Comprehensive Amniotic Fluid Cytokine Profile Evaluation in Women with a Short Cervix: which Cytokine(s) Correlates Best with Outcome?

Sean M. Keeler MD
Lehigh Valley Health Network

Daniel G. Kiefer MD
Lehigh Valley Health Network, Daniel_G.Kiefer@lvhn.org

Orion Rust MD
Lehigh Valley Health Network, Orion.Rust@lvhn.org

Anthony M. Vintzileos MD

Robert O. Atlas MD
Lehigh Valley Health Network

See next page for additional authors

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Authors

Sean M. Keeler MD, Daniel G. Kiefer MD, Orion Rust MD, Anthony M. Vintzileos MD, Robert O. Atlas MD, Eran Bornstein MD, and Nazeeh Hanna MD

Comprehensive amniotic fluid cytokine profile evaluation in women with a short cervix: which cytokine(s) correlates best with outcome?

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OBJECTIVE: The objective of this study was to determine whether an expanded amniotic fluid cytokine profile predicts spontaneous preterm birth in patients with short cervix in the midtrimester.

STUDY DESIGN: Amniocentesis was performed on singleton gestations between 16-24 weeks with a cervical length ≤25 mm. Amniotic fluid from patients who received no surgical or hormonal treatment was assayed for 25 cytokines. Univariate analysis identified cytokine(s) that correlated with the interval between amniocentesis to delivery. Stepwise regression identified which cytokine(s) was most predictive of delivery, followed by the generation of receiver-operator characteristic curves. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated.

RESULTS: Forty-four amniotic fluid samples were analyzed. After stepwise regression, only monocyte chemotactic protein-1 remained significant and was the most predictive of early delivery. With a cutoff of 1320 pg/mL, monocyte chemotactic protein-1 had a 69% sensitivity, 83% specificity, 36% positive predictive value, and 87% negative predictive value to predict spontaneous preterm birth within 1 week of amniocentesis ($P = .015$).

CONCLUSION: Among 25 cytokines, monocyte chemotactic protein-1 was most predictive of spontaneous preterm birth.

Key words: amniotic fluid, cytokine, short cervix

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Preterm birth (PTB) complicates 12.8% of pregnancies in the United States despite early screening with transvaginal ultrasound and cervicovaginal fetal fibronectin.[1-4] The pathophysiology of spontaneous PTB is multifactorial and involves multiple metabolic pathways that ultimately lead to delivery of an immature fetus.[5-7] A sonographically detected short cervix is the final common pathway of the preterm parturition syndrome.[5,6] However, only a proportion of patients with short cervix deliver prematurely.[2,8,9] Therefore, a clinical marker that identifies patients destined for premature parturition would be extremely beneficial.

Inflammation plays a central role in spontaneous term parturition.[10,11] Interleukin-6 (IL-6), IL-8, IL-1β, and monocyte chemotactic protein-1 (MCP-1) are proinflammatory cytokines and have been identified in increased concentrations in cervical tissue, cervicovaginal secretions, myometrium, amniotic fluid, and fetal membranes of patients during normal term parturition.[11-14] Higher levels of proinflammatory cytokines have been shown to stimulate prostaglandin production, uterine activity, and spontaneous labor.[15-24]

High levels of proinflammatory cytokines have been shown to be a significant risk factor for spontaneous preterm delivery.[15,20-22] The inflammatory cascade further mediates leukocyte infiltration, proteolytic enzyme activation such as matrix metalloproteinases, arachidonic acid production, and generation of prostaglandins that cause uterine contractions, cervical remodeling, and ripening.[10-17,22-24] It has been postulated that preterm labor has a similar pathophysiology as term labor: a stimulatory cascade of inflammation (with/without infection) and prostaglandin production occurring at an abnormal time in gestation. Studies have identified inflammatory intraamniotic cytokines in patients in active preterm labor;[25-27] however, no studies have assayed intraamniotic cytokines in patients with a midtrimester short cervix (Medline search using key words "short cervix," "amniotic fluid," and "cytokine"). The purpose of this article was to determine whether an expanded amniotic fluid cytokine profile predicts spontaneous PTB in patients

From the Department of Obstetrics and Gynecology (Drs Keeler and Bornstein), New York University Medical Center, New York, NY; the Department of Obstetrics and Gynecology (Drs Keeler and Rust), Lehigh Valley Health Network, Allentown, PA; the Departments of Obstetrics and Gynecology (Drs Kiefer and Vintzileos) and Pediatrics (Dr Hanna), Winthrop University Hospital, Mineola, NY; the Department of Obstetrics, Gynecology, and Reproductive Medicine (Dr Kiefer), Stony Brook University Medical Center, Stony Brook, NY; and the Department of Obstetrics and Gynecology (Dr Atlas), Mercy Medical Center, Baltimore, MD.

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TABLE 1
Clinical characteristics of study patients (n = 44)

Patient characteristic	n
Age, y	27.4 ± 6.9
Race	
White	25 (56.8%)
Hispanic	10 (22.7%)
African American	9 (20.5%)
Multiparity	29 (65.9%)
Gestational age at entry, wk	20.8 ± 2.1
Prior preterm birth 16-36 wk	17 (38.6%)
Cervical length, mm	10.7 ± 9.3
Amniotic fluid glucose, mg/dL	33.1 ± 14.5[a]
Amniotic fluid glucose (≤14 mg/dL)	5 (12.8%)[a]
Amniotic fluid culture positive	2 (5.1%)[a]
Amniotic fluid WBC, cells/mm^3	172.6 ± 352.9[a]
Membranes at external os	15 (34%)
Bacterial vaginosis	9 (20.5%)

Data are n (%) or mean ± SD.
SD, standard deviation; *WBC*, white blood cells.
[a] n = 39.
Keeler. Comprehensive amniotic fluid cytokine profile evaluation. Am J Obstet Gynecol 2009.

with short cervix in the midtrimester and also which, if any, of the cytokines correlated best with outcome.

Materials and Methods

All studies were performed under informed written consent by using a protocol approved by the Lehigh Valley Health Network Institutional Review Board. Our study cohort consisted of asymptomatic, singleton pregnancies between 16-24 weeks' gestation presenting to the Lehigh Valley Health Network Perinatal Testing Center between April 1998-March 2007 found to have a transvaginal sonographic cervical length (CL) ≤25 mm. Patients with risk factors for spontaneous PTB were screened with serial transvaginal ultrasound beginning at 16 weeks' gestation. Risk factors for PTB included history of spontaneous PTB, second-trimester pregnancy loss, previous cervical surgery (conization or loop excision), or documented uterine anomaly. Also, low-risk, asymptomatic singleton pregnancies were screened for evidence of cervical shortening, with transabdominal ultrasound as part of routine anatomic survey. If the cervix appeared short (≤25 mm) transabdominally, a transvaginal ultrasound was performed. Transvaginal CL measurement was obtained using the standardized technique described by Rust et al.[28]

Patients with a CL ≤25 mm (shortest CL with or without transfundal pressure) were offered enrollment into protocols for treatment of short cervix. All patients underwent an ultrasound-guided transabdominal amniocentesis to exclude intraamniotic infection (low glucose, elevated white blood cell [WBC] count, and aerobic/anaerobic cultures). Five milliliters of unspun amniotic fluid was aliquoted into 15-mL polypropylene tubes and stored at –70°C for future cytokine analysis.

Patients were excluded for the following conditions: any known fetal chromosomal or structural anomaly, multiple gestation, ruptured membranes, clinical chorioamnionitis (defined as maternal fever, fundal tenderness, or mucopurulent vaginal discharge), vaginal bleeding, or the need for an obstetrically indicated delivery. All patients received empiric treatment with indomethacin (100 mg orally, followed by 50 mg every 6 hours) and clindamycin (900 mg intravenous every 8 hours) for 48-72 hours. After this initial treatment, all patients underwent repeat transvaginal ultrasound to exclude rapidly progressing cervical shortening and prolapse of membranes beyond the external cervical os. None of the study patients received hormonal or surgical treatment for short cervix.

We sought to perform a comprehensive amniotic fluid cytokine evaluation by using the Bio-Plex array system (Bio-Rad Laboratories Inc, Hercules, CA) to simultaneously assay and quantify 25 different cytokines. The amniotic fluid samples were analyzed by Bio-Plex array system according to the manufacturer's protocols. Fifty microliters of amniotic fluid was placed in a 96-well filtration plate supplied with the assay kit. Premixed beads (50 μL) coated with target capture antibodies were used (5000 beads per well per cytokine). The data were analyzed using Bio-Plex Manager software (v 3.0) with 5PL curve fitting.

We analyzed amniotic fluid samples for 25 cytokines, including IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, granulocyte colony stimulating factor (G-CSF), interferon gamma (IFN-γ), inducible protein-10 (IP-10), MCP-1, macrophage inflammatory protein (MIP)-1a, MIP-1b, platelet-derived growth factor (PDGF), tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), and regulated on activation, normal T cells expressed and secreted (RANTES). All included patients received no intervention for short cervix and all had outcome data available.

Data on maternal demographics, gestational age at study entry, risk factors for spontaneous PTB, and pregnancy outcome were recorded from the Lehigh Valley Health Network cerclage database. First, we performed a univariate analysis to identify which intraamniotic cytokines were both detected in significant quantities and were significantly correlated with the interval between amniocentesis to delivery. A stepwise regression model was constructed to adjust for the effect of several cytokines on the interval of amniocentesis to delivery. All significant cytokines identified in the univariate analysis were entered into the model in a stepwise fashion to identify which cytokines were most predictive of delivery. Receiver-operator characteristic (ROC) curves were generated to define the best cutoff levels of the cytokine that best predicts preterm delivery, and the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated to predict spontaneous PTB for each cytokine cutoff level. A *P* value of less than .05 was required for significance. The Bonferroni correction was used to control for the simultaneous evaluation of 25 cytokines, requiring a *P* value of less than .002 for statistical significance in the univari-

TABLE 2
Univariate analysis of cytokine concentration, range, percent undetected, and Spearman correlation with amniocentesis delivery interval

Intraamniotic cytokine	Median concentration (pg/mL), n = 44	Cytokine range (pg/mL)	Percent <1 pg/mL	Spearman correlation with delivery interval	*P* value[a]
IL-1β	0.85	0.3-1468	53.5%	−0.742	< .0001
IL-1ra	5414.33	0-29,939	2.3%	−0.482	.0009
IL-2	1.42	0-22	44.2%	−0.281	.0674
IL-4	0.48	0-4	69.8%	−0.167	.2798
IL-5	0.41	0-2	93.0%	−0.091	.5553
IL-6	449.22	3.4-25,902	0%	−0.633	< .0001
IL-7	3.94	0-15.3	11.6%	−0.037	.8329
IL-8	576.68	15.2-25,022	0%	−0.616	< .0001
IL-9	17.08	2-74	0%	−0.091	.0952
IL-10	1.11	0-869	44.2%	−0.711	< .0001
IL-12	1.07	0-29	46.5%	−0.640	< .0001
IL-13	0.96	0-5	55.8%	−0.372	.0128
IL-15	6.60	0-30	4.65%	−0.643	< .0001
IL-17	0.00	0-95	60.5%	−0.674	< .0001
Eotaxin	18.20	0-453	9.3%	−0.474	.0011
G-CSF	370.95	4.5-30,177	0%	−0.603	< .0001
INF-γ	86.62	27-736	0%	−0.440	.0028
MCP-1	539.07	7.8-9171	0%	−0.700	< .0001
MIP-1a	0.00	0-432	58.1%	−0.725	< .0001
MIP-1b	60.19	0-1947	2.3%	−0.629	< .0001
PDGF	327.89	84.6-4559	0%	−0.372	.0137
RANTES	20.23	7.3-7072	0%	−0.622	< .0001
TNF-α	0.00	0-1730	62.8%	−0.708	< .0001
IP-10	26,147.22	0-104,553	2.3%	−0.010	.9962
VEGF	9.95	0-116	27.9%	−0.224	.1534

Shading indicates those cytokines that were detected in sufficient quantities and achieved a statistically significant correlation.
G-CSF, granulocyte colony stimulating factor; *IL*, interleukin; *INF*, interferon; *IP*, inducible protein; *MCP-I*, monocyte chemotactic protein-1; *MIP*, macrophage inflammatory protein; *PDGF*, platelet-derived growth factor; *RANTES*, regulated on activation, normal T cells expressed and secreted; *TNF-α*, tumor necrosis factor alpha; *VEGF*, vascular endothelial growth factor.

[a] Bonferroni correction; $P < .002$ required for significance.

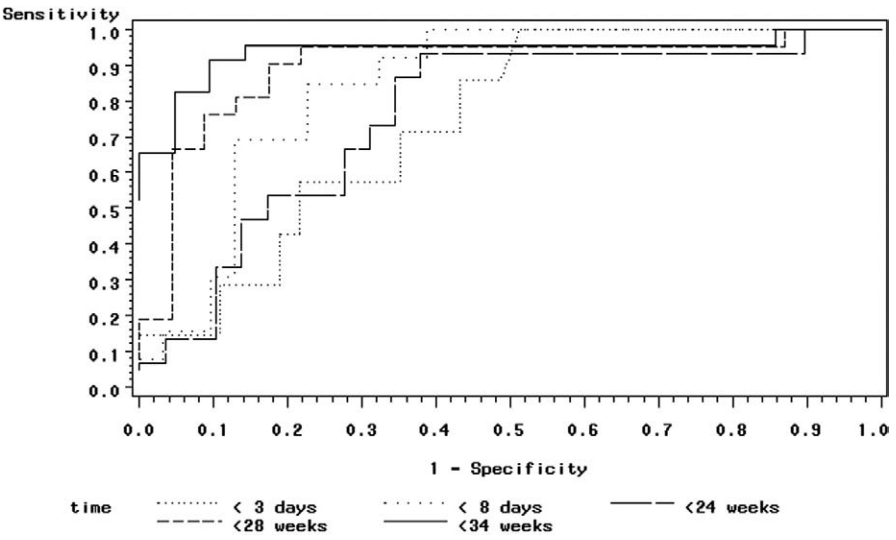
Keeler. Comprehensive amniotic fluid cytokine profile evaluation. Am J Obstet Gynecol 2009.

ate analysis. Calculations were performed using SAS 9.1 (SAS Institute, Cary, NC).

Results

Forty-four amniotic fluid samples were available for analysis and assayed for 25 cytokines. The maternal demographics and risk factors for spontaneous PTB are presented in Table 1. The intervals of amniocentesis to delivery for the 44 patients analyzed are as follows: 7 (15.9%) delivered within 48 hours of amniocentesis, 13 (29.5%) delivered within 7 days, 15 (34.1%) delivered before 24 weeks' gestation, 21 (47.7%) delivered before 28 weeks' gestation, and 23 (52.2%) delivered before 34 weeks' gestation. Univariate analysis identified 15 intraamniotic cytokines that significantly correlated with the interval between amniocentesis and delivery (Table 2). For several cytokines, the significant correlations were driven largely by high cytokine levels at very short delivery intervals. This finding also accounts for how some cytokines (eg, IL-1β) were not detected in large quantities (53.5% of the samples were <1 pg/mL), yet still managed to obtain extremely significant correlations. There were 9 cytokines that were both detected in significant quantities (<10%, <1 pg/mL) and significantly correlated with amniocentesis to delivery interval (Table 2, *shaded lines*). Of these, MCP-1 was the most significantly correlated. Once MCP-1 entered the stepwise regression analysis, it was the only covariate that remained significantly associated with the interval of amniocentesis to delivery. Therefore, subsequent analysis focused on MCP-1.

FIGURE
Area under ROC curves



AUCs for <3 days, <8 days, <24 weeks, <28 weeks, and <34 weeks are 0.743, 0.840, 0.755, 0.894, and 0.940, respectively.

AUC, area under the curve; *ROC*, receiver-operator curve.

Keeler. Comprehensive amniotic fluid cytokine profile evaluation. Am J Obstet Gynecol 2009.

Patients who had a shorter amniocentesis to delivery interval had higher levels of MCP-1. The Figure depicts the ROC curves for MCP-1 based on time of delivery (within 48 hours, 7 days, before 24 weeks, before 28 weeks, and before 34 weeks). Table 3 shows the best MCP-1 cutoff concentrations, areas under the curve, sensitivities, specificities, PPV, and NPV in predicting the timing of spontaneous PTB. In general, those patients with MCP-1 levels above 1500 pg/mL were more likely to deliver early (8/14, 57.1%, delivered before 24 weeks), whereas those with an MCP-1 level <500 pg/mL were more likely to deliver after 28 weeks (19/22, 86.4%, remained undelivered at 28 weeks). With the use of a cutoff of 1320 pg/mL, MCP-1 had a 69% sensitivity, 83% specificity, 36% PPV, and 87% NPV to predict spontaneous PTB within 1 week of amniocentesis ($P = .015$).

Comment

We report the pregnancy outcomes of the only comprehensive amniotic fluid cytokine evaluation in asymptomatic patients with short cervix (≤25 mm) in the midtrimester. An elevated amniotic fluid level of MCP-1 ≥1320 pg/mL was most predictive of spontaneous preterm delivery within 1 week of amniocentesis. Our results are consistent with the findings of an elevated MCP-1 in active preterm labor patients with intact membranes by Esplin et al.[26] They showed that MCP-1 plays a role in the common pathways of labor, regardless of the presence or absence of infection.[26] This suggests that an early elevation of MCP-1 in asymptomatic midtrimester short-cervix patients may be an initiating factor in the preterm parturition syndrome and may confer a similar risk of PTB as a symptomatic patient in active preterm labor.

There are several unique qualities to our study. We sought to identify which cytokines were most predictive of pregnancy outcome by performing a comprehensive amniotic fluid cytokine analysis rather than focusing on an individual cytokine. We used the Bio-Plex array system (Bio-Rad Laboratories Inc) to simultaneously identify 25 different cytokines. The advantage of this method over the enzyme-linked immunosorbent assay kits is that it identifies a wide selection of cytokines and quantitates these over a broad range (from 0.3 pg/mL to >32,000 pg/mL, depending on the cytokine) using only 50 μL of sample. We selected this panel of cytokines, developed by Bio-Rad, because (1) it includes several key proinflammatory and antiinflammatory cytokines, chemokines, and growth factors that were shown to play a key role in placental immune regula-

TABLE 3
MCP-1 predicts spontaneous preterm birth

Delivery	Frequency (%)	Cutoff (pg/mL)	ROC AUC	*P* value	RR (95% CI)	Sensitivity	Specificity	PPV	NPV
≤48 h	7 (15.9)	1539	0.74	.073	1.04 (0.99–1.08)	57	73	29	90
≤7 d	13 (29.5)	1320	0.84	.015	1.07 (1.01–1.12)	69	83	36	87
≤24 wk	15 (34.1)	1517	0.76	.056	1.04 (0.99–1.09)	53	79	57	78
≤28 wk	21 (47.7)	515	0.89	.005	1.16 (1.05–1.29)	82	81	80	83
≤34 wk	23 (52.2)	436	0.94	.007	1.92 (1.19–3.09)	91	86	88	90

AUC, area under the curve; *CI*, confidence interval; *MCP-1*, monocyte chemotactic protein-1; *NPV*, negative predictive value; *PPV*, positive predictive value; *ROC*, receiver-operator curve; *RR*, relative risk.

Keeler. Comprehensive amniotic fluid cytokine profile evaluation. Am J Obstet Gynecol 2009.

tion and parturition; and (2) the same panel of amniotic fluid cytokines used in our study has been studied by previous investigators in early-midtrimester normal pregnancy, and cytokines were detected in amniotic fluid. The widespread availability and ease of use of this instrument allow it to be easily incorporated into many research protocols.[29,30] Finally, we included all asymptomatic patients with midtrimester short cervix receiving no intervention—specifically, patients with intraamniotic evidence of infection (positive culture and/or glucose <14 mg/dL). We included these patients in our analysis, because they represent the entire spectrum (no inflammation, inflammation without infection, and infection) of patients who present with this cervical disorder.[31,32]

MCP-1 is a proinflammatory chemoattractant cytokine responsible for monocyte, macrophage, T lymphocyte, and natural killer cell recruitment into foci of inflammation.[33-35] The source of MCP-1 in our patients is unknown. However, MCP-1 has been located in cervical tissue, placenta, decidua, and chorion.[15,27,36,37] We have previously shown that extremely short CL (≤5 mm) is highly correlated with intraamniotic inflammation.[30] Even in asymptomatic patients, MCP-1 appears to be abundant and plays a leading role in mediating leukocyte infiltration as a potent inducer and regulator of the inflammatory cascade, leading to a pronounced cytokine expression, prostaglandin production, and early cervical remodeling and maturation.

It remains to be seen if MCP-1 is found in similar concentrations in other compartments of the maternal-fetal-placental unit. If so, detection of this cytokine through less invasive means (without amniocentesis) would allow determination of levels of inflammation and perhaps allow tailoring of therapy aimed at the underlying cause of preterm labor or cervical shortening, rather than the symptoms of contractions or cervical maturation. ■

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