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The association between maternal cervicovaginal proinflammatory cytokines concentrations during pregnancy and subsequent early-onset neonatal infection

Jarosław Kalinka[1,*], Paweł Krajewski[1], Wojciech Sobala[2], Małgorzata Wasiela[3] and Ewa Brzezińska-Błaszczyk[4]

[1] Department of Perinatology, I Division of Gynecology and Obstetrics, Medical University of Lodz, Poland
[2] Department of Environmental Epidemiology, Nofer Institute of Occupational Medicine, Lodz, Poland
[3] Department of Medical Microbiology, Medical University of Lodz, Poland
[4] Department of Experimental Immunology, Medical University of Lodz, Poland

Abstract

Objective: The aim of this study was to investigate the relationship between the concentration of selected proinflammatory cytokines (IL-1α, IL-1β, IL-6 and IL-8) in cervicovaginal fluid, as measured in midgestation, and the risk of early-onset neonatal infection (EONI)**.**

Method: Cervicovaginal fluids were obtained from a cohort of 114 pregnant women at 22 to 34 weeks' gestation. The samples were analyzed for the concentrations of selected proinflammatory cytokines using standard enzyme-linked immunosorbent assay technique (ELISA). Lower genital tract microbiology was diagnosed using Gram stain method according to Spiegel's criteria and by culture.

Results: Mean gestational age at the time of sampling was 29.0 weeks. Mean time between sampling and delivery was 9.3 (SD 4.7) weeks. Bacterial vaginosis (BV) was diagnosed in 27.2% of subjects and *M. hominis* and *U. urealyticum* in 22.8% and 26.3%, respectively. Out of 114 women examined, 20 (17.5%) delivered newborns with EONI. Median cervicovaginal concentrations of IL-1α, IL-1β, IL-6 and IL-8 did not differ between women who delivered newborns with EONI as compared to women who delivered newborns without EONI. Women with pathological lower genital tract microflora and low IL-8 concentration (below 25th percentile) during pregnancy presented a significant risk of delivering newborns with EONI (OR = 4.9; 95% CI, 1.1–22.8). Subjects with pathological lower genital tract microflora and a low concentration of more than one cytokine had the highest risk of delivering a newborn with EONI, OR = 16.2, 95% CI, 1.1–234.0.

Conclusions: Cytokine measurement in cervicovaginal fluid in early gestation could be useful for predicting subsequent EONI only among pregnant women with lower genital tract infection. Maternal genital tract immune hyporesponsiveness as represented by low concentrations of proinflammatory cytokines may create a permissive environment for ascending infection and may lead to subsequent EONI.

Keywords: Bacterial vaginosis; early-onset neonatal infection; lower genital tract infections; proinflammatory cytokines.

*Corresponding author:
Jarosław Kalinka, MD PhD
Department of Perinatology
I Division of Gynecology and Obstetrics
Medical University of Lodz
ul. Wileńska 37, 94-029 Łódź, Poland
Phone: +48 42 6868380
Fax: +48 42 6314562
E-mail: j.kalinka@csk.am.lodz.pl

Introduction

Early-onset neonatal infection (EONI) is an important cause of morbidity and mortality among newborns [27]. This infection, appearing in the first two days of life, is usually the result of exposure to microorganisms of maternal origin. Risk factors for acquired bacterial perinatal infection in the neonate include: maternal fever during labor, preterm or prolonged rupture of membranes, preterm onset of labor, *chorioamnionitis,* maternal urinary tract infection, and maternal lower genital tract bacterial colonization [27].

Many bacteria involved in ascending infection and/or intrauterine infection produce phospholipases A_2 and C, proteinases and endotoxins activating placental, decidual, amnion and fetal membrane cells [8, 16, 24]. This in turn may stimulate these cells to produce proinflammatory cytokines, chemokines and prostaglandins. This process has an important role in the initiation of parturition, especially in cases related to chorioamnionitis. Intrauterine infection is accompanied by host-inflammatory response which involves expression of cytokines in fetoplacental tissues [6]. An association between the immuno-inflammatory reaction and neonatal morbidity from bronchopulmonary dysplasia and cerebral palsy has also been described [5].

Interleukin-1 (IL-1) is produced by epithelial cells, fibroblasts and phagocytes and has a crucial role in cervical

ripening by stimulating the production of colagenases and elastases [3, 17]. Interleukin-8 (IL-8) strongly promotes the attraction of neutrophils and their degranulation. IL-1β and IL-8 can also induce hyaluronic acid production by human cervical fibroblasts [17]. IL-6 activates acute phase response and stimulates immunoglobulin production.

Measurements of cytokine concentrations in amniotic fluid and cervicovaginal secretion were used for prediction of selected perinatal events, mainly preterm delivery and intrauterine infection. The influence of intrauterine infection on the concentration of cytokines and prostaglandins in amniotic fluid has been subject to extensive investigations [4, 9, 19]. Some authors have reported increased concentrations of IL-1β, IL-1α, IL-10, TNF-α and IL-6 in pregnant women with intrauterine infection and with threatened preterm labor [4, 9, 23]. The increase in amniotic concentration of some proinflammatory cytokines was found to be a sensitive indicator of intrauterine infection and preterm delivery [11, 22].

In many studies, cytokine measurements in amniotic fluid have been employed to investigate the relationship between cytokine concentration and subsequent preterm delivery [4, 9, 22]. Elevated levels of proinflammatory cytokines in maternal serum and in amniotic fluid during the infection and shortly before parturition have been extensively described. Studies indicate that measurement of cytokines concentration in cervicovaginal fluid could be of some value for the prediction of intrauterine infection and preterm birth especially at a relatively short period before the delivery [2, 20, 21]. In view of invasive character of amniotic fluid aspiration during pregnancy, the determination of cytokines concentration in cervicovaginal fluid has been used. The studies of Jun et al. [11] performed on pregnant women with preterm rupture of membranes revealed a strong correlation between IL-6 concentrations measured in amniotic and in cervicovaginal fluids ($P < 0.001$). This finding was confirmed by the results reported by Rizzo et al. [21]. Further, in the studies by Inglis et al. [10], the cervicovaginal levels of IL-6 and TNF-α were found to correlate with fetal fibronectin concentration. Recently, high levels of interleukin-18 in umbilical blood was shown to correlate with periventricular leukomalacia and cerebral palsy in preterm infants [15]. Also, IL-18 deficiency in mice decreases brain white matter vulnerability [7].

However, data concerning the usefulness of cytokines measurements in cervicovaginal fluid at several weeks before delivery for prediction of various perinatal events are relatively scarce [12, 25]. We hypothesize that there is a relationship between lower genital tract microbial invasion during pregnancy, inflammation, and the risk of early-onset neonatal infection. We also assume that the highest risk pertains to women who have impaired cytokines production in early gestation, which could enhance an ascending microbial invasion of the upper genital tract thus leading to early-onset neonatal infection.

The main objective of this study was to investigate the relationship between concentration of selected proinflammatory cytokines (IL-1α, IL-1β, IL-6 and IL-8) in cervicovaginal fluid, as measured in midgestation, and the risk of subsequent early-onset neonatal infection.

Material and methods

The study population comprised 120 women with singleton pregnancies at 22–34 weeks' gestation recruited from patients of the hospital at the Department of Perinatology, Medical University of Lodz, Poland, between May 2001 and December 2002. The exclusion criteria were as follows: preterm premature rupture of membranes, antibiotic therapy within 4 weeks prior to examination, vaginal bleeding, serious maternal diseases and any immunological disorders. Of 120 women enrolled, six women were lost to follow up and excluded from further analysis, thus the final study group comprised 114 women.

This prospective cohort study was approved by the Biomedical Ethics Committee of the Medical University of Lodz, Poland. (Decision No. RNN/215/00). Each participant provided a written informed consent to participate in the study.

A standard questionnaire covering medical, socio-economic, demographic and constitutional aspects, as well as tobacco smoking, was administered to every subject and verified based on medical records. Routine ultrasound examination of fetal biometry was performed. The gestational age at the time of sampling was based on the last menstruation and verified by early ultrasound crown-rump length of the fetus.

For the assessment of biocenosis in the lower genital tract, vaginal and cervical swabs were collected from the pregnant women under study. At first, bacteriological tests of cervical swabs were made to screen for *C. trachomatis, M. hominis* and *U. urealyticum.* The *C. trachomatis* antigen was detected by direct immunofluorescence assay (Chlamydia Direct IF-Bio-Merieux, Lyon, France). For isolation and identification of genital mycoplasmas the commercially available Mycoplasma DUO kits (Sanofi Diagnostics, Pasteur, Marnes la Coquette, France) were used.

Bacterial vaginosis was diagnosed by Gram stain of vaginal smear according to Spiegel's criteria [26]. Based on microbiological results, three groups were distinguished: grade I – normal (predominantly lactobacillus morphotypes), grade II – intermediate (mixed lactobacillus morphotypes and other Gram-positive and Gram-negative bacteria), grade III – BV- (few or absent lactobacillus morphotypes, but a greatly increased number of *G. vaginalis* and other Gram-negative small rods characteristic for anerobic bacteria).

Cervicovaginal fluids were obtained by Dacron swabs from the posterior fornix. Then, Dacron swabs were placed in a glass probe containing 2 mL of phosphate-buffer saline solution and stored at −70°C. The samples were analyzed for the concentrations of selected cytokines by commercially available standard enzyme-linked immunosorbent assay kit (Endogen).

The early-onset neonatal infection (EONI) is defined as an infection occurring within the first 48 h of birth. In this study, EONI was defined as the presence of at least one of the following signs: three of the clinical signs of infection or positive blood, or urine culture, or laboratory findings suggesting infection. Clinical evidence indicative of infection was any of: respiratory signs

(tachypnea, apnea, irregular respirations, hypoxia); temperature instability (38°C); gastrointestinal signs (vomiting, diarrhea, abdominal distension, poor feeding, ileus); neurological signs (lethargy, irritability, tremor or seizures, hypotonia or hyporeflexia, high-pitched cry, swelling of fontanel); cardiovascular signs (hypotension, tachycardia, metabolic acidosis); skin changes (pallor or skin mottling, petechiae or purpura, cold or clammy skin, cyanosis, jaundice). Laboratory microbiological evidence was positive blood culture, or positive culture of urine, cerebrospinal fluid or other normally sterile site. Laboratory evidence included any of the following: abnormal white blood cell count (low or high), left shift, elevated band/neutrophil ratio; thrombocytopenia [27].

To evaluate the risk of EONI associated with cytokines concentration the crude and adjusted odds ratios (OR) and 95% confidence intervals (CI) were calculated. Uterine contractions were considered as a possible confounder. We considered lower genital tract pathological microflora as an effect modifier and estimated the risk of EONI in this subgroup. Median concentrations of cytokines were compared using LAV (least absolute value) regression model. Observed differences were considered statistically significant at standard level 0.05. Statistical analysis was carried out using STATA 8 software (Stata Corp. College Station, TX).

Results

The characteristics of the examined population are presented in Table 1. The mean gestational age at the time of cytokine measurement was 29.0 (SD 4.0) weeks.

According to Gram stain results, bacterial vaginosis was diagnosed among 31 women (27.2%). Among 49 women, pathological vaginal microflora was detected, defined as the presence of *M. hominis* or *U. urealyticum* or bacterial vaginosis.

Out of 114 analyzed women, 20 (17.5%) had newborns with EONI. Mean gestational age at the time of delivery was 38.3 weeks and mean birth weight was 3146 g. Mean time between cytokines measurements and the time of delivery was 9.3 weeks.

Table 1 The characteristic of examined population.

Patients' characteristics	n = 114
Mean gestational age at entry to study (weeks)	29.0 (SD 4.0)
Mean maternal age (years)	27.3 (SD 4.7)
Mean maternal weight (kg)	56.5 (SD 8.0)
Parity	
nulliparous	69 (60.5%)
multiparous	45 (39.5%)
Mean number of deliveries	1.54 (SD 0.96)
Smoking	
Yes	18 (15.8%)
No	96 (84.2%)
Bacterial vaginosis	31 (27.2%)
M. hominis	26 (22.8%)
U. urealyticum	30 (26.3%)

In the univariate analysis, the significant risk factors of EONI were as follows: lower genital tract colonization by *M. hominis* (OR = 2.8; 95% CI, 1.0 –7.9*), U. urealyticum* (OR = 3.7; 95% CI, 1.4–10.1). Pregnant women with lower genital tract pathological vaginal microflora also presented higher risk of delivering a baby with EONI but this observation did not reach a statistical significance (OR = 2.3; 95% CI, 0.9–6.2). Interestingly, respiratory tract infection in mothers during the 2nd trimester seems to have a protective effect on delivering a baby with EONI (Table 2).

No significant differences were found in median cervicovaginal concentrations of the examined cytokines, between women who delivered an infant with or without EONI (Table 3).

Median concentrations of IL-1α, IL-1β and IL-8, but not IL-6, were significantly higher among women with pathological vaginal microflora compared to women with normal flora (Table 4).

Because of the wide range and lack of normal distribution of cytokine concentrations we dichotomized the concentrations into those $<25^{th}$ percentile and those $\geq 25^{th}$ percentile. The 25th percentile values for the evaluated cytokines were as follows: for IL-1α – 8 pg/mL; IL-1β – 0.8 pg/mL; IL-6 – 2 pg/mL and for IL-8 – 198 pg/mL.

Women who delivered a baby with EONI tended to have more frequently all cytokines values below 25th percentile (data not shown).

We made an additional univariate and multivariate analysis comparing relationship between cervicovaginal cytokines concentrations with subsequent EONI in the subgroup (n = 49) of women with lower genital tract pathological microflora only (Table 5). After controlling for confounding factors, women with pathological microflora of lower genital tract and IL-8 concentration below the 25th percentile presented a significant risk of subsequent EONI (OR = 4.9; 95% CI, 1.1–22.8). A similar tendency was noted for low IL-1α concentration.

Significantly higher risk of delivering newborns with EONI was observed among women with lower genital tract pathological microflora and low concentration (below 25th percentile) of more than one cytokine (OR = 9.3, 95% CI, 1.4–63.2). After adjusting for confounding factors, the calculated OR was even higher, 16.2 (Table 6).

Discussion

This study aimed at assessing the usefulness of determinations of selected proinflammatory cytokines in the maternal cervicovaginal fluid as an early marker of EONI. It should be stressed that cytokine concentrations were measured at approximately the 29th week of pregnancy, i.e., about 8–10 weeks before the delivery. This relatively

Table 2 Selected risk factors of early-onset neonatal infection (EONI)in examined population.

	n = 114	Early-onset neonatal infection		
		n	OR	95% CI
Bacterial vaginosis				
No	83	14	1	
Yes	31	6	1.2	0.4–3.4
M. hominis				
No	88	12	1	
Yes	26	8	2.8	1.0–7.9
U. urealyticum				
No	84	10	1	
Yes	30	10	3.7	1.4–10.1
Pathological microflora				
No	65	8	1	
Yes	49	12	2.3	0.9–6.2
Mothers respiratory tract infection < 22 weeks				
No	94	17	1	
Yes	20	3	0.8	0.2–3.0
Mothers respiratory tract infection > 22 weeks				
No	96	20	1	
Yes	18	0	0	0–0.8
Previous surgery				
No	96	14	1	
Yes	18	6	2.9	0.9–9.1

Table 3 Median cervicovaginal concentrations of analyzed cytokines among women who delivered a baby with EONI and healthy infants (non-EONI).

Cytokines	EONI n = 20 Median (inter quartile range)	Healthy infants n = 94 Median (inter quartile range)	P-value
IL-8 (pg/mL)	387 (143–630)	503 (198–826)	0.59
IL-6 (pg/mL)	6.0 (0.1–13.3)	6.9 (1.9–16.2)	0.70
IL-1α (pg/mL)	28.9 (3–131.8)	23.5 (5.2–84.6)	0.67
IL-1β (pg/mL)	11.8 (0.9–32.4)	10.8 (0.1–35.3)	0.84

Table 4 Median cervicovaginal concentrations of selected cytokines among women with lower genital tract pathological microflora and normal flora.

Cytokines	Lower genital tract pathological flora n = 49 Median (inter quartile range)	Lower genital tract normal flora n = 65 Median (inter quartile range)	P-value
IL-8 (pg/mL)	592 (298–829)	302 (91–710)	0.001
IL-6 (pg/mL)	9.1 (1.5–27.1)	5.1 (0.7–11.6)	0.12
IL-1α (pg/mL)	48.3 (8.2–136.8)	18.1 (3.5–56.8)	0.009
IL-1β (pg/mL)	21.5 (1.9–69.1)	6.2 (0.2–16)	0.003

long interval allowed the exclusion of possible influence of uterine contraction activity and labor on the concentrations of cytokines. We did not find any significant differences in median cytokines concentrations, as measured in midgestation, between women who delivered newborns with and without EONI. Our study indicates that pregnant women with low cervicovaginal concentrations (below 25th percentile) of IL-8 and IL-1α who, at the same time, have a lower genital tract pathological microflora, have a higher risk for delivering a baby with EONI. We also noted that an increased risk of EONI relates to women for whom the concentrations of more than one of the cytokines were below the 25th percentile.

During the course of ascending lower genital tract infection, microorganisms may cross the cervical barrier

Table 5 Early-onset neonatal infection in relation to cervicovaginal cytokines concentrations among a subgroup of women with pathological microflora during pregnancy.

Cytokines	n=49	Early-onset neonatal infection				
		n	OR[a]	95% CI	OR[b]	95% CI
IL-8						
>25%	39	7	1		1	
<25%	10	5	4.6	1.0–20.2	4.9	1.1–22.8
IL-6						
>25%	37	8	1		1	
<25%	12	4	1.8	0.4–7.6	2.6	0.6–12.1
IL-1α						
>25%	38	7	1		1	
<25%	11	5	3.7	0.9–15.6	4.6	0.8–27.3
IL-1β						
>25%	38	9	1		1	
<25%	11	3	1.2	0.3–5.5	2.2	0.4–13.7

[a] Crude odds ratios.
[b] Odds ratios adjusted for uterine contractions, mothers respiratory tract infections after 22 weeks and previous surgery.

Table 6 The risk of early-onset neonatal infection according to number of low cervicovaginal cytokines concentrations (<25th percentile) among women with lower genital tract pathological microflora.

Number of cytokines <25 percentile		Early-onset neonatal infection				
	n=49	n	OR[a]	95% CI	OR[b]	95% CI
None	34	6	1		1	
One	9	2	1.3	0.2–8.1	1.4	0.2–11.4
Two or more	6	4	9.3	1.4–63.2	16.2	1.1–234.0

[a] Crude odds ratios.
[b] Odds ratios adjusted for uterine contractions, mothers respiratory tract infections >22 weeks and previous surgery.

and reach the decidua. If this inflammatory process is not sufficient to signal the onset of labor, microorganisms can cross the intact membranes into the amniotic cavity, where they can also stimulate the production of inflammatory mediators; the umbilical cord and fetus in some cases could also be infected [6]. Microorganisms that gain access to the fetus may elicit the systemic inflammatory response syndrome and then, after labor, EONI. [1, 5]. EONI may be a clinical post-birth manifestation of FIRS (fetal inflammatory response syndrome).

Park et al. [18] tried to determine whether amniotic fluid concentrations of tumor necrosis factor-alpha (TNFα) could be of value for the prediction of early-onset neonatal sepsis in patients with preterm labor and intact membranes. Patients delivering neonates with early-onset neonatal sepsis had significantly higher median amniotic fluid TNFα concentrations than patients delivering neonates without early-onset neonatal sepsis. The authors concluded that amniotic fluid TNFα is a better independent predictor of early-onset neonatal sepsis than placental histological findings or amniotic fluid culture. However, it is worth noting that in contrast to our study, all the patients delivered preterm neonates within a very short time (72 h) after cytokines sampling by transabdominal amniocentesis.

Our findings are also supported by the report of Simham et al. [25] who investigated the possible relationship between cervicovaginal concentration of IL-1β and IL-8 in women at 8–20 weeks gestation who had subsequently developed chorioamnionitis. They found an increased risk for this pathology in women who had low concentrations of one of the proinflammatory cytokines in early pregnancy. This risk was significantly higher in women for whom two or three of the examined cytokines were found to have low levels early in pregnancy (OR=4.5).

Also, results of our previous study [12] indicate that pregnant women with low cervicovaginal concentrations of proinflammatory cytokines who, at the same time, have a pathological vaginal microflora, present an increased risk for delivering before the 37th week of gestation. The highest risk of preterm delivery was observed among women with low concentrations of IL-1α and IL-1β with OR 10.7 and 4.9, respectively. Lower, but still elevated, risk was found for women with genital tract infection and low levels of IL-6 (OR=2.2) and IL-8 (OR=2.7). The highest risk of preterm delivery (OR=11.8) was noted among the women for whom the concentrations of more than one of the cytokines studied were below the 25th percentile.

The condition underlying the observed increase in the risk for *chorioamnionitis* [25], preterm delivery [12], and delivery of newborns with EONI among women with low cytokines levels in early pregnancy may be the susceptibility to genitourinary tract infections due to decreased

cytokine concentrations which could enhance development of the ascending infection. The findings of our study provided evidence for this probable mechanism: the increased risk for delivering newborns with EONI was found only in the group of women with lower genital tract infection who had low cervicovaginal concentrations of proinflammatory cytokines. The latter condition may also imply an improper reactivity of the maternal immune system that should normally result in diminishing the growth of pathological bacteria in the genitourinary tract early at pregnancy. The more the cytokines were found to have low concentration levels, the higher was the risk for EONI. This finding may indicate that the probability of pregnancy and neonatal complications increases with a growing extent of immunological disorders.

Microorganisms interact with a family of toll-like receptors (TLRs) belonging to the innate immune system, which triggers intracellular activation of nuclear factor kappa B and various kinases, leading to the production and release of cytokines and anti-microbial peptides [6]. There are also findings indicating that systemic activation of TLR-4, via bacterial endotoxins, may produce toxic effects on the immature central nervous system and induce white matter injury in fetal sheep [14].

EONI, an infection that appears in the first two days of neonatal life, is usually the result of exposure to microorganisms of maternal origin [26]. Among various risk factors associated with EONI, maternal lower genital tract bacterial colonization during pregnancy is one of the most important. The results of this study confirm that lower genital tract colonization by *M. hominis* and *U. urealyticum* are the risk factors of subsequent early-onset neonatal infection. In our study EONI was diagnosed among 17.5% of newborns, in accord with the results of other authors [5].

As in some populations, abnormal lower genital tract microflora during pregnancy is a common phenomenon [13], and cytokines measurements in cervicovaginal fluid early in gestation could also help identify the subgroup of women with lower pathological microflora who are at risk of subsequently delivering a newborn with EONI.

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