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The cytokine network in women with an asymptomatic short cervix and the risk of preterm delivery

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Problem: To characterize the amniotic fluid (AF) inflammatory-related protein (IRP) network in patients with a sonographic short cervix (SCx) and to determine its relation to early preterm delivery (ePTD).

Method of study: A retrospective cohort study included women with a SCx (≤25 mm; n=223) who had amniocentesis and were classified according to gestational age (GA) at diagnosis and delivery (ePTD <32 weeks of gestation).

Results: (i) In women with a SCx ≤ 22 1/7 weeks, the concentration of most IRPs increased as the cervix shortened; those with ePTD had a higher rate of increase in MIP-1α, MCP-1, and IL-6 concentrations than those delivering later; and (ii) the concentration of most IRPs and the correlation between several IRP pairs were higher in the ePTD group than for those delivering later.

Conclusion: Women with a SCx at 16-22 1/7 weeks have a unique AF cytokine network that correlates with cervical length at diagnosis and GA at delivery. This network may aid in predicting ePTD.

KEYWORDS
amniocentesis, cervical insufficiency, cytokine, macrophage inflammatory protein, network analysis, preterm birth

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1 | INTRODUCTION

A sonographic short cervix is a strong predictor of spontaneous preterm delivery.[1–15] The earlier the diagnosis of a short cervix, the more likely the patient will deliver preterm.[16–33] Compelling evidence supports the view that this condition is syndromic in nature and has multiple etiologies,[13,34–37] such as a decline in progesterone action,[38–48] prior cervical surgery,[49–57] intra-amniotic inflammation and/or infection,[58–63] and genuine cervical insufficiency.[35,64] Patients with a mid-trimester sonographic short cervix can be offered treatment with vaginal progesterone[65–73] and, for those with a history of preterm birth, a cervical cerclage[74–80] may be placed.

A subset of patients with a mid-trimester sonographic short cervix (cervical length ≤25 mm) may have microbial invasion of the amniotic fluid (MIAC) with or without inflammation.[62,81,82] Sterile intra-amniotic inflammation appears to be more common than MIAC (10% vs 2.2%, respectively) and is associated with an increased risk of spontaneous preterm delivery <34 weeks of gestation.[82] The presence of intra-amniotic inflammation (both microbial and sterile) is associated with adverse pregnancy outcomes[35,83–91] in patients presenting with preterm labor and intact membranes,[92–108] preterm pre-labor rupture of the membranes (PPROM),[109–114] a sonographic short cervix,[60,62,82,115] and cervical insufficiency.[58,59,64,116,117] Thus, we and others have explored the behavior of cytokines and other inflammatory-related markers in amniotic[82,100,106–108,118–128] or vaginal[129–140] fluid.

Disease states are generally caused by the interaction of a group of correlated molecules (or network) and not by the effect of a single molecule.[141–147] For example, in the context of inflammation, one cytokine can induce the expression of others, which can modulate the immune response and lead to the development of feedback regulatory networks.[148] Thus, to understand the pathogenesis of inflammatory-related diseases, cytokines should ideally be studied as groups of interacting molecules/proteins.[149–151] Indeed, recent evidence suggests that biomarkers derived from network analysis are able to achieve better diagnostic performance than those derived from a single-molecule approach.[61,152–159]

Women with microbial-associated or sterile intra-amniotic inflammation had a more coordinated cytokine network than those without intra-amniotic inflammation.[127] Using a network approach, we were able to demonstrate that microbial and sterile intra-amniotic inflammation differ in their cytokine networks.[127] Therefore, the objective of this study was to examine the amniotic fluid inflammatory-related protein network in asymptomatic patients with a mid-trimester sonographic short cervix according to gestational age at the time of diagnosis and delivery.

2 | MATERIALS AND METHODS

2.1 | Study population

A retrospective cohort study was conducted to include 223 patients with an asymptomatic sonographic short cervix. Amniotic fluid samples of participants were selected from the clinical database and Bank of Biological Materials of Wayne State University, the Detroit Medical Center, and the Perinatology Research Branch of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) if the patients met the following criteria: (i) singleton gestation, (ii) asymptomatic sonographic cervical length ≤25 mm, (iii) transabdominal amniocentesis performed for molecular microbiological studies, and (iv) available pregnancy outcomes. Exclusion criteria were as follows: (i) rupture of membranes or preterm labor symptoms before amniotic fluid collection, (ii) chromosomal or structural fetal anomaly, or (iii) placenta previa. A subset of patients in this study was included in a prior study,[82] which provides a description of the molecular microbiologic findings of the amniotic cavity and amniotic fluid IL-6 concentrations. All patients provided written informed consent, and the use of biological specimens as well as clinical and ultrasound data for research purposes was approved by the Institutional Review Boards of Wayne State University and NICHD.

Clinical definitions, methods of sonographic assessment of the cervix, sample collection, and the detection of microorganisms as well as amniotic fluid IL-6 concentrations were previously reported.[82] Briefly, spontaneous preterm labor was defined by the presence of regular uterine contractions occurring at least twice every 10 minutes, associated with cervical changes before 37 completed weeks of gestation. Early spontaneous preterm delivery was defined as spontaneous preterm delivery before 32 completed weeks of gestation. Patients classified as having spontaneous preterm delivery included those with spontaneous preterm labor, preterm pre-labor rupture of the membranes (PPROM), and those whose labor was induced or augmented due to clinical chorioamnionitis.[160] Patients who were unavailable for a follow-up or who had inaccessible delivery data were excluded.

Intra-amniotic inflammation was characterized by an amniotic fluid IL-6 concentration ≥2.6 ng/mL.[82,100,106–108,114,118,120,122–124] Microbial invasion of the amniotic cavity was defined according to the results of amniotic fluid culture and broad-range polymerase chain reaction (PCR) coupled with electrospray ionization mass spectrometry (PCR/ESI-MS) analysis.[105,113] Acute histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes, and acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or Wharton's jelly, using previously described criteria.[86,161–170]

2.2 | Multiplex determination of inflammatory-related proteins

Amniotic fluid concentrations of 33 inflammatory-related proteins were determined using a multiplex bead array assay developed by the investigators as previously described in detail (Methods S1).[127]

2.3 | Statistical analysis

The goal of the statistical analysis was to (i) assess the relation between the risk of preterm delivery and gestational age at amniocentesis, (ii) assess the relation between inflammatory-related protein concentration and cervical length, (iii) evaluate differences in inflammatory-related

protein concentration between groups (delivery ≤32 or >32 weeks of gestation), and (iv) evaluate differences in the correlation of pairs of proteins between groups and build correlation networks. To accomplish these goals, women were divided into three groups based on their gestational age at amniocentesis with the two cutoff points representing tertiles of the distribution: 16 5/7 to 22 1/7, 22 2/7 to 26 1/7, and 26 2/7 to 31 5/7 weeks of gestation. In addition to creating equal-sized subgroups of patients, this subclassification of samples is based on (i) previous observations that the populations of patients diagnosed with a short cervix are different, depending upon whether the diagnosis is made earlier as opposed to later in gestation[81] and also (ii) the observation that pro-inflammatory protein concentrations may vary with gestational age at amniocentesis.[127]

The association between the interval of gestational age at amniocentesis and risk of preterm delivery was tested using a chi-square test for trends in proportions. To test for differential analyte concentrations between groups within each gestational-age interval, a linear model was fit to the log (base 2) of the analyte concentration as a function of the group indicator (patient delivered ≤32 or >32 weeks of gestation) while adjusting for gestational age at amniocentesis. Similarly, to test the association between analyte concentration and cervical length, a linear model was fit to the log (base 2) of the analyte concentration as a function of cervical length and the group indicator (patient delivered ≤32 or >32 weeks of gestation) while adjusting for gestational age at amniocentesis. An interaction term was allowed between the group indicator and cervical length to accommodate possible differences in the rate of change of the analyte concentration and cervical length between groups.

Significance test *P* values for the group (delivery ≤32 or >32 weeks of gestation) coefficient as well as for the cervical length coefficient were adjusted with the Benjamini-Hochberg method over all 33 analytes to compute *q*-values. The significance of differences in concentration was determined based on a *q*-value <.1 and a fold change >1.5. For changes in cervical length, the minimum fold change cutoff of 1.5 was defined as more than a 1 cm change in cervical length.

The difference in concentration correlation of each possible pair of analytes (eg, IL-1α and IL-6, IL-1α and IL-33) between groups was assessed as follows. A linear model was fit to the log-transformed data of each protein as a function of gestational age using samples of each group separately in each gestational-age interval. The residuals (actual value−fitted value) were then used to compute the Pearson correlation for each pair of analytes (called partial correlations). To test for differences in partial correlations between groups within each interval, the partial correlations were first converted into an intermediary statistic using Fisher's *z* transformation. Under the null hypothesis (partial correlations are equal between groups), the standardized differences in *z* values among groups were assumed to follow a standard normal distribution. Significant differences in partial correlations were inferred based a *P* value <.01, and the magnitude of correlation differences was at least .2.

A network of differential correlation between groups of women was constructed for each gestational-age interval by linking/connecting the proteins with significant differences in partial correlations (also referred to as perturbed correlation). For each node (protein) in the network, we calculated the degree (number of perturbed correlations of a given protein) and the average absolute difference in correlations as previously described.[127]

To identify confounding variables that could impact the analyses described above, we have used chi-square tests to determine the association of covariates (administration of steroids, antibiotics, and progesterone before amniocentesis) and preterm delivery within each interval. Covariates with a significant association with the outcome were adjusted for in the differential concentration and differential correlation analyses.

3 | RESULTS

3.1 | Characteristics of the study population

Two hundred and twenty-three patients who had a sonographic short cervix (≤25 mm) were included in this study. Demographic and clinical characteristics of the study population are displayed in Table 1. The median gestational age at amniocentesis was 24 3/7 weeks. Forty-four percent (99/223) of patients with a short cervix delivered at term. Most patients did not have intra-amniotic inflammation (75.3% [168/223]). Among patients with intra-amniotic inflammation, the frequency of sterile intra-amniotic inflammation was higher than microbial-associated intra-amniotic inflammation (10.3% [23/223] and 2.2% [5/223], respectively) (Table 1). Patients with a sonographic short cervix were classified according to gestational age at delivery, and 28.7% (64/223) delivered ≤32 weeks of gestation while the remaining 71.3% (159/223) delivered >32 weeks of gestation.

The samples included in this study were collected before the publication of randomized trials reporting that vaginal progesterone reduces the rate of preterm delivery and neonatal morbidity; therefore, none of our patients received vaginal progesterone. Eight percent (18/221) of patients underwent cerclage. There were no significant differences in the frequencies with which 17-alpha-hydroxyprogesterone caproate, antibiotics, and antenatal corticosteroids were administered within 7 days before amniocentesis between women with a short cervix who delivered ≤32 weeks of gestation and those who delivered >32 weeks of gestation (Table 1).

The prevalence of intra-amniotic inflammation (defined as an amniotic fluid IL-6 concentration ≥2.6 ng/mL), including both microbial-associated and sterile intra-amniotic inflammation, was significantly higher in patients with a sonographic short cervix who delivered ≤32 weeks of gestation than in those who delivered >32 weeks of gestation (*P*<.05 for all; Table 1). Additionally, the median amniotic fluid IL-6 concentrations (ng/mL) and the frequency of acute inflammatory lesions of the placenta were significantly higher and more frequent in patients with a short cervix who delivered ≤32 weeks of gestation than in those who delivered >32 weeks of gestation (IL-6: median [IQR]; 1.11 [0.31-4.15] vs 0.59 [0.28-0.99]; *P*=.002; acute inflammatory lesion of the placenta: 73.3% [44/60] vs 33.3% [48/144]; *P*<.001; Table 1).

TABLE 1 Clinical and demographic characteristics of the study population

Variable	Overall cohort (N=223)	Gestational age at delivery		*P*-value[a]
		≤32 wk of gestation (N=64)	>32 wk of gestation (N=159)	
Maternal age (y)	23.5 (20-28)	25 (21-32.8)	23 (20-27)	.03
Pre-pregnancy body mass index (kg/m^2)[b]	25.8 (21.5-32.6)	29.1 (23.0-35.4)	24.7 (21.1-31.0)	.004
Race, % (n)				
African American	91.5% (204)	93.7% (60)	90.6% (144)	.64
Hispanic	3.1% (7)	3.1% (2)	3.1% (5)	
Caucasian	2.7% (6)	1.6% (1)	3.1% (5)	
Asian	0.9% (2)	1.6% (1)	0.6% (1)	
Other	1.8% (4)	0	2.5% (4)	
Gestational age at amniocentesis (wk)	24.4 (21.1-27.4)	21.7 (19.9-24.5)	25.4 (22.9-28.4)	<.001
Sonographic cervical length at diagnosis (mm)	13 (7-18)	7 (2.3-14)	14 (10-19)	<.001
Diagnosis				
No intra-amniotic inflammation, % (n)	75.3% (168)	59.4% (38)	81.8% (130)	<.001
Microbial invasion of the amniotic cavity, % (n)	12.1% (27)	9.4% (6)	13.2% (21)	.43
Sterile intra-amniotic inflammation, % (n)	10.3% (23)	25.0% (16)	4.4% (7)	<.001
Microbial-associated intra-amniotic inflammation, % (n)	2.2% (5)	6.3% (4)	0.6% (1)	.01
Intra-amniotic inflammation (ELISA IL-6 ≥2.6 ng/mL), % (n)	12.6% (28)	31.3% (20)	5.0% (8)	<.001
Treatment				
Cerclage, % (n/N)	8.1% (18/221)	9.4% (6/64)	7.6% (12/157)	.67
Progesterone supplementation before amniocentesis, % (n/N)	1.4% (3/214)	0%	2.0% (3/152)	.27
Administration of antenatal corticosteroids within 7 d before amniocentesis, % (n)	11.7% (26)	12.5% (8)	11.3% (18)	.80
Antibiotics before amniocentesis, % (n)	5.8% (13)	6.3% (4)	5.7% (9)	.87
Amniotic fluid				
Amniotic fluid IL-6 concentration (ng/mL)	0.65 (0.29-1.28)	1.11 (0.31-4.15)	0.59 (0.28-0.99)	.002
Amniotic fluid glucose concentration (mg/dL)[c]	30 (25-35) (N=221)	31 (24.3-36) (N=64)	30 (25-35) (N=157)	.52
Amniotic fluid white blood cell count (cells/mm^3)[d]	2 (0-7.5) (N=218)	2 (0-10) (N=63)	1 (0-6) (N=155)	.06
Placenta				
Acute inflammatory lesion of the placenta, % (n/N)	45.1% (92/204)	73.3% (44/60)	33.3% (48/144)	<.001
Acute histologic chorioamnionitis, % (n/N)	44.1% (90/204)	73.3% (44/60)	31.9% (46/144)	<.001
Acute funisitis, % (n/N)	32.4% (66/204)	51.7% (31/60)	24.3% (35/144)	<.001
Delivery				
Amniocentesis to delivery interval (d)	68 (36-95)	23 (10-35)	85 (64-105)	<.001
Gestational age at delivery (wk)	35.7 (30.6-38.7)	26.6 (22.1-29.5)	38.0 (35.1-39.1)	<.001
Delivery ≥37 wk of gestation, % (n)	44.4% (99)	0%	62.3% (99)	<.001

ELISA, enzyme-linked immunosorbent assay; IL, interleukin.
Data are given as median (interquartile range) and percentage (n).
Acute inflammatory lesion of the placenta is defined as acute histologic chorioamnionitis and/or acute funisitis.
[a]Comparison between patients with the diagnosis of a short cervix who delivered before or after 32 wk of gestation.
[b]Missing data: 18 cases.
[c]Missing data: two cases.
[d]Missing data: five cases.

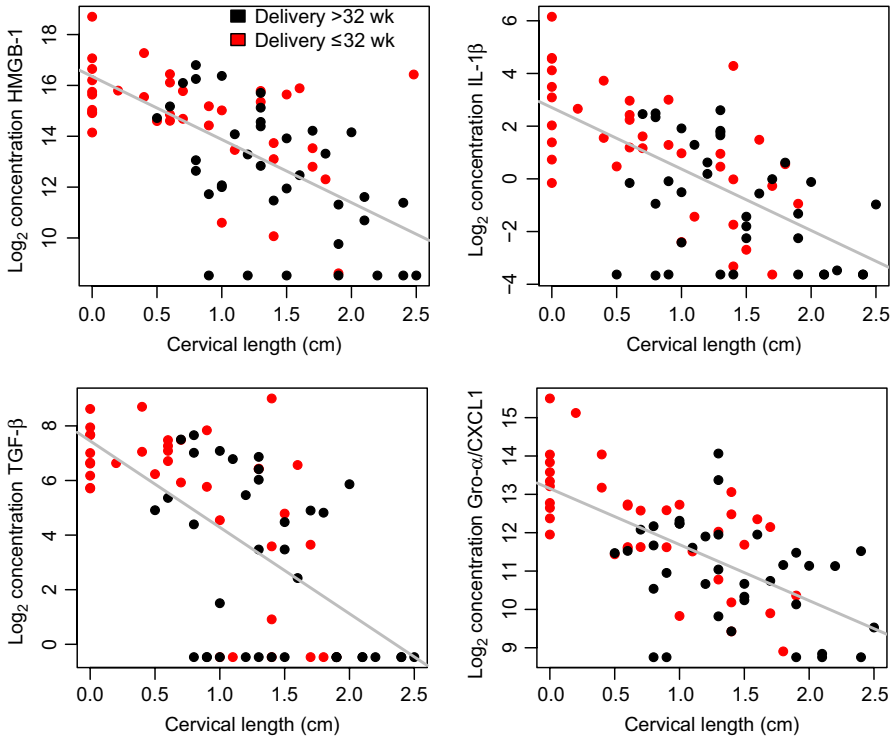

FIGURE 1 Association between pro-inflammatory protein concentration and cervical length for patients diagnosed with a short cervix at 16 5/7 to 22 1/7 weeks of gestation. The figure shows the concentration ($\log_2$, thereof) as a function of cervical length for 4 of the 33 proteins shown in Table 2. The concentration of these proteins is higher in women with a short cervix regardless of whether they delivered ≤32 weeks of gestation (red) or >32 weeks of gestation (black)

3.2 | The risk of preterm delivery according to the presence of a sonographic short cervix and gestational age at diagnosis

Patients with a short cervix who delivered ≤32 weeks of gestation had a significantly lower gestational age at amniocentesis/diagnosis with a short cervix, and they had a shorter sonographic cervical length than those who delivered >32 weeks of gestation (P<.001 for all; Table 1). When patients were stratified according to the tertiles of gestational age at amniocentesis, the rate of delivery ≤32 weeks of gestation was 50% in those with an asymptomatic short cervix identified at 16 5/7 to 22 1/7, 21% in those identified at 22 2/7 to 26 1/7, and 15% in those at 26 2/7-31 5/7 weeks of gestation (chi-square test for trend P<.0001).

3.3 | Potential effects of confounding variables

We examined whether the administration of steroids, antibiotics, and progesterone before amniocentesis could have had a confounding effect on the differential concentration analysis of proteins. The association of these covariates with the "group indicator" (whether a patient delivered ≤32 or >32 weeks of gestation) for each gestational-age interval at amniocentesis was assessed and found to be significant only for steroids (P=.014) in the gestational-age window between 26 2/7 and 31 5/7 weeks at amniocentesis. Therefore, we adjusted for exposure to steroids in the third interval of gestational age at amniocentesis and found little effect on the differential concentration and correlation analysis results, as described below.

3.4 | The inflammatory protein concentrations and sonographic cervical length

We tested the association between inflammatory-related protein concentrations and cervical length within the group of patients in which the diagnosis of a short cervix/gestational age at amniocentesis was made early in pregnancy (16 5/7 to 22 1/7 weeks) while controlling for the gestational age at amniocentesis. This set of patients included an equal number of deliveries ≤32 and >32 weeks of gestation. The concentration of all inflammatory-related proteins were inversely related to cervical length (ie, the shorter the cervix, the higher the concentration of the proteins; q<.1 for all). Only IP-10 and IL-18 did not reach the required minimum of a 1.5-fold change in abundance per cm change in cervical length to be considered statistically significant. Fold changes for significant proteins ranged from 1.5- to 8.5-fold per cm (IL-33 and IL-8, respectively; see Figure 1 and Table 2). For three proteins (MIP-1α, MCP-1, IL-6), the rate of change in abundance with cervical length was higher in patients who delivered ≤32 weeks compared to those who delivered >32 weeks of gestation (Figure 2).

3.5 | Differences in amniotic fluid inflammatory-related protein concentrations between patients with a sonographic short cervix who delivered before and after 32 weeks of gestation

The amniotic fluid concentrations of 33 inflammatory-related proteins were compared between patients with a sonographic short cervix who delivered ≤32 and >32 weeks of gestation. As the amniotic

TABLE 2 Changes in protein concentration with cervical shortening

	Overall change with CL			Interaction between group and CL		
Protein	Fold change[a]	*P*	*q*	Fold change[b]	*P*	*q*
MCP-1	−2.3	.000	.000	−2.4	.003	.098
MIP-1α	−2.7	.000	.000	−2.7	.006	.098
IL-6	−5.0	.000	.000	−4.6	.009	.098
MIP-1β	−3.7	.000	.000	−3.5	.014	.115
MIP-3α	−5.4	.000	.000	−2.7	.135	.735
HMGB-1	−5.0	.000	.000	2.0	.177	.735
I-TAC/CXCL-11	−4.5	.000	.000	2.4	.190	.735
Gro-a/CXCL1	−2.5	.000	.000	−1.5	.226	.735
Calgranulin C	−2.1	.041	.043	−2.5	.241	.735
IL-8	−8.5	.000	.000	−4.0	.241	.735
IL-1α	−2.7	.001	.001	−2.0	.273	.735
IL-15	−4.3	.000	.000	1.9	.292	.735
Eotaxin	−4.2	.000	.000	1.7	.299	.735
IL-12	−4.4	.000	.000	1.9	.330	.735
RANTES	−2.3	.004	.005	1.8	.334	.735
IL-1β	−4.9	.000	.000	−1.6	.392	.808
IL-18	−1.4	.004	.005	1.2	.444	.862
IL-16	−2.5	.000	.000	1.2	.535	.897
IL-10	−4.0	.001	.002	−1.7	.558	.897
GM-CSF	−3.0	.001	.001	1.5	.571	.897
IFN-γ	−3.7	.000	.000	1.3	.658	.971
IP-10	−1.4	.042	.043	1.1	.742	.971
M-CSF	−3.0	.000	.000	1.2	.749	.971
IL-2	−3.9	.000	.000	1.1	.809	.971
IL-33	−1.5	.013	.014	1.1	.819	.971
IL-7	−6.2	.000	.000	1.1	.846	.971
IL-4	−2.2	.000	.000	1.1	.860	.971
IL-13	−6.3	.000	.000	−1.1	.920	.971
TGF-β	−7.8	.000	.000	−1.1	.940	.971
TNF-α	−4.0	.000	.000	1.0	.942	.971
Calgranulin A	−4.8	.000	.000	1.1	.949	.971
Lactoferrin	−2.4	.000	.000	1.0	.971	.971

CL, cervical length.

[a]Refers to the change in average protein concentration per cm increase in cervical length. Minus sign denotes decrease in concentration with increasing cervical length (or increase with cervical shortening). Both groups of patients (delivery ≤32 wk and >32 wk of gestation) were included in this analysis.

[b]Refers to the ratio of change in the average protein concentration per cm of cervical length in patients with delivery ≤32 wk than for those who delivered >32 wk. Minus sign denotes that the decrease in protein concentration and cervical length (or increase in concentration with cervical shortening) is greater in patients who delivered ≤32 wk compared to those who delivered >32 wk.

fluid concentration of these analytes has a nonlinear relationship with gestational age and their discriminatory power may vary as a function of duration of pregnancy, we assessed differential concentration within three separate intervals, adjusting for gestational age at amniocentesis.

3.5.1 | Amniotic fluid concentration of inflammatory proteins in patients with a short cervix diagnosed between 16 5/7 and 22 1/7 weeks of gestation

All inflammatory proteins but five (IL-18, IL-33, IP-10, MIG, and MIP-3α) had a significantly higher mean concentrations in patients

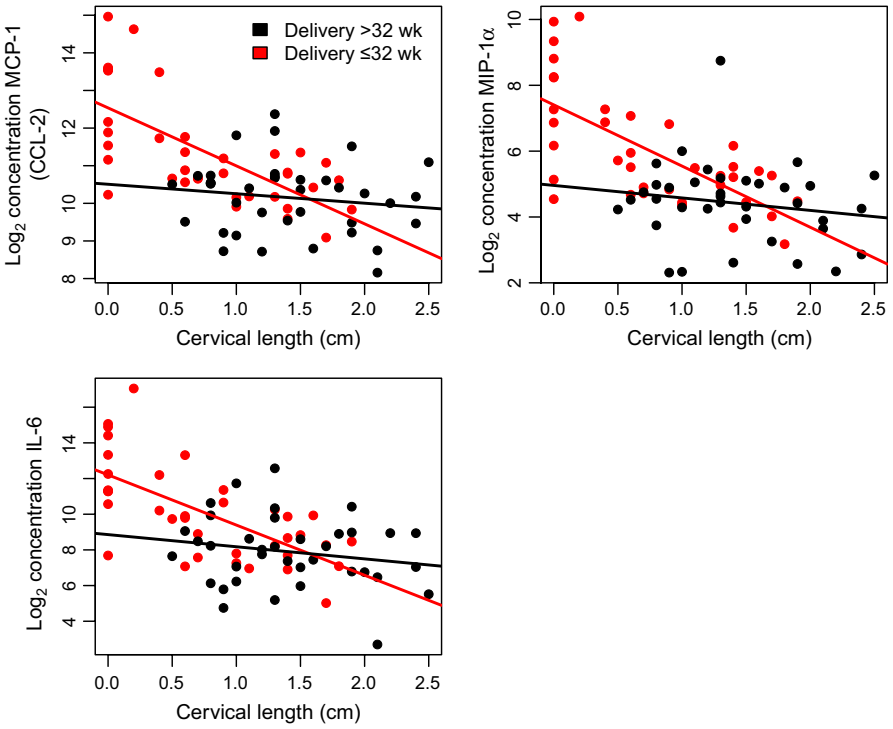

FIGURE 2 Three proteins that increase in abundance with cervical shortening a higher rate in women who delivered ≤32 weeks of gestation (red) compared to those who delivered >32 weeks of gestation (black). As in Figure 1, only patients diagnosed with a short cervix at 16 5/7 weeks to 22 1/7 weeks of gestation are included in this analysis. Solid lines represent the best linear fit of the $\log_2$ protein concentration as a function of gestational age at amniocentesis

who delivered ≤32 weeks of gestation than in those who delivered >32 weeks of gestation (fold change >1.5; q<.1). The highest changes of amniotic fluid protein concentration were observed for IL-8, MIP-1β, IL-6, and IL-10 (5.6-, 5.1-, 4.5-, and 4.4-fold, respectively; Table 3). Of the 28 inflammatory-related proteins whose concentrations were significantly different between groups in this interval, 18 would remain significant when the analysis was restricted to patients without intra-amniotic inflammation (see proteins in boldface, Table 3).

3.5.2 | Amniotic fluid concentration of inflammatory proteins in patients with a short cervix diagnosed between 22 2/7 and 26 1/7 weeks of gestation

In this gestational-age window, the differences in amniotic fluid concentrations of inflammatory-related proteins between groups were of a lesser magnitude than those in the first gestational-age interval (16 5/7 to 22 1/7 weeks); for nine analytes, the mean amniotic fluid concentration was significantly higher in patients who delivered ≤32 weeks of gestation compared to those who delivered >32 weeks of gestation (fold change >1.5; q<.1). The three analytes with the highest concentration were IL-8, IL-6, and MIP-1β (5.4-, 4.2-, and 2.6-fold, respectively, Table 3).

3.5.3 | Amniotic fluid concentration of inflammatory proteins in patients with a short cervix diagnosed between 26 2/7 and 31 5/7 weeks of gestation

Differences in the amniotic fluid concentrations of the inflammatory-related proteins between groups was less than that of the other two gestational-age groups. The only protein with a significant difference in median concentration between patients who delivered ≤32 weeks of gestation compared to those who delivered >32 weeks of gestation was IL-8 (fold change of 2.3 and nominal P value <.05, Table 3). However, this significance was lost when adjusting for the administration of steroids, which was a confounder variable in the analysis of patients in this interval of gestational age (as described above).

3.6 | Amniotic fluid cytokine network analysis according to gestational age at diagnosis of a short cervix

As the concentration of pro-inflammatory proteins changes with gestational age at amniocentesis (see Figure 3, top panel for IL-10 and MIP-1β), differences in correlation patterns of pairs of proteins between groups were assessed within three intervals of gestational age, after removing the linear trend with gestational age from log (base 2) protein concentration (Figure 3, bottom panel). The example presented in Figure 3 shows that in the interval of 16 5/7 to 22 1/7 weeks, the partial correlation between MIP-1β and IL-10 is .65 in patients who delivered ≤32 weeks and only .05 in patients who delivered >32 weeks (difference in correlation coefficient of .65-.05=.60, P=.0055, see Table S1).

Patients with a sonographic short cervix diagnosed between 16 5/7 and 22 1/7 weeks of gestation who delivered ≤32 weeks of gestation had a higher concentration of inflammatory-related proteins than those who delivered >32 weeks of gestation (22 perturbed proteins). IL-8, MIP-1β, and MIP-1α had the highest number of perturbed correlations (degrees of 6, 5, and 5, respectively), while IL-8, MIP-1β, and IL-6 showed the largest magnitude of change between groups (Figure 4A).

TABLE 3 Differential protein concentration analysis for different intervals of gestational age at amniocentesis

	16 5/7-22 1/7 wk			22 2/7-26 1/7 wk			26 2/7-31 5/7 wk		
Protein	**FC**	***P***	***q***	**FC**	***P***	***q***	**FC**	***P***	***q***
Calgranulin A	**3.8**	.0006	.002	1.4	.0382	.097	−1.2	.4503	.803
Calgranulin C	3.3	.0023	.004	1.3	.4633	.493	1.4	.4294	.803
Eotaxin	**2.4**	.0021	.004	1.3	.0937	.155	1.1	.5669	.813
GM-CSF	**2.5**	.0023	.004	1.3	.3937	.433	2.2	.0686	.479
Gro-a/CXCL1	**2.2**	.0003	.001	1.6	.0058	.030	1.2	.3670	.803
HMGB-1	**3.0**	.0008	.002	1.8	.0064	.030	1.1	.7594	.878
I-TAC/CXCL-11	2.4	.0062	.009	1.4	.0484	.114	1.2	.4836	.803
IFN-γ	**2.4**	.0050	.008	1.3	.0942	.155	1.1	.7037	.878
IL-10	**4.4**	.0001	.000	1.4	.0650	.134	1.1	.6757	.878
IL-12	**2.9**	.0005	.001	1.3	.1875	.229	1.2	.5176	.803
IL-13	1.9	.0774	.088	1.5	.0884	.155	1.1	.7981	.878
IL-15	1.8	.0183	.023	1.1	.5204	.537	1.4	.1785	.793
IL-16	**2.8**	.0000	.000	1.4	.0361	.097	−1.0	.9394	.969
IL-18	1.4	.0231	.028	1.3	.0290	.095	1.3	.0870	.479
IL-1α	**2.7**	.0006	.002	1.2	.5498	.550	2.1	.0721	.479
IL-1β	3.0	.0034	.006	1.9	.0193	.071	1.2	.5224	.803
IL-2	2.0	.0144	.020	1.3	.1661	.211	1.2	.3397	.803
IL-33	1.8	.3545	.354	1.8	.1050	.156	1.2	.6707	.878
IL-4	**1.9**	.0033	.006	1.3	.0935	.155	−1.2	.2510	.793
IL-6	**4.5**	.0002	.001	4.2	.0000	.000	1.0	.9970	.997
IL-7	**3.0**	.0020	.004	1.4	.1122	.156	1.2	.4243	.803
IL-8	**5.6**	.0003	.001	5.4	.0000	.000	2.3	.0331	.479
IP-10	1.4	.0881	.097	1.5	.1180	.156	1.2	.4149	.803
Lactoferrin	**2.8**	.0000	.000	1.4	.0318	.095	1.6	.3441	.803
M-CSF	**2.8**	.0000	.000	1.3	.3386	.385	1.4	.2240	.793
MCP-1	2.4	.0000	.000	2.0	.0027	.022	1.3	.2644	.793
MIG	1.4	.2105	.217	1.4	.2975	.351	−1.1	.7902	.878
MIP-1α	**2.9**	.0000	.000	1.9	.0075	.031	1.5	.0756	.479
MIP-1β	**5.1**	.0000	.000	2.6	.0016	.018	1.4	.0860	.479
MIP-3α	1.9	.1479	.157	1.9	.0046	.030	−1.3	.2384	.793
RANTES	1.9	.0326	.038	1.4	.1172	.156	−1.2	.5353	.803
TGF-β	2.5	.0167	.022	1.3	.1060	.156	−1.0	.9385	.969
TNF-α	2.4	.0109	.016	1.3	.0592	.130	1.1	.7260	.878

FC, fold change. *P*, nominal *P*-values; *q*, false discovery rate adjusted *P*-values.
Negative values represent a decrease in patients who delivered ≤32 wk compared to those who delivered >32 wk. Bolded fold changes are for proteins for which the differential abundance remains significant if only the patients with no intra-amniotic inflammation are used in the analysis.

Patients with a sonographic short cervix diagnosed between 22 2/7 and 26 1/7 weeks of gestation who delivered ≤32 weeks of gestation had higher correlation among 16 amniotic fluid inflammatory-related proteins than those who delivered >32 weeks of gestation (35 perturbed correlations). RANTES, IL-8, IL-6, MCP-1, and MIP-1β had the highest number of perturbed correlations (degree of 11, 8, 8, 8, and 6, respectively), whereas IL-6 and IL-8 demonstrated the highest magnitude of change in concentration (Figure 4B).

Among patients with a sonographic short cervix diagnosed between 26 2/7 and 31 5/7 weeks of gestation, those who delivered ≤32 weeks of gestation had similar concentrations and concentration correlations of amniotic fluid cytokines to those who delivered >32 weeks of gestation (only 5 perturbed correlations).

4 | DISCUSSION

4.1 | Principal findings of the study

(i) The earlier the short cervix was diagnosed, the higher the rate of preterm delivery ≤32 weeks of gestation, reaching 50% in the

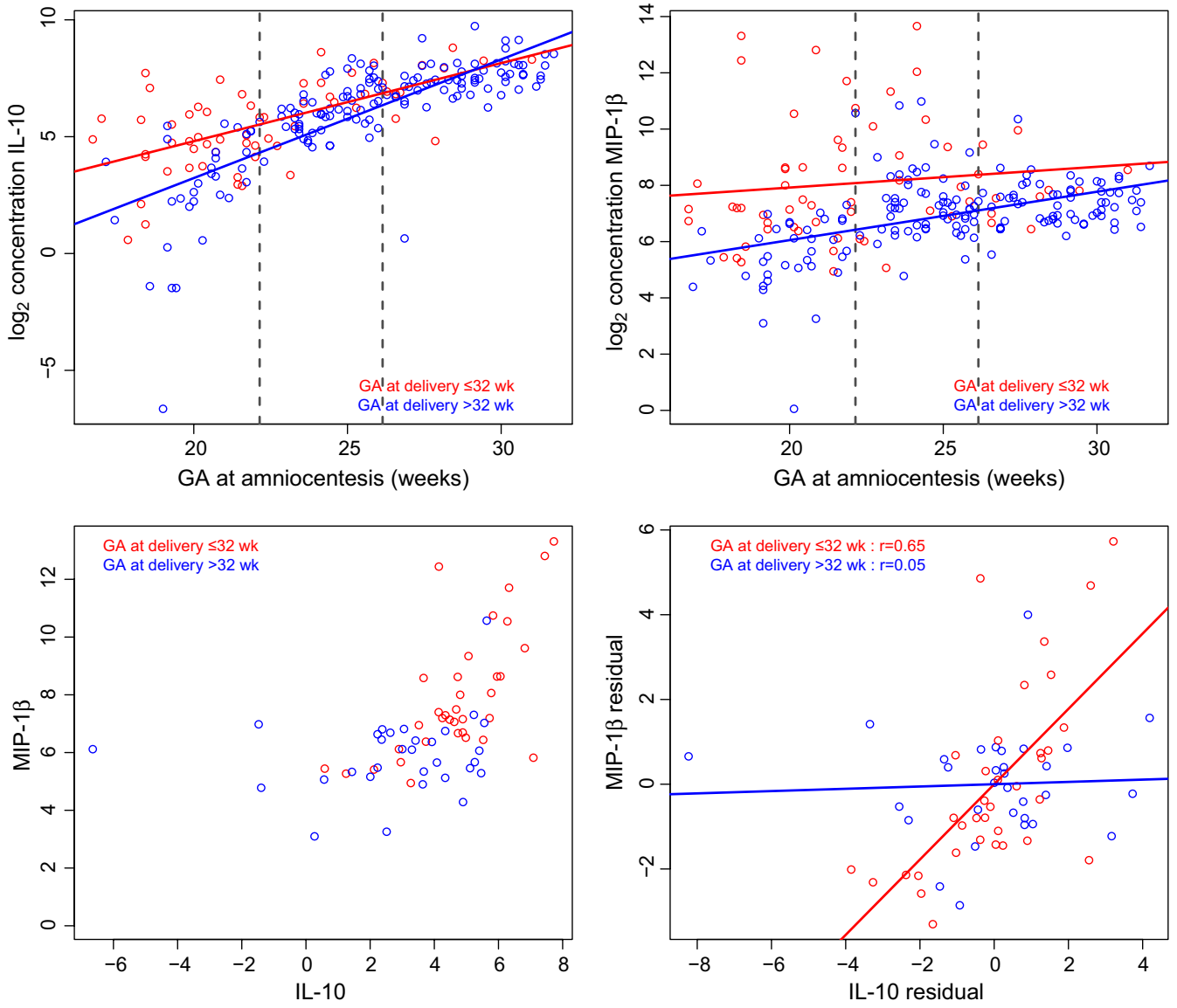

FIGURE 3 Differential correlation analyses. The figure shows $\log_2$ concentration (pg/mL) of IL-10 (upper left panel) and MIP-1β (upper right panel) as a function of gestational age at amniocentesis in patients who had a sonographic short cervix and who delivered ≤32 weeks (red) and those >32 weeks (blue) of gestation. Dashed lines denote the two tertiles of the distribution of gestational age at amniocentesis designating patients into three intervals, and the scatterplot of the $\log_2$ concentration of IL-10 and MIP-1β in the first interval is shown (bottom left panel). A linear model was fit to the $\log_2$ concentration of each analyte as a function of gestational age in each group within each gestational-age interval, and residuals were used to compute partial correlations between analytes (bottom right panel). The partial correlation between the two analytes was significantly increased in patients who delivered ≤32 weeks than those >32 weeks of gestation

interval of 16 5/7 to 22 1/7 weeks. (ii) In patients with a short cervix diagnosed at 16 5/7 to 22 1/7 weeks, the concentration of most inflammatory-related proteins increases as the cervix shortens in both delivery groups, yet the rate of increase in MIP-1α, MCP-1, and IL-6 is higher in women who delivered ≤32 weeks compared to those who delivered >32 weeks of gestation. (iii) The concentration of most inflammatory-related proteins studied herein was higher in patients who delivered ≤32 weeks compared to those who delivered >32 weeks of gestation, yet the magnitude of the difference decreases with increasing gestational age at amniocentesis and diagnosis of a short sonographic cervix. (iv) The concentration correlation of several pairs of inflammatory-related proteins was overall higher in patients who delivered ≤32 weeks compared to those who delivered >32 weeks of gestation.

4.2 | Network of protein correlation perturbations related to preterm delivery in patients diagnosed with a short cervix between 16 and 22 weeks of gestation

Patients with a sonographic short cervix and a subsequent early preterm delivery had more coordinated amniotic fluid cytokine concentrations than those who had late preterm deliveries. IL-8 and MIP-1β

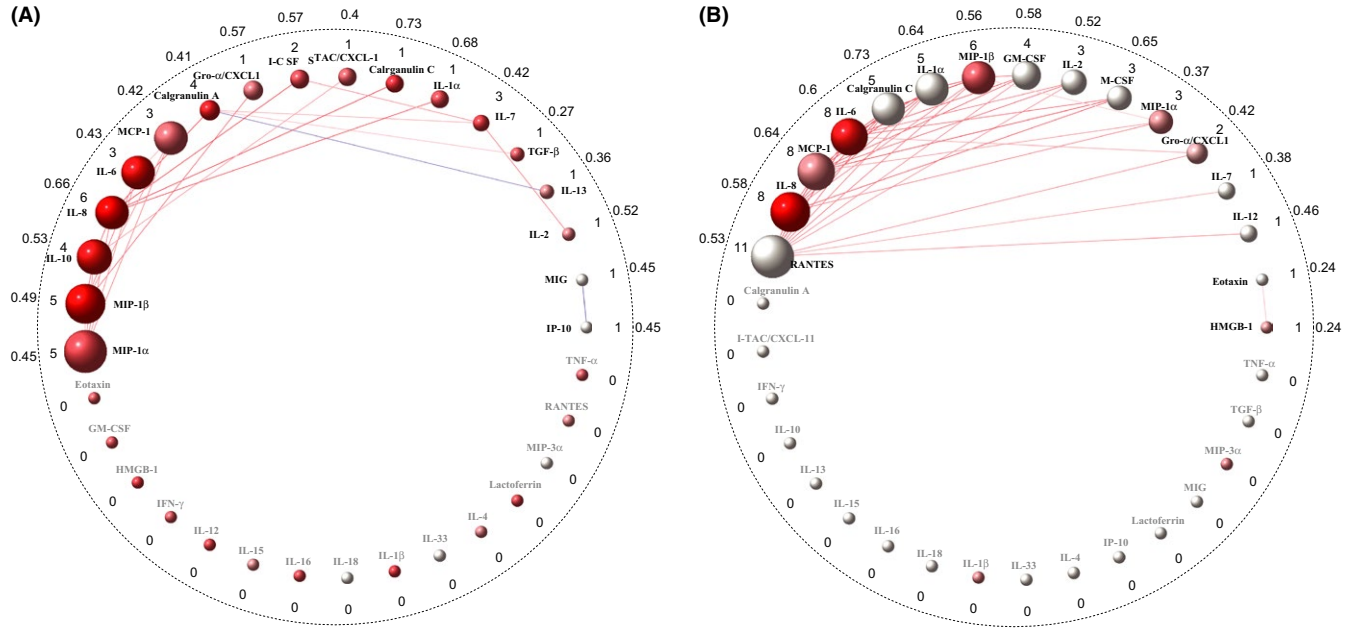

FIGURE 4 Network of perturbed cytokine concentration correlations between patients who had a sonographic short cervix and who delivered ≤32 weeks of gestation and those who delivered >32 weeks in the first (left panel, A) and second (right panel, B) gestational-age intervals. Each node (sphere) represents one of the 33 analytes, with a link (line) between two nodes representing a significantly perturbed correlation. The node color represents the direction of concentration change between delivery ≤32 weeks compared to delivery >32 weeks (red=increased, blue=decreased, and white=no change), while the color intensity is proportional to the magnitude of concentration change. The color of links gives the direction of correlation change (red=increased correlation and blue=decreased correlation), while the color intensity is proportional to the magnitude of correlation change. The numbers inside/outside of the dotted black circle represent the node degree/average of the absolute difference in correlations

are the key mediators derived from both differential correlation and concentration analysis. Interestingly, MIP-1α is the top-ranked protein obtained from the differential correlation approach despite its small magnitude of change in concentration (fold change=1.56; *P*=.00016).

The two top-ranked proteins, IL-8 and MIP-1β, obtained from the analysis of both correlation network and differential concentration have been implicated in the pathogenesis of intra-amniotic inflammation/infection in preterm labor,[171–173] preterm pre-labor rupture of the membranes,[173] and clinical chorioamnionitis.[174] Both IL-8 (neutrophil attractant/activating peptide-1) and MIP-1β (CCL4) are chemotactic cytokines that activate neutrophils and other human granulocytes in response to inflammation or infection.[175,176] These chemokines are mainly produced by macrophages, mononuclear cells, and lymphocytes.[176,177] Previous studies demonstrated that amniotic fluid concentrations of IL-8 and MIP-1β increase in patients with preterm labor who had intra-amniotic inflammation/infection and clinical chorioamnionitis at term with proven intra-amniotic infection, and these chemokines can predict the likelihood of spontaneous preterm delivery.[171–174,178–184] In addition, both chemokines are significantly higher in patients with a mid-trimester short cervix who delivered <34 weeks than in those who delivered ≥34 weeks of gestation,[152,156] and their concentrations correlated with cervical length.[61]

By contrast, MIP-1α (or CCL-3) is the top-ranked protein identified from correlation analysis but not differential concentration analysis. Interestingly, this cytokine has been implicated in the pathogenesis of intra-amniotic inflammation/infection in patients with preterm delivery and clinical chorioamnionitis.[174] MIP-1α is produced by macrophages[185] and activates human granulocytes in response to inflammation and infection.[186–194] These observations suggest that proteins ranked by network correlation analysis are of value and have biological plausibility in the pathogenesis of intra-amniotic inflammation/infection in patients with a sonographic short cervix, despite having a small magnitude of change from a simple comparison of mean concentrations.

4.3 | Network of protein correlation perturbations related to preterm delivery in patients diagnosed between 22 2/7 and 26 1/7 weeks of gestation

The top-ranked proteins obtained from differential concentration analysis (IL-8, IL-6, and MIP-1β) are similar to those derived from correlation analysis. Moreover, IL-8 and MIP-1β are also the top-ranked proteins in the network of perturbed inflammatory-related protein concentrations observed between 16 5/7 and 22 1/7 weeks of gestation. RANTES and MCP-1, two additional proteins, are key mediators during this interval derived from correlation analysis. MCP-1 is significantly lower in patients who delivered ≤32 weeks than in those who delivered >32 weeks when a sonographic short cervix was diagnosed between 22 2/7 and 26 1/7 weeks of gestation (fold change=1.97, *P*=.022). The amniotic fluid concentrations of RANTES were not significantly different (*P*=.156). Interestingly, RANTES had no perturbed correlations of inflammatory-related protein concentration in women with a short cervix diagnosed between 16 5/7 and 22 1/7 weeks, but

it had the highest number of perturbed correlations in women with a short cervix diagnosed between 22.2 and 26.1 weeks of gestation (n=11), suggesting that gestational age is a factor that may modulate the nature of the cytokine response.

It is well established that amniotic fluid IL-8, IL-6, and MIP-1β are involved in the pathogenesis of intra-amniotic inflammation/infection in preterm delivery,[96,101,108,171–173,179,182,183,195–197] preterm pre-labor rupture of the membranes,[111,180,181,198–201] clinical chorioamnionitis,[174] and cervical insufficiency.[202–204] In patients with a mid-trimester short cervix, the concentrations of these cytokines/chemokines in the amniotic cavity are correlated with sonographic cervical length[61] and gestational age at delivery.[152] Additionally, all three cytokines are included in the amniotic fluid inflammatory score model, which can predict gestational age at delivery.[152]

In the current study, the information obtained from network analysis is of value, because there is evidence that suggests that amniotic fluid MCP-1 (also known as CCL-2) and RANTES play a role in intra-amniotic inflammation/infection in patients with preterm labor,[132,156,205,206] preterm pre-labor rupture of the membranes,[132] a sonographic short cervix,[61,156] spontaneous labor at term,[207,208] and histological chorioamnionitis.[205]

Keeler et al.[156] reported that patients with a mid-trimester sonographic short cervix demonstrated that an elevation of amniotic fluid MCP-1 concentration (>1320 pg/ml) had a 69% sensitivity, 83% specificity, 36% positive predictive value, and 87% negative predictive value for preterm birth within one week of amniocentesis. Moreover, among the other 25 cytokines, MCP-1 was the most predictive cytokine of spontaneous preterm delivery in patients with a mid-trimester short cervix.[156] MCP-1 is capable of recruiting macrophages and other leukocytes into sites of inflammation.[209–212] Similarly, RANTES is a pro-inflammatory chemokine secreted from T cells, which recruit monocytes, lymphocytes, basophils, and eosinophils in the host response to inflammation/infection.[213–217]

Collectively, patients with a sonographic short cervix who subsequently delivered early had a more coordinated cytokine network than those who had a late preterm delivery. The ranked proteins derived from correlation analysis are informative, have known biological properties relevant to parturition, and can be potentially useful in the future for the development of a biomarker pipeline to identify patients with a sonographic short cervix who subsequently deliver early.

4.4 | Network of protein correlation perturbations related to preterm delivery in patients diagnosed between 26 2/7 and 31 5/7 weeks of gestation

In this interval, the number of perturbed correlations is small (n=5), and none of the inflammatory-related protein concentrations differed between patients who delivered early or late preterm. However, we found a connection between the antimicrobial peptide lactoferrin and T-cell-associated cytokines IL-16 and IL-13. Elevated IL-16 and lactoferrin amniotic fluid concentrations in women with preterm labor and intra-amniotic infection/inflammation were previously reported by our group and other investigators.[218–220]

4.5 | Gestational age at the diagnosis of a sonographic short cervix and the behavior of the cytokine network

It is known that the earlier the diagnosis of a short cervix is made, the greater the degree of intra-amniotic inflammation and the earlier the delivery.[17–21,23–27,30,61,62] Additionally, Moroz and Simhan[221] reported a significant association between cervical shortening and maternal systemic inflammation (determined by C-reactive protein; r^2=.44, *P*=.001) in women with a sonographic short cervix (≤25 mm) diagnosed between 21 and 28 weeks of gestation. However, this association was not observed in patients without a short cervix.[221] We present herein for the first time that the earlier the occurrence of a short cervix, the more orchestrated is the inflammatory response associated with it in women who subsequently deliver prior to 32 weeks of gestation. These findings suggest that the risk of early spontaneous preterm delivery in asymptomatic women with a sonographic short cervical length changes as a function of gestational age at diagnosis and is associated with the magnitude and character of the intra-amniotic inflammatory processes characterized by network analysis.

4.6 | Importance of the study of protein networks in the "great obstetrical syndromes"

The improved understanding of the immune response and its soluble mediators (cytokines) coupled with the application of molecular biology has led to substantial gains in the description of the behavior of multiple inflammatory-related proteins in health and disease. The initial emphasis in the study of inflammatory molecules was on the individual changes in the concentration and expression of these molecules. This is understandable as discoveries of virtually every cytokine occur one at a time. Now, decades later, a more comprehensive, detailed map of the protein inflammatory network is available, as well as an improved understanding of the nature of the protein-protein interaction and biological function of these molecules. A major advance in the understanding of the biology of the inflammatory response indicates that cytokines are organized in complex, redundant networks; further, there is the realization that global analysis is required to improve insights into the biology and that this approach is superior to a simple catalogue of the changes in the concentrations of individual cytokines. Indeed, the cellular response during inflammation represents the interaction between the input derived from several cytokines that activate different receptors on the cell surface, leading to the generation of several intracellular processes. In addition, each cytokine can attach to different receptors on the cell surface, and this has implications on the type of cellular response, cellular activation, number, and profile of cytokine receptors expressed on the cell membrane.[222–224] Thus, to understand the effect of different cytokines, an integrative model of cytokine activity is needed. There are several reports documenting that information derived from correlation network analysis can (i) improve classification of disease,[61,152,156] (ii) chart the cytokine interaction in terms of its effect on specific inflammatory cell types (B and T cells, macrophages) in single and

multiple cell interactions,[223,224] (iii) identify new interfaces between signaling molecules in uni- or multiscale models that incorporate several cell populations,[225,226] (iv) be the basis of hypothesis-generating studies,[227] and (v) identify potential therapeutic targets.[228,229] This has been the case with breast cancer[158] and chronic fatigue syndrome.[230] The description of the cytokine network presented herein is novel and may assist in identifying the key cytokines involved in the amniotic fluid inflammatory response at different gestational-age windows that, in turn, may have diagnostic, prognostic, and therapeutic implications.

4.7 | Strengths and limitations of the study

A major strength of the study is that it is the first to investigate the inflammatory protein network in asymptomatic women at risk for preterm birth because of a sonographic short cervix. Second, the study focuses on amniotic fluid, the biological fluid in which major changes in cytokine activity are observed in the context of a short cervix. Third, the characterization of the protein inflammatory network was not restricted to cytokines, but also included antimicrobial proteins, such as lactoferrin, that have been implicated in premature labor. Fourth, the results of the cytokine network were unknown at the time of patient management and, therefore, could not have biased the clinicians and investigators. As with any other studies, replication of the findings is desirable.

5 | CONCLUSIONS

We have characterized the amniotic fluid pro-inflammatory protein network in women with an asymptomatic short cervix who are at risk for early preterm birth (<32 weeks of gestation) for the first time. Importantly, the shorter the cervical length, the greater are the perturbations in the amniotic fluid inflammatory network and the higher the risk of early preterm delivery. Characterization of the amniotic fluid inflammatory network has implications for the taxonomy of disease for patients with a short cervix and identification of those at risk for early premature birth.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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