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Cervicovaginal fluid cytokines as predictive markers of preterm birth in symptomatic women

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Objective

Here, we investigated whether cytokines in the cervicovaginal fluid (CVF) can be predictive markers of preterm birth (PTB).

Methods

A multi-center prospective cohort study was conducted on 59 singleton pregnant women hospitalized for preterm labor (PTL) and/or preterm premature rupture of membranes (pPROM) between 22 weeks and 36 weeks 6 days of gestation from 2014 to 2015. The levels of 13 inflammatory cytokines (macrophage inflammatory protein [MIP]-1α, MIP-1β, tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6, IL-8, IL-17α, granulocyte colony stimulating factor [G-CSF], IL-7, IL-4, IL-5, IL-10, and IL-13) were measured using a multiplex bead-based immunoassay and that of fetal fibronectin (fFN) was measured using enzyme-linked immunosorbent assay (ELISA). Statistical analyses were performed using Student's *t*-test, Mann-Whitney *U* test, Pearson's correlation, and receiver operating characteristic (ROC) curve analysis in SPSS version 20.0.

Results

Among the 13 cytokines assessed, the levels of 3 cytokines (MIP-1α, IL-6, and IL-7) were negatively correlated with gestational age at delivery (P=0.028, P=0.002, and P=0.018, respectively). Sensitivities of MIP-1α, IL-6, and IL-17α were 70%, 80%, and 75%, respectively, and their specificities were 57%, 65%, and 69%, respectively. The sensitivity and specificity of fFN were 33% and 95%, respectively.

Conclusion

In symptomatic women diagnosed with PTL and/or pPROM, cytokines from cervicovaginal fluid, especially IL-6 and IL-17α, could be better predictive markers of PTB than fFN.

Keywords: Cytokines; Interleukin-6; Interleukin-17A; Biomarkers; Preterm birth

Introduction

Preterm birth (PTB), defined as birth before 37 weeks of gestation, is a major cause of neonatal morbidity and mortality [1,2]. Over the past decade, the prevalence of preterm births has steadily increased worldwide. Morbidity and mortality of infants born before 34 weeks of gestation are more than those of infants born after 34 weeks. Therefore, antenatal steroid administration is recommended for patients with preterm labor (PTL) and preterm premature rupture of membranes (pPROM). However, prediction methods and therapies

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for the same are not well studied.

PTB can be classified into two categories: spontaneous and iatrogenic. Spontaneous PTB accounts for up to 75% of all PTBs [3]. About 45% of PTBs are caused by PTL with intact membranes, while 30% are caused by the pre-labor rupture of fetal membranes. Causes of spontaneous PTB include activation of inflammatory reactions in the gestational tissues and secretion of inflammatory cytokines as an immune response to ascending infection of the genital tract and abnormal vaginal microorganisms [4-7].

Many studies aimed at predicting PTB have been conducted. Commonly used screening tests include risk scoring, cervical length measurement, and detection of biochemical markers. Risk scoring involves quantification of risk factors for spontaneous PTB and/or pPROM, including PTB history, cervical conization history, low socioeconomic status, low body mass index, smoking, drug use, anxiety, multifetal gestation, gestational diabetes, and gestational hypertension [6-9]. Tests for fetal fibronectin (fFN) and phosphorylated insulin-like growth factor binding protein-1 (IGFBP1) are now commercially available [10,11]. The level of fFN in cervicovaginal fluid (CVF), leaked due to the disruption of maternal choriodecidual tissues and through matrix remodeling, was used as a diagnostic marker for predicting PTB using a non-invasive method. Lockwood et al. described the clinical utility of fFN to predict spontaneous PTB in symptomatic women with high sensitivity (81.7%) and specificity (82.5%), based on a fFN threshold concentration of ≥50 ng/mL [3,8]. However, its specificity and sensitivity were confounded by unprotected vaginal intercourse, digital examination, bleeding, or contamination with amniotic fluid [1,12]. Cervical length measurement using transvaginal sonograms in patients with PTL and pPROM have also been used to predict PTB; however, it requires a skilled ultrasonographer and shows limited correlation with CVF [13-15].

Increased levels of inflammatory cytokines in CVF signify intra-amniotic infection that causes PTB [16]. Several inflammatory cytokines, including interleukin (IL)-1, -2, -4, -6, -8, -10, -12 and -17, tumor necrosis factor-alpha (TNF-α), interferon gamma (INF-γ), regulated on activation, normal T cell expressed and secreted (RANTES), and C-reactive protein (CRP), have been detected in the CVF, amniotic fluid, and blood of asymptomatic and symptomatic pregnant women [3,4,17,18]. IL-6 and matrix metalloproteinase-8 (MMP-8) levels in the amniotic fluid were significantly correlated with pPROM [19,20]. We also found that the levels of cytokines such as IL-1b, IL-6, IL-7, IL-7a, and TNF-α were significantly increased in the amniotic fluid of patients with cervical insufficiency [16]. Thereafter, the objective of our study was to determine whether inflammatory cytokines could be measured using non-invasive methods. Recent studies have shown that IL-6, IL-8, macrophage inflammatory protein (MIP)-1α, and MIP-1β levels in the CVF of patients with pPROM were correlated with those in the amniotic fluid [21]. IL-1β, IL-6, IL-8, and TNF-α levels were also elevated in patients with intact membranes in PTL [22]. There have also been studies in which IL-6 was shown to be a useful marker for predicting preterm delivery.

In the preset study, we expected that the levels of inflammatory cytokines in the CVF would be elevated in patients with PTL and pPROM. We investigated whether the levels of cytokines in the CVF could be a better predictive marker of PTB than that of fFN in CVF.

Materials and methods

1. Study population

A multi-center (Ewha Womans University Mokdong Hospital, Samsung Medical Center, Konkuk University Hospital, Seoul St. Mary's Hospital, and Seoul Metropolitan Government-Seoul National University Boramae Medical Center) prospective cohort study was conducted in singleton pregnant women suffering from complications of PTL and/or pPROM between 22 weeks and 36 weeks 6 days of gestation, from 2014 to 2015. The characteristics (age, parity, and body mass index at admission) of the mother were analyzed. To diagnose pPROM, sterile speculum exam was conducted for detecting amniotic fluid pooling in vaginal cavity and nitrazine test was done. Uterine activity was assessed by cardiotocography. PTL was diagnosed in patients with regular uterine contraction and 4 or more contractions in 20 minutes, or 8 or more in 60 minutes as detected by cardiotocography. A total of 65 women with singleton pregnancies were included in this study. Patients with preeclampsia, hemolysis, elevated liver enzymes, a low platelet count (HELLP) syndrome, incompetent internal os of cervix (IIOC), and placenta previa were excluded. Out of the 59 participants, 19 were diagnosed with pPROM, and the rest with PTL (Fig. 1); no patient was suspected of clinical chorioamnionitis, defined according to

the criteria of Gibbs et al. [23], which includes the presence of fever (>37.8°C) and two or more of the following associated clinical findings: uterine tenderness, malodorous vaginal discharge, maternal leukocytosis, maternal tachycardia, and fetal tachycardia. Gestational age was estimated by fetal biometry, assessed using a sonogram during the first trimester. Cervical length was measured at the time of admission by transvaginal ultrasound. Vaginal transducers were placed in the anterior fornix to examine the endocervical canal. Calipers were used to measure the length of the cervical canal from internal to external os. Women with pPROM at less than 35 weeks of gestation were treated with corticosteroids, tocolytics, and antibiotics. After 34 weeks, all patients with pPROM underwent induced delivery, when there was no labor pain, or cesarean section, if it was indicated. Those with PTL were treated with corticosteroids and tocolytics. We checked pregnancy outcome in terms of gestational age at delivery, birth weight, and Apgar score of the newborn at 1 and 5 minutes.

2. Cervicovaginal fluid collection and laboratory study

CVF was collected from the posterior vaginal fornix with a sterile cotton swab and stored at –80°C within 30 minutes of collection, until further analysis. The levels of 13 inflammatory cytokines (MIP-1α, MIP-1β, TNF-α, IL-1β, IL-6, IL-8, IL-17α, granulocyte-colony stimulating factor [G-CSF], IL-7, IL-4, IL-5, IL-10, and IL-13) in the CVF were measured using the Human Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore, Burlington, MA, USA). The intra-assay and inter-assay variations of all analyses were less than 10% and 12%, respectively. fFN in CVF was also sampled similarly and measured using a human fFN enzyme-linked immunosorbent assay (ELISA) kit (CUSABIO, Houston, TX, USA).

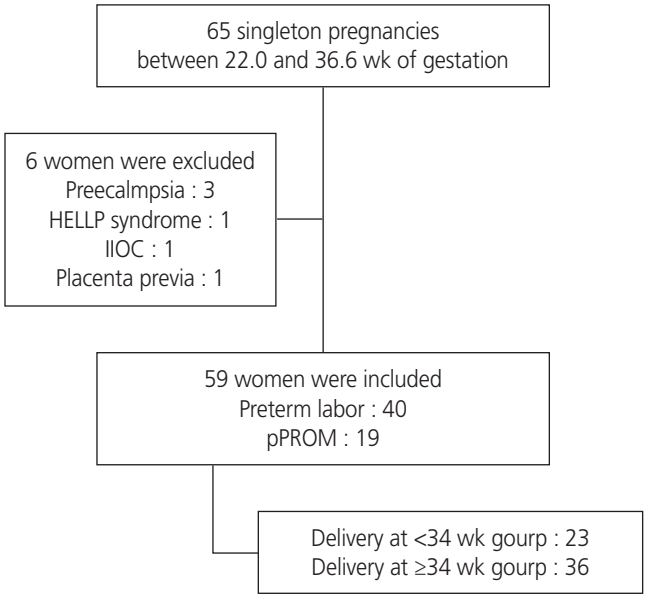

Fig. 1. Flow chart of participants in the cohort study. HELLP, hemolysis, elevated liver enzymes, and a low platelet count; IIOC, incompetent internal os of cervix.

3. Statistical analysis

Statistical analyses were performed using the Statistical Package for Social Sciences (version 20; SPSS Inc., Chicago, IL, USA). Clinical characteristics were compared using Student's *t*-test for continuous variables and χ^2 test for categorical variables. $P<0.05$ was considered statistically significant. The relationship between the level of cytokines and gestational age at delivery was analyzed using Pearson's correlation. The levels of cytokines were analyzed by the Mann-Whitney *U* test and compared between preterm delivery group (delivery at <34 weeks) and normal controls (delivery at ≥34 weeks). Receiver operating characteristic (ROC) curves of cytokine levels in early preterm birth were generated and the area under the curve (AUC) was determined.

Results

1. Characteristics of the study population

The demographic and clinical data of the study participants are presented in Table 1. The study group consisted of 23 pregnant women who delivered before 34 weeks of gestation and the controls (n=36) delivered after 34 weeks of gestation. There was no significant difference in age, parity, body mass index, and cervical length between the two groups ($P=0.519$, $P=0.730$, $P=0.487$, and $P=0.381$, respectively). fFN in the CVF showed a significant difference between the two groups ($P=0.017$). The study group was divided into PTL- and pPROM- subgroups. Frequency analysis was performed at the time of admission using the χ^2 test for the diagnosis. There was no statistical difference in the distribution between the two groups, with a *P*-value of 0.77. Gestational age at the time of diagnosis was significantly different for the two groups ($P=0.002$). The white blood cell count, birth weight, and Apgar score of the newborns showed sig-

nificant differences between the two groups (P<0.001).

2. Cytokine levels in the cervicovaginal fluid in the study and control groups

Among the 13 cytokines considered for the study, 9 (MIP-1β, MIP-1α, TNF-α, IL-1β, IL-6, IL-8, IL-17α, G-CSF, IL-7) were detected in the CVF of the 2 groups. Fig. 2 graphically shows the correlation of these 9 cytokines with the gestational age at delivery; 4 of these (MIP-1α, MIP-1β, IL-6, and IL-7) showed a significant negative correlation (P=0.003, P=0.010, P=0.035, and P=0.041, respectively). The levels of these 9 cytokines were compared between the study group and control group and are shown in Table 2. The levels of 3 cytokines (MIP-1α, IL-6, and IL-17α) were significantly higher in patients with PTL and/or pPROM who delivered before 34 weeks of gestation, compared to controls (P=0.028, P=0.002, and P=0.018, respectively). ROC curves were constructed for the 5 cytokines (MIP-1α, MIP-1β, IL-6, IL-7, and IL-17α) showing significant differences between the two groups and AUC values and optimal concentration thresholds for predictive utility were determined. Based on these thresholds, the sensitivities and specificities for the prediction of PTB were determined (Fig. 3 and Table 3). Sensitivities of MIP-1β, MIP-1α, IL-6, IL-17α, and IL-7 were 47%, 70%, 80%, 75%, and 35%, respectively, and their specificities of were 82%, 57%, 65%, 69%, and 94%, respectively; the areas under the ROC curves of these cytokines were 0.676, 0.677, 0.788, 0.715, and 0.643, respectively. The sensitivity and specificity of CVF fFN were 33% and 95%, respectively. Forty patients who were diagnosed with PTL at the time of admission were analyzed. The levels of IL-6 and IL-7 showed significant increase in the study group with P-values of 0.023 and 0.040, respectively (Supplementary Table 1). The ROC curves for these 2 cytokines were constructed and AUC values and optimal concentration thresholds for predictive utility were determined (Supplementary Table 2). Sensitivities of IL-6 and IL-7 were 83% and 83%, respectively, and their specificities were 76% and 64%, respectively. The sensitivity and specificity of CVF fFN were 33% and 70%, respectively. Subgroup analysis of 19 patients who were diagnosed with pPROM at the time of admission compared the cytokines of PTB and control group. IL-1β showed a significant difference with P-value 0.043 (Supplementary Table 3).

Table 1. Clinical characteristics of the study population (n=59)

Characteristics	Delivery at <34 wk (n=23)	Delivery at ≥34 wk (n=36)	P-value[a]
Maternal age (yr)	31 (23–38)	32 (19–46)	0.519
Nulliparity	6 (16.7)	3 (13.0)	0.730[d]
BMI (kg/m^2)	24 (20–33)	25 (18–32)	0.487
Fetal fibronectin (ng/mL)	133 (11–699)	40 (11–142)	0.017[b]
WBC (cell/mL)	13,596 (8,130–24,280)	10,063 (6,440–16,640)	<0.001[c]
Cervical length (mm)	20 (0–44)	23 (2–69)	0.381
<25 mm group	12 (57.1)	18 (54.5)	>0.990[d]
≥25 mm group	9 (42.9)	15 (45.5)	
Diagnosis at admission			
PTL group	12 (52.2)	28 (77.8)	0.077[d]
pPROM group	11 (47.8)	8 (22.2)	
Gestational age at admission (wk)	28 (22–33)	31 (20–36)	0.002[b]
Gestational age at delivery (wk)	29 (23–33)	36 (34–41)	<0.001[c]
Birth weight (g)	1,323 (570–2,320)	2,640 (780–3,740)	<0.001[c]
Apgar score at 1 min	6 (0–10)	8 (4–10)	<0.001[c]
Apgar score at 5 min	7 (0–10)	9 (8–10)	<0.001[c]

Data are expressed as median (range) for continuous variables and number (%) for categorical variables.
BMI, body mass index; WBC, white blood cell; PTL, preterm labor; pPROM, preterm premature rupture of membranes.
[a]Continuous variable were analyzed by Student's t-test; [b]Significant difference between 2 group (P<0.05); [c]Significant difference between 2 group (P<0.001); [d]Categorical variable was analyzed by χ^2 test.

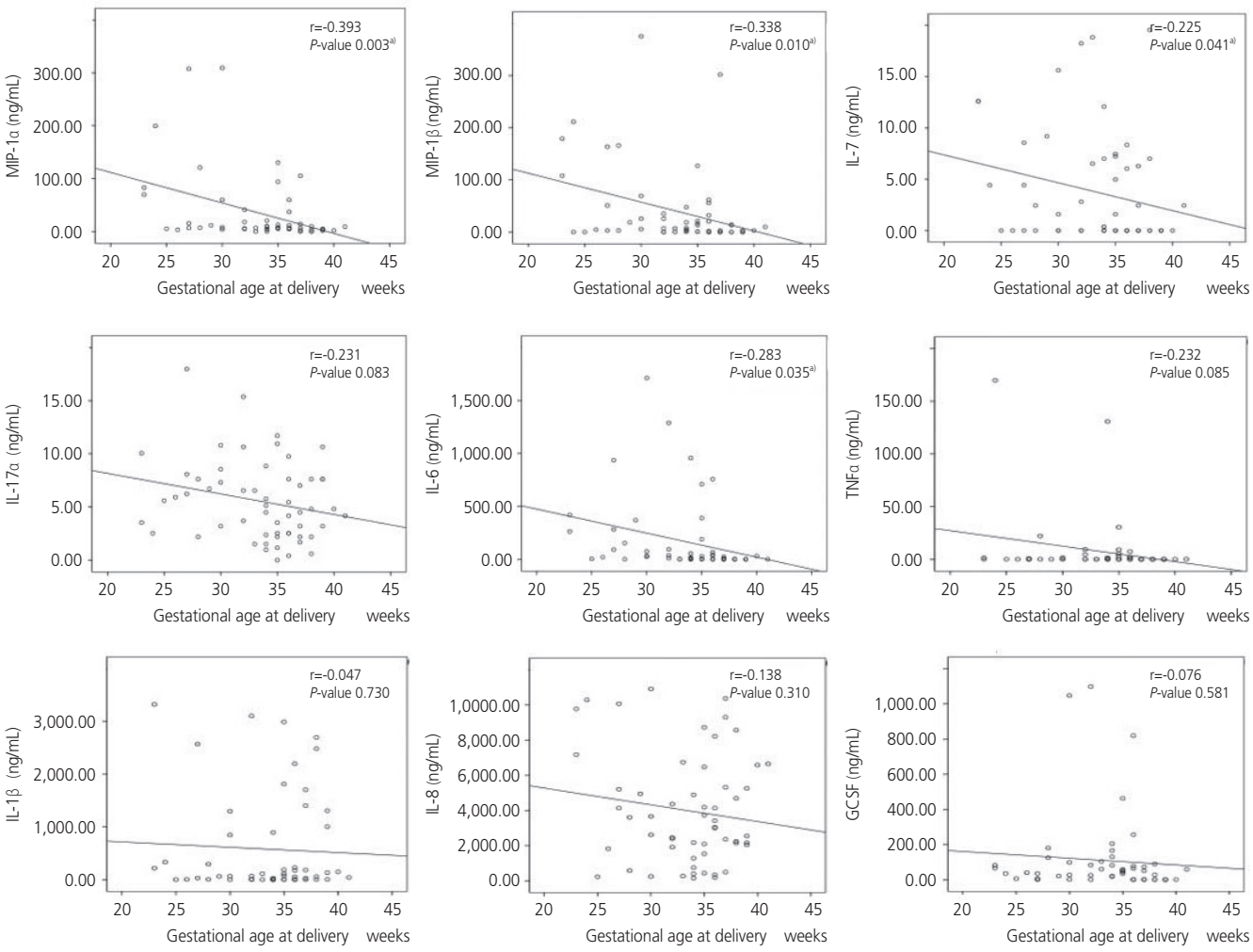

Fig. 2. The relationship between cytokine level and gestational age at delivery. Using Pearson correlation. MIP, macrophage inflammatory protein; TNF, tumor necrosis factor; IL, interleukin; G-CSF, granulocyte colony stimulating factor. [a]Significantly negative correlated with the gestational age at delivery (P<0.05).

Table 2. Cervicovaginal fluid cytokine levels in patients in the study group and control group

Cytokine	Delivery at <34 wk (n=23)	Delivery at ≥34 wk (n=36)	*P*-value[b]
MIP-1β	18.56 (0–375.00)	2.89 (0–301.56)	0.067
MIP-1α	11.62 (0–309.47)	5.73 (0–130.48)	0.028[a]
TNF-α	0 (0–169.67)	0 (0–130.66)	0.661
IL-1β	65.35 (0–3,321.48)	67.08 (0–2,990.27)	0.738
IL-6	83.27 (0–1,712.41)	1.44 (0–956.15)	0.002[a]
IL-8	3,663.22 (234.78–10,911.85)	3,007.72 (154.79–10,365.90)	0.472
IL-17α	6.54 (1.51–17.97)	4.17 (0–11.69)	0.018[a]
G-CSF	38.80 (0–1,097.48)	38.74 (0–818.30)	0.225
IL-7	2.81 (0–18.83)	0 (0–19.55)	0.064

Data are expressed as the median (range) (pg/mL).
MIP, macrophage inflammatory protein; TNF, tumor necrosis factor; IL, interleukin; G-CSF, granulocyte colony stimulating factor.
[a]Significant difference between 2 groups (P<0.05); [b]Using Mann-Whitney U test.

Discussion

Our study showed that elevated levels of multiple cytokines (MIP-1α, MIP-1β, IL-6, IL-7, and IL-17α) in the CVF were associated with PTB. Among these, IL-6 and IL-17α showed better sensitivity than the more commonly used marker fFN. This indicated that IL-6 and IL-17α can be used as predictive markers for PTB.

Fetal fibronectin, involved in maintaining the integrity of the fetal chorion and maternal decidua, predicts spontaneous PTB when detected in CVF between 22 and 34 weeks of gestation. Meta-analysis of Leitich et al. [24], found the sensitivity and specificity of fFN in predicting PTB in mothers with PTL to be 63% and 86%, respectively. In our study, however, fFN showed 33% sensitivity of and 95% specificity as a predictor of PTB. As shown in Table 3, the sensitivities of IL-6 and IL-17α were higher than those of fFN by 80% and 75%, respectively. The AUC of fFN was 0.662, while those of IL-6 and IL-17α were 0.788 and 0.715, respectively. The results for fFN may be confounded by unprotected vaginal intercourse, digital examination, bleeding, or contamination with amniotic fluid [1,12]. We did not consider the amniotic fluid contamination of CVF as a confounding factor and believe that it affected the outcome for pPROM patients. Therefore, in the subgroup analysis, the fFN and cytokine levels of the PTL patients, but not of the pPROM patients, were compared. In subgroup analysis, the sensitivity of IL-6 and IL-7 both were 83%, which was higher than the 33% sensitivity of fFN.

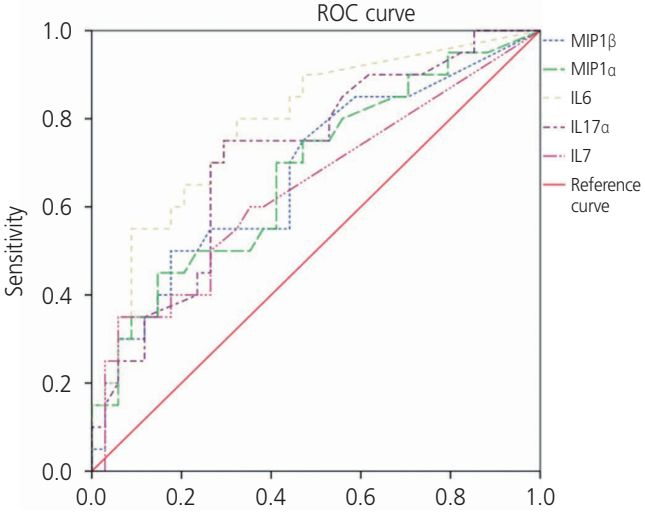

Fig. 3. ROC curves of 5 cytokines in the prediction of early preterm birth (delivery at <34 weeks). ROC, receiver operating characteristic; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor; IL, interleukin; G-CSF, granulocyte colony stimulating factor.

Several protein mediators and cytokines have been studied in the amniotic fluid, blood, urine, and CVF [1,25,26]. Studies considering the association of elevated inflammatory cytokines with chorioamnionitis and PTL were initiated in the amniotic fluid [19]. However, since amniocentesis itself is an invasive method, many studies sought to measure the same non-invasively in the blood, urine, and CVF. The composition of human CVF reflects the local biochemical environment of the gestational tissues. Therefore, CVF proteins, such as fFN, IGFBP1, defensins, lactoferrin, sialidase, granulocyte elastase, human chorionic gonadotropin, IL-1β, IL-6, IL-8, IL-18, IL-1 receptor antagonists, and TNF have been utilized to predict PTB [27-32]. Particularly, IL-6 is strongly associated with intra-amniotic inflammation and microbe-associated intra-amniotic inflammation; hence, measurement of IL-6 in CVF can be used as an easy, noninvasive, rapid method for point-of-care assessment [33,34]. IL-6 induces T-lymphocytes to synthesize C-reactive protein and to promote the differentiation of B-cells. It is a pro-inflammatory cytokine seen widely in de-

Table 3. Receiver operating characteristic curve analysis

Cytokine	AUC	95% CI	*P*-value	Cut-off	Sensitivity	Specificity
MIP-1β	0.676	0.525–0.828	0.032	17.51	47	82
MIP-1α	0.677	0.527–0.828	0.031	6.46	70	57
IL-6	0.788	0.661–0.915	0.000	23.46	80	65
IL-17α	0.715	0.574–0.857	0.009	5.53	75	69
IL-7	0.643	0.485–0.802	0.081	0.98	35	94
Fibronectin	0.662	0.494–0.830	0.073	74.50	33	95

AUC, area under a receiver operating characteristic curve; CI, confidence interval; MIP, macrophage inflammatory protein; IL, interleukin.

cidual tissue, placenta, fetal membrane, and amniotic fluid, and regulates immune adaptation to allow the pregnancy to progress. Preterm labor and pPROM are thought be because of the inflammatory condition caused by infection. Our study has showed that IL-6 is superior to conventional fFN assays. IL-17α as a protective cytokine in host defense against bacterial and fungal infections, was shown to be associated with intra-amniotic inflammation [35]. No study has analyzed IL-17 in the CVF; however, IL-17α was significantly elevated in the amniotic fluid of patients with cervical insufficiency [16]. MIP-1α and MIP-1β are produced in the macrophages in response to infection and inflammation. They subsequently activate human granulocytes and the level of MIP-1α in the CVF is associated with amniotic fluid infection [21,36,37]. MIP-1α has been studied in the CVF of patients with pPROM, but not PTL. In this study, we found that elevation of the MIP-1α level in the CVF was associated with PTL. IL-7 is a cytokine that can stimulate IL-17 production by cells involved in both innate and adaptive immunity. Elevated serum IL-7 is also associated with PTL [38].

Although our sample size was small, the strength of this study is that it was conducted as a multi-center prospective study in 5 centers in Korea. This study directly compared cytokines and fFN in the CVF from the same cohort of patients. When selecting a patient group, the ones with multiple gestation, gestational diabetes, and gestational hypertension were excluded. However, factors such as cervical conization history, socio-economic status, pre-pregnancy body mass index, smoking, drug use, and anxiety that could be confounding factors could not be excluded. The gestational age at diagnosis is also a confounding factor that may affect the gestational age at delivery, but no analysis has been done to correct this.

In conclusion, CVF cytokines, especially IL-6, IL-17α, could be useful predictive markers of PTB in patients with PTL and/or pPROM; however, studies with larger cohort will be required for validation of these findings.

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Conflict of interest

No potential conflict of interest relevant to this article was reported.

Ethical approval

This study was approved by the Institutional Review Board Committee of Ewha Womans University Mokdong Hospital (certificate No. EUMC 2014-06-010).

Patient consent

The patients provided written informed consent for the publication and the use of their images.

Supplementary materials

Supplementary Tables associated with this article can be found online at https://doi.org/10.5468/ogs.19131.

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