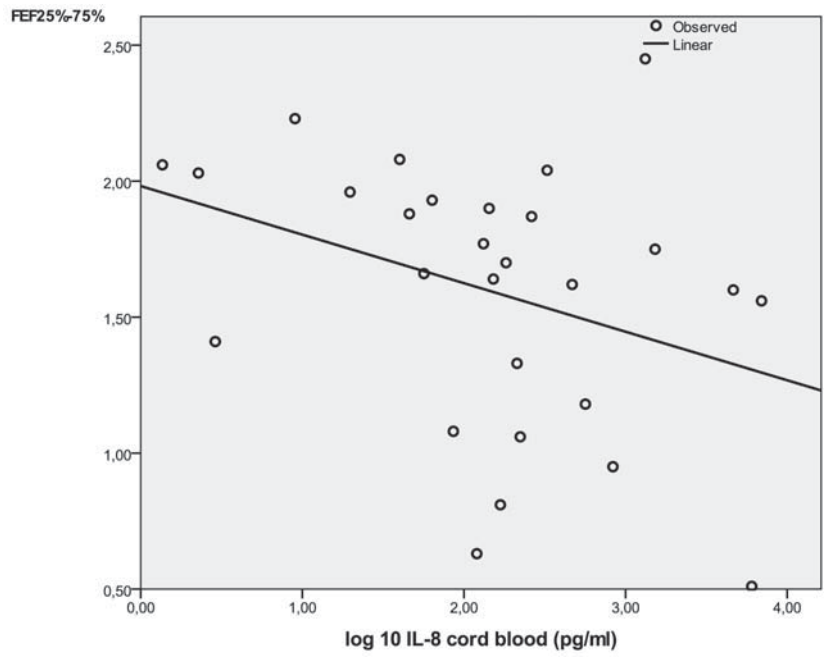


**Figure 2. Correlation between FEF25-75% and log IL-8 concentration in cord blood.**
**FEF25-75%, forced mid-expiratory flow; IL, interleukin.**

on, and late onset sepsis were major risk factors for BPD development. (11) According to an overview from Beer and Zimmerman, (12) the increased incidence of BPD after prenatal inflammation was confirmed only in 6 of 18 studies; furthermore, in only one study were the results adjusted for gestational age. (13) Results of long-term outcomes with respect to lung function of school-age children and adolescents born preterm are also conflicting. In some studies, respiratory function variables reflecting airflow were significantly diminished (14-17) when compared to healthy, term-born children and adolescents, while in the study by Narang et al. no significant difference was found. (15)

The question is: can prenatal inflammatory exposure affect lung function in later life? So far, we are aware of only two studies investigating the association between CA and pulmonary outcome in childhood. (16,17) The study by Watterberg on children aged 18-22 months showed no difference in the use of supplemental oxygen, bronchodilators, and systemic or inhaled steroids between infants with or without CA. In the study by Kumar, it was established that CA and prematurity have a joint predisposing effect on recurrent wheezing and physician-diagnosed asthma at a mean age of 2.2 years.

The aim of this prospective observational study was to evaluate the effects of interleukin 6 (IL-6) and interleukin 8 (IL-8) in cord blood and gastric aspirate on lung function 8 years after preterm birth.

## Methods

Study design and subjects

Infants with GA < 30 wks born at the Ljubljana Maternity Hospital, a tertiary referral centre for premature infants in Slovenia, during the period from September 1st, 2000, to June 30th, 2002, were prospectively enrolled in the study with the aim to analyze prenatal and postnatal risk factors for the development of BPD. The eligibility criteria for the study were: 1) inborn infant, 2) written informed parental consent obtained, 3) cord blood and placenta available, and 4) absence of obvious congenital malformation. BPD was defined as a requirement for supplemental oxygen at 36 weeks postmenstrual age. (18) Details including gestational

**Table 1. Perinatal characteristics of the study group and non-responder group. Values are expressed as a - mean (standard deviation), b - median [mininum-maximum], c -median Šinterquartile range] or percentage.**

| | study group n=30 | non-responder preterm group n=98 | p |
|---|---|---|---|
| gestational age (wks)[a] | 26.6 (1.8) | 27.2 (1.6) | 0.07 |
| birth weight (g) [a] | 955 (254) | 1041 (231) | 0.05 |
| male sex (%) | 40 | 38 | 0.80 |
| 1-minute Apgar score [b] | 6 [5-7] | 6 [4-7] | 0.26 |
| 5-minute Apgar score [b] | 7 [6-8] | 7 [6-8] | 0.12 |
| prenatal dexamethasone (%) | 68 | 87 | 0.56 |
| ventilation (days) [c] | 16 (0-77) | 13 (0-137) | 0.39 |
| oxygen (days) [c] | 40 (0-162) | 35 (0-149) | 0.47 |
| postnatal dexamethasone (%) | 33 | 30 | 0.81 |
| BPD (%) | 33 | 18 | 0.59 |
| IL-8 cord blood (pg/ml) [b] | 168 [51-662] | 289 [85-1017] | 0.57 |
| IL-6 cord blood (pg/ml) [b] | 6 [1-27] | 5 [2-22] | 0.43 |
| IL-8 gastric aspirate (ng/ml) [b] | 25 [0.4-394] | 22 [0.5-279] | 0.25 |
| IL-6 gastric aspirate (ng/ml) [b] | 4 [0.1-12] | 2.5 [0.4-14] | 0.50 |
| chorioamnionitis (%) | 47 | 40 | 0.29 |

BPD, bronchopulmonary dysplasia; IL, interleukin.

**Table 2. Lung function tests at 8 years after preterm birth compared to term controls, p value for % predicted (except for FEV1/FVC, measured value).**

| | preterm infants (30) | | term controls (31) | | p |
|---|---|---|---|---|---|
| | measured | % predicted | measured | % predicted | |
| FEV 1, (L) | 1.5 (0.3) | 86.7 (14.5) | 1.7 (0.4) | 90.9 (10.5) | 0.21 |
| FVC, (L) | 1.7 (0.3) | 97.7 (10.8) | 1.9 (0.4) | 99.5 (9.8) | 0.54 |
| FEV1/FVC, (%) | 82.4 (16.9) | - | 87 (21.0) | - | 0.16 |
| FEF25-75%, (L/s) | 1.6 (0.5) | 79.2 (19.6) | 2.1 (0.5) | 87.0 (21.0) | 0.02 |

age, sex, birth weight, prenatal steroids, and surfactant treatment, ventilatory support and days of postnatal oxygen requirement were also collected. Gestational age was determined by the best obstetric estimate using the last menstrual period and/or early ultrasound assessment. After 8 (range 7-9) years, children were called back for lung function follow-up. Infants with severe mental handicap were excluded from the follow-up (figure 1). A control group with 31 age and sex-matched children born at term was added for the comparison of pulmonary function tests, measurement of fraction of nitric oxide in air (FeNO), and six-minute walking test (6MWT). The National Medical Ethics Committee approved the study. Parents or guardians of each participating child gave written informed consent.

At the time of birth, blood was collected from the umbilical cord, then centrifuged and stored at –70$^{O}$C until cytokine immunoassay was performed. Commercial ELISA kits (Endogen-Pierce, Boston, USA) were used for determination of the IL-6 and IL-8 levels. Gastric aspirate sample was collected at the time of delivery with a 5F orogastric feeding tube. Because no reliable correction factor was available, (19) cytokine concentrations in gastric aspirates were expressed as volume concentrations (pg/ml or ng/ml). The placenta, including fetal membranes and umbilical cord, was examined microscopically for the presence of inflammation. CA was defined as acute when ten or more polymorphonuclear leukocytes were found per high-power field in the amniotic and chorionic membranes, in the chorionic plate, and/or in the umbilical cord.

61 participants attended the respiratory function unit at the University Children's Hospital in Ljubljana for a 1.5-hour visit during which they were examined by a doctor and underwent lung function testing, FeNO measurement, and fitness assessments with 6MWT.

Fraction of exhaled nitric oxide

FeNO was measured following the American Thoracic Society/European Respiratory Society's recommendations. We used single breath measurement with computerized system device NioxMino, Aerocrine, Sweden, with flow rate of

0.05 l/min for at least 6 seconds. FeNO measurements were done prior to spirometric manoeuvres, only in children without signs and symptoms of acute respiratory infection for 6 weeks before testing. We asked children not to eat or drink for at least 1 hour before measurement. (20) Due to flow dependency of FeNO values, we encouraged the use of constant expiratory flow rates by using a visual computer program. Nose clips were not used. Three recordings were made. FeNO was calculated as the mean of three measurements with a coefficient of variation of less than 10%.

Six-Minute Walk test

The fitness assessment was measured by 6MWT. It was chosen because it is a simple method for assessing exercise capacity. All tests were done on an indoor 50- m-long, flat hard ground. Only one technician, trained in using the standard protocol, performed all the tests. (21,22)

Pulmonary Function Tests

The American Thoracic Society guidelines were used to perform tests. (23) Only one respiratory technician performed the measurements, and he was blinded to the clinical details of the participant. We used a flow-volume spirometry (Vitalograph, Ltd, Birmingham, UK). The best of three manoeuvres was recorded. Measured variables reflecting airflow were: forced expired volume in one second (FEV1) and forced mid-expiratory flow (FEF25-75%). Lung volumes included forced vital capacity (FVC) and vital capacity (VC). Results were expressed as percentages of the standardised values predicted for normal children according to sex and height. We used Polgar's and Promadhat's prediction equations for lung-function measurements. (24)

Statistical analysis

The sample size was opportunistic, so a power calculation was not performed. Numerical data are presented as mean ± standard deviation (SD) or median with 1st and 3rd quartile and analyzed by independent-samples t-test if normally distributed, or by Mann-Whitney U test if skewed. Categorical data are expressed as proportions and analyzed by chi-squared or Fisher's exact tests as appropriate. For post-hoc comparison of categorical variables, Keppel modification of Bonferroni correction was used.

The raw data for cytokine concentrations was log-transformed to obtain a normal distribution before correlation was calculated. The strength of association between variables was obtained

**Table 3. Characteristics of the study population at 8 years compared to term controls, results as mean (SD).**

| | preterm infants (30) | term controls (31) | p |
|---|---|---|---|
| age | 8.8 (0.8) | 8.5 (0.5) | 0.85 |
| male sex | 12 | 12 | 1.0 |
| height (cm) | 128.7 (9.7) | 134.1 (10.0) | 0.04 |
| weight (kg) | 27.2 (8.3) | 30.4 (7.1) | 0.11 |
| eNO (ppb) | 18.2 (14.2) | 12.1 (4.5) | 0.04 |
| 6-min WT (m) | 510 (72) | 525 (61) | 0.39 |

**Table 4. Correlation between measured leveles of IL-6 and IL-8 (logarithmic values) in cord blood (cb) and gastric aspirate (ga) at birth and lung volumes and flows at the age of 8 years. (r = Spearman`s coefficient).**

| | | IL-8cb (pg/ml) | IL-6cb (pg/ml) | IL-8ga (ng/ml) | IL-6ga (ng/ml) | $0^2$ (days) | ventilation (days) |
|---|---|---|---|---|---|---|---|
| FVC % | r | -0.02 | -0.16 | -0.05 | -0.04 | -0.35 | -0.24 |
| | p | 0.89 | 0.41 | 0.78 | 0.84 | 0.05 | 0.20 |
| FEV1 % | r | -0.22 | -0.15 | -0.15 | -0.16 | -0.47 | -0.42 |
| | p | 0.25 | 0.45 | 0.44 | 0.39 | 0.009 | 0.02 |
| FEV1/FVC | r | -0.38 | -0.07 | -0.237 | -0.32 | -0.08 | -0.14 |
| | p | 0.04 | 0.73 | 0.22 | 0.09 | 0.69 | 0.46 |
| $FEF_{25-75\%}$ | r | -0.44 | -0.23 | -0.31 | -0.33 | -0.51 | -0.57 |
| | p | 0.02 | 0.22 | 0.10 | 0.08 | 0.04 | 0.01 |
| 6min WT | r | -0.29 | -0.32 | -0.23 | 0.04 | -0.12 | -0.24 |
| | p | 0.13 | 0.09 | 0.23 | 0.83 | 0.52 | 0.20 |
| eNO | r | -0.01 | 0.26 | 0.24 | -0.06 | 0.04 | 0.07 |
| | p | 0.95 | 0.19 | 0.22 | 0.77 | 0.83 | 0.97 |

by the Spearman rank-order method. A statistical analysis was performed using PASW 18 software (SPSS Inc., Chicago, IL, USA). A P value of $< 0.05$ was considered as significant.

## Results

Perinatal characteristics of the study group versus the non-responder preterm group are shown in table 1. None of the tested variables showed a statistically significant difference between the groups.

The characteristics of the study group at the age of 8 years are shown in table 2 where the values are also compared to the term control group. Children born preterm were shorter at the average age of 8 years when compared to the term control group, but there was no difference in body weight. The values of pulmonary-function test measurements in children born preterm were lower than in their term pairs but only in FEF25-75% was the difference statistically significant. The 6MWT showed no difference between both groups. The Fe-NO was lower in the term control group compared to the preterm study group.

The concentration of IL-6 and IL-8 in cord blood and in gastric aspirate inversely correlated to all parameters of lung function at the age of 8 years; however, only the correlations between the concentration of IL-8 in cord blood to FEV1/FVC and to FEF25-75% were statistically significant. There was no correlation between the length of ventilator days ($r = -0.012$, $p = 0.5$)/oxygen treatment days ($r = 0.16$, $p = 0.39$) and the concentration of IL-8 in cord blood. The length of mechanical ventilation was significantly inversely correlated to FEV1%, FVC%, and FEF25-75%, and the length of oxygen supplementation was in inverse correlation to FEV1% and to FEF25-75% .

The 6MWT and FeNO at the age of 8 yrs did not correlate either to the prenatal IL-8 concentration or to the length of respiratory support or the length of oxygen supplementation.

## Discussion

In our study of preterm infants with GA < 30 wks who were prospectively followed to the average age of 8 years, we found significantly lower values of FEF25-75% when compared to term-born controls. Besides the length of ventilation and oxygen support, the concentration of IL-8 in cord blood of the preterm study group was also associated with lower FEF25-75% at the age of 8 years. IL-8 in cord blood also negatively correlated to FEV1/FVC at the same age.

BPD is one of the important long-term consequences of preterm birth. The length of ventilation and the length of oxygen treatment are well known risk factors for BPD. Clinical, histological, microbiological or biochemical CA is well established as a major risk factor for preterm birth. (25) The recent systematic review and meta-analysis of the association between CA and BPD showed that CA was significantly associated with BPD; however, the authors found strong evidence of publication bias, and at the end they concluded that, despite a large body of evidence, CA by itself cannot be definitively considered a risk factor for BPD. However, in pregnancies complicated with CA the concentrations of IL-8 and IL-6 are significantly higher than in those without histological CA. (26,27) Ureaplasma spp. are the most common organisms isolated from amniotic fluid obtained from women who present with preterm labor either with intact membranes or preterm premature rupture of membranes, or a short cervix associated with microbial invasion of the amniotic cavity. (26-30) Casper DC and al. found a positive correlation between the amount of Ureaplasma parvum and the magnitude of inflammatory response inside the amniotic cavity observed by elevated IL-8 levels. (27) The production of IL-8 is inducible by proinflammatory cytokines (IL-1, tumor necrosis factor (TNF)-alpha), bacteria, bacterial products, and viruses. Environmental conditions such as hypoxia also induce IL-8 formation. The results from our original study, from 2000-2002, did not identify either CA or the cytokine level as a risk factor for BPD development.

Pulmonary blood-vessel formation within the mesenchyme is under the influence of growth factors from endodermal cells (VEGF) as well as some from within the mesenchyme (angiopoetin). (28) Several studies have examined how premature delivery and changes in oxygen tension, inflammatory cytokines, and other signals can decrease VEGF expression and signalling, thereby altering lung structure. Experimentally, hyperoxia, which impairs alveolar and vascular growth and inhibits alveolarisation in neonatal rats, also down-regulates lung VEGF and its receptors (VEGFR) expression, and pharmacologic inhibition of VEGFRs inhibits lung vascular and alveolar growth in newborn rats. (29,30) The effects of recombinant human VEGF 165 (rh-VEGF) treatment of newborn rats during or after exposure to hyperoxia on vessel growth were studied. Despite increased capillaries growth there was no positive effect on alveolarisation because newly formed capillaries were immature and leaky, and led to lung edema. Despite its central role in vascular formation, it was shown that VEGF works in concert with other factors such as angiopoetins which stabilise capillaries network. (31,32) Accordingly, combined lung VEGF and angiopoetin1 gene transfer preserved alveolarisation and enhanced angiogenesis with more mature capillaries that were less permeable, and the vascular leakage seen in VEGF-induced capillaries was reduced. (33) We speculate that the long-term effect of IL-8 in the new-born lung is mediated through abnormal vascular development which disables normal alveolar development. It is likely that vascularisation of the new-born lung under IL-8 stimulation is similar to angiogenesis described in tumor-producing vessels with thin walls, tortuous shape, absence of pericytes, and variations in diameter. (34) As IL-8 up-regulates VEGF m-RNA in endothelial cells (the process requires activation of transcription factor NFkappa and does not involve HIF1 alpha), the effect could be actually the same as in the studies where angiogenesis was stimulated with rh-VEGF, resulting in the

growth of hyper-permeable capillaries which cannot lead to normal alveolarisation. (35) FEF25-75% was the only parameter of lung-function measurement which was significantly lower in the study group when compared to healthy pairs at the age of 8 years. According to the results of this study, it is possible that prenatal exposure to IL-8 leads to long-term consequences in small airway development. It is also interesting that in the sputum of adult patients with chronic obstructive pulmonary disease (COPD) the levels of VEGF, angiogenin, IL-8 and TNF alpha were significantly higher when compared to healthy smokers and non-smokers. Moreover, they were also able to demonstrate significant negative correlation between FEV1 and VEGF, IL-8 and angiogenin among smokers. (36) It seems that IL-8, together with other angiogenic factors, plays an important role either in COPD or BPD airway remodelling.

The question has been raised whether or not survivors of BPD and prematurity have a risk of developing a COPD-like phenotype with aging. We are aware of a low number of children who responded to follow-up and also that the control group of term-born children did not have measured cytokines at birth; however, to the best of our knowledge this data is one of the first reports that intrauterine inflammatory response could have long term consequences on lung function. It is likely that the long-term effect is mediated through IL-8. Larger studies are necessary.

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