Significance of Cervicovaginal Inflammatory Cytokines During Spontaneous Onset of First Stage of Labor: A Data-Driven Approach from a Pseudo-Longitudinal Study

Daniel Eduardo Sandoval-Colin[1], Myrna Souraye Godines-Enriquez[2], Aurora Espejel-Núñez[2], Jorge Beltrán-Montoya[2], Denisse Alejandra Picazo-Mendoza[1], Nidia Carolina Espinosa-Maldonado[3], Juan Carlos de la Cerda-Ángeles[4], Omar Yaxmehen Bello-Chavolla[5], Noemí Meraz-Cruz[1], Erika Chavira-Suárez[1], and Felipe Vadillo-Ortega[1]

[1]National Autonomous University of Mexico
[2]National Institute of Perinatology
[3]National Institute of Genomic Medicine
[4]Ministry of Health of Mexico
[5]Instituto Nacional de Geriatría

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Abstract

Objective: To evaluate the ability of cervicovaginal cytokines to describe and predict the inflammatory processes associated to spontaneous labor onset both, term and preterm. Design: Pseudo-longitudinal study. Setting: Two Ministry of Health-affiliated hospitals in Mexico City from 2018 to 2020. Population or Sample: Women with singleton pregnancies at different moments during spontaneous onset of first stage of labor between 12 and 41 weeks of gestation. Methods: Women were grouped in five stages going from the absence of uterine activity and cervical changes (Stage 0) to the regular uterine contractions with cervix dilation >3 cm (Stage 4 or established labor). Main Outcome Measures: Cervicovaginal cytokine concentrations between term and preterm labor, cytokine trajectories throughout spontaneous labor onset and predictive accuracy of IL-6 for spontaneous labor. Results: Of 144 women with spontaneous labor 96 delivered at term and 48 preterm, both groups displayed similar cytokine concentrations. We found positive correlations between pro-inflammatory cytokines and clinical manifestations of labor (study stages) using individual cytokines and score-based data by principal-component analysis (IFN-, TNF-α, IL-1β, IL-6) as dependent variables. The risk of delivery increased as IL-6 concentrations increased (HR 202.09, 95% CI 24.57-1662.49, P<0.001). IL-6 was a significant predictor for spontaneous labor within 12 days (AUC=0.785, 95% CI 0.693-0.877) regardless of gestational age. Conclusions: Cervicovaginal cytokines, particularly IL-6, reflect and predict the intrauterine inflammatory sequence associated to initial labor progression. This study provides a new insight into cervicovaginal inflammatory biomarkers usability for labor diagnosis and birth prediction. Keywords: Labor, cytokines, preterm birth, IL-6.

Introduction

The mechanisms of normal human parturition are still incompletely understood; however, they include a network of biological pathways, including endocrine and paracrine signaling, with participation of the innate immune response.[1–5] The inflammatory response associated to human labor is characterized by the highly compartmentalized secretion of cytokines and chemokines by resident and selectively recruited immune cells that infiltrate the reproductive tissues at the end of gestation and are enriched in the maternal-fetal interface.[3,6,7] Initial activation resulting in local secretion of inflammatory mediators under sterile conditions in normal spontaneous human labor is not completely characterized; however, it has been linked to activation

of inflammasomes in response to endogenous danger signals/alarmins/damage-associated molecular patterns (DAMPs).[9–11]

The current generation of biomarkers for risk evaluation of preterm birth (PTB) have limited clinical usefulness,[10] and accurate prediction of preterm labor (PTL) is difficult due to the multifactorial etiology of PTB as well as existence of various pathophysiological pathways.[11–13] Therefore, a single predictor of PTB may have variable utility in symptomatic women compared to asymptomatic women with or without PTL risk factors. It is surprising that despite the accepted concept of selective inflammation associated with spontaneous onset labor in human, local inflammatory mediators are not routinely used as clinical biomarkers for the diagnosis and prediction of labor. Longitudinal reconstruction of the inflammatory response during normal human labor has many limitations and here, we explore cervicovaginal fluid (CVF) cytokines as a proxy of changes occurring in the intrauterine/choriodecidual compartment during initial stage of human labor. We hypothesize that cervicovaginal inflammatory cytokines can be used as biomarkers to predict inflammatory intrauterine processes associated with the onset of labor.

Methods

This was a pseudo-longitudinal study (a variant of repeated cross-sectional study) conducted from January 2018 to January 2020 in two Ministry of Health-affiliated hospitals in Mexico City: a tertiary-level unit and a secondary-level unit. This study was approved by the pertinent Institutional Review Board (IRB; Register 2010/010/3117). Written informed consent was obtained from each patient prior to inclusion.

Women at different moments of spontaneous onset of the first stage of labor were included. Women with multiple pregnancy, chronic diseases, obstetric complications, and history of sexual intercourse or vaginal medication within the last 48 hours were excluded. Women with clinical or microbiological evidence of urinary tract infection or infectious vulvovaginitis, as well as those with clinical or laboratory evidence of premature rupture of membranes (PROM) and/or incomplete follow-up were eliminated.

Obstetrics and Gynecology specialists, previously standardized, evaluated the presence of clinical signs of spontaneous labor. Uterine activity was recorded with a tocodynamometer. Cervical changes and membrane integrity were assessed by vaginal examination. Gestational age was determined by the date of the last menstrual period. Clinical and demographic characteristics were obtained of all participants.

To represent initial changes associated to first phase of spontaneous labor, pregnant women between 12 and 41 weeks of gestation (WoG) were classified into one of the following five groups:

- **Stage 0** : Women with no clinical evidence of myometrial activity or cervical changes and intact fetal membranes. This stage represents the total absence of labor.
- **Stage 1** : Women with minimal sporadic uterine activity without cervical changes and intact fetal membranes. None of these women gave birth within posterior 10 weeks.
- **Stage 2** : Women with self-perceived uterine contractions with [?]1 contraction event in 30 minutes, cervical softening and dilation <2 cm, and intact fetal membranes. This stage is considered as the initial or latent phase of spontaneous labor. Only women who gave birth within posterior 24 hours after classification were included in this group.
- **Stage 3** : Women with painful uterine contractions [?]2 events in 30 minutes, cervical dilation 1-3 cm and intact fetal membranes. Prelude phase of active labor. Only women delivering within posterior 12 hours after classification were included in this group.
- **Stage 4** : Women with effective and regular uterine contractions (three painful events in 10 minutes), cervix dilation >3 cm and intact fetal membranes. This stage included women in active labor. Only women delivering within posterior 6 hours after classification were included in this group.

CVF samples were obtained by speculum examination. A Dacron tipped swab was placed into the posterior fornix and transferred to transporting buffer at 4^{o}C (0.05M Tris-base, 0.15M NaCl, 1% BSA, 0.1% Tween-20 and protease inhibitors). Samples were centrifuged at 2000 x g at 4^{o}C for 15 minutes and supernatant fluid was collected and stored at -80^{o}C.

Cytokines in the CVF were measured with the Milliplex Human Cytokine/Chemokine Magnetic Bead Panel and the MAGPIX Reader (Millipore, Burlington, MA, USA); including: (1) chemokines: CXCL8 (IL-8 or interleukin-8); (2) pro-inflammatory: interleukin-2 (IL-2), interleukin-12p70 (IL-12p70), interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6); (3) anti-inflammatory: interleukin-10 (IL-10), interleukin-4 (IL-4) and interleukin-1 receptor antagonist (IL-1RA).

Statistical analysis

We compared groups according to study stages and sub-categories of preterm birth using Welch's One-way Analysis of Variance (ANOVA) or Kruskal-Wallis rank-sum test according to variable distribution, and Mann-Whitney U for term and preterm delivery groups. Categorical variables and frequencies were compared between groups using chi-squared tests. A p-value <0.05 was established as the threshold for statistical significance. For details on variable transformation and imputation for benefit in models' assumptions, see Supporting Information.

To characterize a cytokine profile in CVF that describes clinical manifestations in labor stages, a multiple principal-component analysis (PCA) was used using cytokine concentrations centered with mean 0 and standard deviation of 1. To choose the components to retain we used the scree plot criterion.

We selected cytokines which better described stages and principal component scores were extracted to develop explanatory models (linear mixed-effects models) to predict pro-inflammatory signaling associated with the onset of labor using stages as independent variable and gestational age (sample collection week) as the moderating variable.

Model diagnostics were conducted using R^2 and minimization of the Bayesian Information Criterion (BIC); multicollinearity was assessed using tolerance and Variance Inflation Factor (VIF). Predictors were subjected to homoscedasticity and linearity tests; model diagnostics were performed by assessing normality of the residuals. Model parameters were expressed using β coefficients and 95% CI.

To test IL-6 diagnostic performance for identification of spontaneous labor, we calculated an optimal cut-point using maximization of Youden's J index, and estimated the respective sensitivity, specificity, predictive values, and likelihood ratios using the*OptimalCutpoints* R package. In addition, we evaluated their time-varying diagnostic performance using time-dependent ROC curves applying the Kaplan-Meier estimator at different time-points using the*timeROC* R package.

Intervals between sampling and delivery were calculated and the estimated cut-point was evaluated by Kaplan–Meier analysis and log-rank test using the *survminer* R package. Cox regression analysis was used to assess whether IL-6 was associated with risk of delivery at any gestational age. Schoenfeld residuals were used to test the proportional hazards assumption. The predictors were tested on homoscedasticity and linearity assumptions. Finally, a post-estimation simulation of the Cox models was performed to evaluate adjusted hazard ratio estimates across IL-6 values using the *simPH* R package. All statistical analyses were performed using R statistical software (Version 4.0.2).

Results

One hundred and forty-four women with spontaneous labor were included (Figure S1), of whom ninety-six delivered at term and forty-eight preterm. Clinical variables are described in **Table 1** and**Table 2** . Subcategories of preterm birth, based on gestational age, were similar with respect to age, multiparity and history of PTB, with a mean gestational week of 35.20±1.10, 31.0±0.24, and 22.10±0.52 for late preterm, very preterm, and extremely preterm delivery, respectively. Likewise, we did not identify differences in stages of labor according to term and preterm delivery groups (Table S1 and S2).

Cytokine concentrations were similar between women who delivered at term and preterm (**Figure 1A;** Table S3). However, when we stratified by subcategories of preterm birth, we identified that women who delivered extremely preterm had significantly higher concentration of pro-inflammatory cytokine IFN-γ compared with

those with late preterm, but interestingly, they were not different with those who delivered at term (**Figure 1B;** Table S3).

Since we did not identify differences of cytokine concentrations in CVF between women who developed term and preterm labor, we analyzed them as a single group for every stage. We compared cytokine concentrations between stages of labor (**Figure 1C;** Table S4), and identified that pro-inflammatory cytokines, excluding IL-2, and the anti-inflammatory cytokine IL-10, increased in correlation with the sequence of spontaneous labor. The main concentration changes occurred with respect to Stage 0 and the final stages (Stage 3 and 4). In particular, IL-6 concentration showed a clear peak at Stage 4. We did not identify a clear trend in the concentrations of chemotactic cytokine IL-8, the pro-inflammatory cytokine IL-2, and the anti-inflammatory cytokines IL-4 and IL-1RA.

To test the hypothesis that CVF cytokines may characterize the initial clinical manifestations of spontaneous labor regardless of gestational age, we performed PCA of cytokines concentrations that showed a rising trend across labor stages. We identified a pro-inflammatory profile defined by IFN-γ, TNF-α, IL-1β and IL-6, and we extracted two principal components, explaining 79.9% of the total variance (**Figure 2A** ; Table S5). Principal component 1 explained 56.3% of the total variance and was mainly composed by, in descending order of loading: TNF-α, IFN-, IL-1β and IL-6 (Table S6). Principal component 2 accounted for 23.6% of the total variance and was mainly composed by IL-6, IL-1β, TNF-α and IFN-γ. We compared labor stages with extracted scores and identified that PC1 plus PC2 scores better distinguish each stage (**Figure 2B**) than an independent pro-inflammatory cytokine.

Linear mixed-effects models were fitted by age, multiparity, and number of previous PTBs to examine the effect of labor stages and their interaction with gestational age (week of sample collection) on pro-inflammatory cytokine concentrations. We hypothesized that throughout pregnancy and during stages of labor, women who delivered preterm might have had higher cytokine concentrations levels than those who delivered at term; therefore, we considered subcategories of delivery as a random effect within the model.

We identified positive linear effects of labor stages on concentrations of IFN-γ, TNF-α and IL-1β (**Figure 3A-C;** Table S7), and positive nonlinear effects on concentrations of IL-6 (**Figure 3D;** Table S7). However, the variance explained by random effect was null for TNF-α, IL-1β and IL-6 model, and 27.21% for IFN-γ model (Table S7).

When we assessed the moderating variable effect, we found no correlation between gestational age (sample collection week) and IFN-γ, TNF-α and IL-1β concentrations (**Figure 3F-H**). In contrast to IL-6 concentrations we found a nonlinear relationship with gestational age (**Figure 3I**), in addition to statistically significant stage*sample collection interaction (Table S7), suggesting there is an estimated value for each stage of the relationship between sample collection (gestational age) and IL-6 concentration. Consequently, the relationship between labor stages and IL-6 concentration could be independent from gestational age.

Furthermore, we fitted a model with the pro-inflammatory profile, and found a nonlinear relationship between labor stages and principal-component scores (**Figure 3E** ; Table S7). As with IL-6 model, we found that this correlation is independent from gestational age (**Figure 3J** ; Table S7). In addition, we identified that mean scores of term, late preterm and very preterm groups were similar, but far lower compared to extremely preterm group (variance explained by random effect was 86.90%; Table S7).

We previously identified that IL-6 was significantly correlated with changes in labor stages, showing one of the highest average effects on cytokine concentration for a one- unit increase in stages of labor. Therefore, we calculated an optimal cut-point to test the diagnostic performance of IL-6 for the identification of spontaneous labor (Stage 4, effective and regular uterine contractions, and dilation >3 cm) regardless of gestational age (**Table 3**).

The identified cutoff was 34.60 pg/mL and the area under the ROC curve (AUC) was 0.862 (95% CI 0.758-0.967). It is worth noting the negative predictive value: 97.93% (95% CI 92.11-98.71), which indicates that 97.93% of pregnant women are not in established labor with an IL-6 concentration <34.60 pg/mL (truly

negative screening test).

To corroborate the assumptions for using an IL-6 cut-point, we assumed homogeneity of outcome on either side of the cut-point (a discontinuous relationship) when X axis is a time variable.[14]Our time variable is the stage, and the discontinuous relationship we wish to achieve is with respect to Stage 4 and all other stages, thus confirming the validity of dichotomization in this setting (**Figure 1C**).

Subsequently, we calculated sampling-to-delivery intervals (censoring those exceeding 120 days) to estimate the probability that a pregnant woman did not give birth according to the estimated cut-point (log-rank test: P =0.0011, **Figure 4A**), and fitted a Cox proportional-hazards model by age, multiparity, and number of previous PTBs to examine the association between IL-6 concentrations and the development of delivery from any gestational age. A nonlinear effect was identified indicating a relationship between IL-6 concentrations and increased risk of delivery (HR 202.09, 95% CI 24.57-1662.49, P <0.001; HR [second-degree] 109.74, 95% IC 14.12-853.07,P <0.001; Table S8). Simulated adjusted hazard ratio estimates showed that hazard ratios exponentially increase in according to the identified cut-point (log scale: 3.54 pg/mL, **Figure 4B**).

Once the time-dependent adjustment has been applied and we ensured that all women included for analysis developed spontaneous labor leading to delivery, we evaluated the time-varying diagnostic performance of IL-6 with time-dependent ROC curves. Event status is observed at each time point yielding different sensitivity and specificity values throughout follow-up (intervals between sampling and delivery). At each time point$t$, each individual is classified as a case or control. A case is defined as any individual experiencing the event (laboring woman) between baseline t =0 and time t and a control as an individual who remains event-free (non-laboring woman) at time t .

Using these definitions, we identified at 12 days an AUC of 0.785 (95%CI 0.693-0.877, **Table 4**), where the diagnostic/predictive performance decreases as time increases. As in previous analyses, the strengths of this marker (at 12 days) are the specificity (81.0%, SE±3.94) and the negative predictive value (84.38%, SE±3.72).

Discussion

Main Findings

We used a standardized clinical-based reconstruction of the first stage of spontaneous labor to explore CVF cytokine expression as a proxy of inflammatory processes occurring in the intrauterine/choriodecidual compartment. Here, we show that the progression of clinical manifestations is correlated with the activation of the inflammatory response during labor, since there is a positive correlation between CVF pro-inflammatory cytokine levels, specifically IL-1β and IL-6, and initial clinical manifestations of labor. These results support the hypothesis that the cervicovaginal milieu can reflect and predict the inflammatory intrauterine processes associated to human labor and can be used to explore potential biomarkers for term and preterm labor;[15–17,10] in addition to being a minimally invasive technique.

Interpretation

Labor-associated inflammasome activation in the intrauterine compartment, mainly in the choriodecidual interphase, might correlates with the progressive concentration increase of IL-1β in the CVF.[9,18] This cytokine has been proposed as a key signal for the amplification cascade leading to the production of secondary labor mediators such as PGs[16, 19–21] and, in conjunction with IL-6, inducing the expression of oxytocin receptors on myometrial cells[22] and the secretion and activation of MMPs in the chorioamniotic membranes and cervix.[23]An effort to discard the presence of cervicovaginal or intrauterine infection in participating women, allowed us to propose that concentrations of danger signals or alarmins and cytokines are associated with sterile inflammation.[24–28]Interestingly, cytokine concentrations were similar between term deliveries and preterm deliveries, highlighting the concept of a common labor-associated inflammatory response in both conditions.

A multitude of maternal biomarkers, including cytokines, have been shown to be associated with the occur-

rence of spontaneous preterm birth (sPTB), but their predictive accuracy has only moderately good, thus precluding their use as a screening test in clinical practice.[29,30] The great heterogeneity in inclusion criteria and gestational age at the time of evaluation represent other peculiar limitations to most studies.[31] In this setting, it has been difficult to quantify the strength of the association between a given marker and sPTB and the diagnostic performance of that marker in identifying women at risk of childbirth. We explored the possibility of using CVF cytokines or a combination of them to improve our knowledge and understanding between the inflammatory response associated with childbirth and the clinical manifestations of childbirth. An initial approach resulted in the pro-inflammatory profile extracted by PCA, pointing out the importance of multiple biomarker modeling for optimal predictive efficacy and the coherence of the statistical results with the proposed biological phenomenon.[32]

IL-6 by itself has proved to be a good marker to diagnose and predict spontaneous labor regardless of gestational age, highlighting the importance of being an independent predictor. IL-6 is one of the most studied biomarkers in sPTB and preterm premature rupture of membranes (pPROM),[33,34] however, multiple cutoffs have been proposed for both clinical entities.[29,30] Therefore, cutoffs for normal and abnormal cytokines in disease states need to be better established to support and distinguish diagnoses, as well as estimate prognoses more accurately. Evidence that IL-6 is a helpful marker to identify spontaneous labor was found and must be followed by a clinical trial evaluating it as a diagnostic tool for characterization of labor progression and furthermore, to identify women at risk of delivering in a limited window of time. The characterization of IL-6 reference values can be better accomplished with an easily accessible compartment such as CVF.

Strengths and Limitations

This study explored CVF cytokine trajectories throughout spontaneous labor onset and considers symptom status, sub-categories of delivery and time-dependent properties biomarkers. In addition, this study attempts to address gestational age heterogeneity and suggests a pragmatic way that could make diagnosis and prediction of spontaneous labor easier with greater accuracy and at a lower cost. Timely detection of spontaneous labor is fundamental to identify those mothers at higher risk of delivering and plan appropriate management. Conversely, predicting spontaneous delivery to identify women at low risk would avoid the use of unnecessary and sometimes costly interventions.[31]

Amongst the limitations to be acknowledged, the research design limits the ability to establish real temporal relationships, which might require further confirmation in a longitudinal setting following the same women.

A common characteristic of cytokine data sets is undetectable values or when concentrations exceed a certain threshold on the top of the measurement scale, and thus the issue of "censored data" needs to be addressed, which generally make statistical analysis challenging. The literature recommends different methods to deal with censored data.[35] We used simple substitution for IL-1Ra (the only cytokine with censored values) due to the large proportion of censored values. However, simple substitution is not advisable as it may lead to strongly biased results.[36] To overcome this, we performed multiple imputation using chained equations to account for variability of missing data; we performed a sensitivity analysis of complete-cases versus multiple imputed data and observed no significant results discrepancies.

Conclusion

The regulation of cytokine expression in reproductive tissues is closely related to both the maintenance of pregnancy and the process of labor induction. Labor is unlikely to occur spontaneously in patients with low cytokine levels, again suggesting that inflammation is an essential component in the initiation of labor. We demonstrated how inflammatory cervicovaginal cytokines can reflect and predict the inflammatory "common pathway" that leads to labor and propose that these biomarkers (particularly IL-6) could be used in clinical practice for diagnosis and prediction of spontaneous labor. Thus, a better understanding of cytokine dynamics during pregnancy and their connection with labor onset will help in the clinical assessment of prematurity, the most important challenge to modern obstetrics.

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Disclosure of interests

The authors have no conflicts of interests or potential conflicts of interest relevant to this article to disclose.

Contribution to authorship

FVO developed formulation of overarching research goals and aims, verified the overall replication/reproducibility of results/experiments and other research outputs, and acquired financial support for the project leading to this publication. MSGE and FVO were responsible for the research activity planning and execution, including mentorship external to the core team. MSGE, FVO, JBM, NCEM, JCCA, NMC and ECS provided study materials, reagents and contributed to transportation and manage of patient's samples. DESC, MSGE and AEN contributed to designing of the study. DESC, DAPM and FVO prepared the published work, specifically writing the initial draft. DESC managed activities to scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later reuse. OYBC and DESC applicated statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data. OYBC contributed to critical review and revision of the published work. All authors contributed to data collection. All of them approved the final version of this manuscript as submitted, and all authors agree to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity are appropriately resolved. The corresponding author (FVO) attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

Details of Ethics Approval

The study was approved by our local Ethics Review Board (Ministry of Health, Mexico): Register 2010/010/3117, 9 August 2017.

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References

1. Li T, Yi T, Zhao J, Zhao X, He X. Combined proinflammatory biomarkers have better predictive value for term labor than single markers. Med Sci Monit 2019;25:4513–20. Doi: 10.12659/MSM.917298

2. Unal ER, Cierny JT, Roedner C, Newman R, Goetzl L. Maternal inflammation in spontaneous term labor. Am J Obstet Gynecol 2011;204:223.e1-223.e5. Doi: 10.1016/j.ajog.2011.01.002

3. Gomez-Lopez N, Vega-Sanchez R, Castillo- Castrejon M, Romero R, Cubeiro-Arreola K, Vadillo-Ortega F. Evidence for a Role for the Adaptive Immune Response in Human Term Parturition. Am J Reprod Immunol 2013;69:212–30. Doi: 10.1111/aji.12074

4. Gomez-Lopez N, StLouis D, Lehr MA, Sanchez-Rodriguez EN, Arenas-Hernandez M. Immune cells in term and preterm labor. Cell Mol Immunol 2014;11:571–81. Doi: 10.1038/cmi.2014.46

5. Menon R, Bonney EA, Condon J, Mesiano S, Taylor RN. Novel concepts on pregnancy clocks and alarms: Redundancy and synergy in human parturition. Hum Reprod Update 2016;22:535–60. Doi: 10.1093/humupd/dmw022

6. Young A, Thomson AJ, Ledingham M, Jordan F, Greer IA, Norman JE. Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term. Biol Reprod 2002;66:445–449. Doi: 10.1095/biolreprod66.2.445

7. Osman I, Young A, Ledingham MA, et al. Leukocyte density and pro-inflammatory cytokine expression in human fetal membranes, decidua, cervix and myometrium before and during labour at term. Mol Hum Reprod 2003;9:41–45. Doi: 10.1093/molehr/gag001

9. Gomez-Lopez N, Motomura K, Miller D, Garcia-Flores V, Galaz J, Romero R. Inflammasomes: Their Role in Normal and Complicated Pregnancies. J Immunol 2019;203(11):2757-2769. Doi: 10.4049/jimmunol.1900901

10. Motomura K, Romero R, García-Flores V, et al. The alarmin interleukin-1α causes preterm birth through the NLRP3 inflammasome. Mol Hum Reprod 2020;26(9):712-726. Doi: 10.1093/molehr/gaaa054

11. Faro J, Romero R, Schwenkel G, et al. Intra-amniotic inflammation induces preterm birth by activating the NLRP3 inflammasome. Biol Reprod 2019;100(5):1290-1305. Doi: 10.1093/biolre/ioy261

12. Combs CA, Gravett M, Garite TJ, et al. Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. Am J Obstet Gynecol 2014;210:125.e1–125.e15. Doi: 10.1016/j.ajog.2014.06.060

13. Oh KJ, Kim SM, Hong JS, et al. Twenty-four percent of patients with clinical chorioamnionitis in preterm gestations have no evidence of either culture-proven intraamniotic infection or intraamniotic inflammation. Am J Obstet Gynecol 2017;216:604.e1–604.e11. Doi: 10.1016/j.ajog.2017.02.035

14. Harrell FEJ, Slaughter JC. Biostatistics for Biomedical Research. Power. 2015;1–158.

15. Simhan HN, Bodnar LM, Kim KH. Lower genital tract inflammatory milieu and the risk of subsequent preterm birth: an exploratory factor analysis. Paediatr Perinat Epidemiol 2011;25(3):277–82. Doi: 10.1111/j.1365-3016.2010.01176.x

16. Heng YJ, Liong S, Permezel M, Rice GE, Quinzio MKWD, Georgiou HM. The interplay of the interleukin 1 system in pregnancy and labor. Reprod Sci 2014;21:122–30. Doi: 10.1177/1933719113492204

17. Ashford KB, Chavan N, Ebersole JL, et al. Patterns of Systemic and Cervicovaginal Fluid Inflammatory Cytokines throughout Pregnancy. Am J Perinatol 2018 Apr;35(5):455–62. Doi: 10.1055/s-0037-1608677

18. Romero R, Grivel JC, Tarca AL, et al. Evidence of perturbations of the cytokine network in preterm labor. Am J Obstet Gynecol 2015;213:836.e1–836.e18. Doi: 10.1016/j.ajog.2015.07.037

19. Romero R, Durum S, Dinarello CA, Oyarzun E, Hobbins JC, Mitchell MD. Interleukin-1 stimulates prostaglandin biosynthesis by human amnion. Prostaglandins 1989;37:13–22. Doi: 10.1016/0090-6980(89)90028-2

20. Hertelendy F, Romero R, Molnar M, Todd H, Baldassare JJ. Cytokine-initiated signal transduction in human myometrial cells. Am J Reprod Immunol 1993;30:49–57. Doi: 10.1111/j.1600-0897.1993.tb00601.x

21. Belt AR, Baldassare JJ, Molna´r M, Romero R, Hertelendy F. The nuclear transcription factor NF-kappaB mediates interleukin-1beta-induced expression of cyclooxygenase-2 in human myometrial cells. Am J Obstet Gynecol 1999;181:359–366. Doi: 10.1016/s0002-9378(99)70562-4

22. Sivarajasingam SP, Imami N, Johnson MR. Myometrial cytokines and their role in the onset of labour. J Endocrinol 2016;231(3):R101-R119. Doi: 10.1530/JOE-16-0157

23. Vadillo-Ortega F, Estrada-Gutiérrez G. Role of matrix metalloproteinases in preterm labour. BJOG 2005;112 Suppl 1:19-22. Doi: 10.1111/j.1471-0528.2005.00579.x

24. Seong HS, Lee SE, Kang JH, Romero R, Yoon BH. The frequency of microbial invasion of the amniotic cavity and histologic chorioamnionitis in women at term with intact membranes in the presence or absence of labor. Am J Obstet Gynecol 2008;199(4):375.e1-5. Doi: 10.1016/j.ajog.2008.06.040

25. Bollapragada S, Youssef R, Jordan F, Greer I, Norman J, Nelson S. Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. Am J Obstet Gynecol 2009;200(1):104.e1-11. Doi: 10.1016/j.ajog.2008.08.032

26. Mittal P, Romero R, Tarca AL, et al. Characterization of the myometrial transcriptome and biological pathways of spontaneous human labor at term. J Perinat Med 2010;38(6):617-43. Doi: 10.1515/jpm.2010.097

27. Romero R, Miranda J, Chaiworapongsa T, et al. A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. Am J Reprod Immunol 2014;71:330–358. Doi: 10.1111/aji.12189

28. Romero R, Miranda J, Chaiworapongsa T, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. Am J Reprod Immunol 2014;72:458–474. Doi: 10.1111/aji.12296

29. Lucaroni F, Morciano L, Rizzo G, et al. Biomarkers for predicting spontaneous preterm birth: an umbrella systematic review. J Matern Fetal Neonatal Med 2018;31(6):726-734. Doi: 10.1080/14767058.2017.1297404

30. Monastero RN, Pentyala S. Cytokines as Biomarkers and Their Respective Clinical Cutoff Levels. Int J Inflam 2017;2017:4309485. Doi: 10.1155/2017/4309485

31. Conde-Agudelo A, Papageorghiou AT, Kennedy SH, Villar J. Novel biomarkers for the prediction of the spontaneous preterm birth phenotype: a systematic review and meta-analysis. BJOG 2011 Aug;118(9):1042-54. Doi: 10.1111/j.1471-0528.2011.02923.x

32. Lamont RF, Richardson LS, Boniface JJ, et al. Commentary on a combined approach to the problem of developing biomarkers for the prediction of spontaneous preterm labor that leads to preterm birth. Placenta 2020;98:13–23. Doi: 10.1016/j.placenta.2020.05.007

33. Santhanam U, Avila C, Romero R, et al. Cytokines in normal and abnormal parturition: elevated amniotic fluid interleukin-6 levels in women with premature rupture of membranes associated with intrauterine infection. Cytokine. 1991;3:155–163. Doi: 10.1016/1043-4666(91)90037-e

34. Menon R, Torloni MR, Voltolini C, et al. Biomarkers of spontaneous preterm birth: an overview of the literature in the last four decades. Reprod Sci 2011;18:1046–1070. Doi: 10.1177/1933719111415548

35. Genser B, Cooper PJ, Yazdanbakhsh M, Barreto ML, Rodrigues LC. A guide to modern statistical analysis of immunological data. BMC Immunol 2007;26(8):27. Doi: 10.1186/1471-2172-8-27

36. Ballenberger N, Lluis A, von Mutius E, Illi S, Schaub B. Novel Statistical Approaches for Non-Normal Censored Immunological Data: Analysis of Cytokine and Gene Expression Data. PLoS One 2012;7(10):e46423. Doi: 10.1371/journal.pone.0046

Tables

Table 1. General characteristics of women included in the study, as well as a comparison between study stages.

Parameter	Overall sample (N=144)	Stage 0 (n=35)	Stage 1 (n=15)	Stage 2 (n=34)	Stage 3 (n=36)	Stage 4 (n=24)
Age, years	29.0 (22.0-35.0)	31.0 (25.50-35.50)	26.0 (21.50-31.50)	29.0 (20.50-34.80)	28.50 (22.0-35.0)	25.0 (17.80-33.0)
Delivery, WoG*	37.30 (36.0-38.70)	37.20 (35.80-39.0)	37.0 (36.20-38.40)	37.0 (36.0-38.20)	37.10 (35.30-38.0)	38.30 (37.30-39.0)
Sample collection, WoG*	30.87±6.52	25.90±5.98	26.0±8.48	31.0±4.57	33.10±3.94	37.70±2.19

Parameter	Overall sample (N=144)	Stage 0 (n=35)	Stage 1 (n=15)	Stage 2 (n=34)	Stage 3 (n=36)	Stage 4 (n=24)
Term delivery, n (%)	96 (66.70)	23 (65.70)	10 (66.70)	22 (64.70)	20 (55.60)	21 (87.50)
Preterm delivery, n (%)	48 (33.30)	12 (34.30)	5 (33.30)	12 (35.30)	16 (44.40)	3 (12.50)
Preterm delivery, n (%)	Preterm delivery, n (%)	Preterm delivery, n (%)	Preterm delivery, n (%)	Preterm delivery, n (%)	Preterm delivery, n (%)	Preterm delivery, n (%)
32-36 WoG	35 (24.30)	9 (25.70)	2 (13.30)	8 (23.50)	14 (38.90)	2 (8.33)
28-31 WoG	10 (6.94)	3 (8.57)	1 (6.67)	4 (11.80)	1 (2.78)	1 (4.17)
<28 WoG	3 (2.08)	NA	2 (13.30)	NA	1 (2.78)	NA
Gestations, n (%)	Gestations, n (%)	Gestations, n (%)	Gestations, n (%)	Gestations, n (%)	Gestations, n (%)	Gestations, n (%)
1	49 (34.0)	7 (20.0)	5 (33.30)	13 (38.20)	12 (33.30)	12 (50.0)
[?]2	95 (66.0)	28 (80.0)	10 (66.70)	21 (61.80)	24 (66.70)	12 (50.0)
History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)
Negative	131 (91.97)	31 (88.60)	15 (100.0)	30 (88.20)	32 (88.90)	23 (95.83)
Positive	13 (9.03)	4 (11.40)	NA	4 (11.80)	4 (11.10)	1 (4.17)

Data are presented as either mean±SD or median (IQR) according to variable distributions. WoG, weeks of gestation; PTB, preterm birth. * P <0.05, Chi-squared test or Fisher exact test for categorical variables, Kruskal-Wallis rank-sum test or Welch's ANOVA for continuous variables. NA, "not available", insufficient subjects.

Table 2. General characteristics of women included in the study stratified by term and preterm delivery, as well as sub-categories of preterm birth.

			Sub-categories of Preterm Birth	Sub-categories of Preterm Birth	Sub-categories of Preterm Birth
Parameter	**Term (n=96)**	**Preterm (n=48)**	**32-36 WoG (n=35)**	**28-31 WoG (n=10)**	**<28 WoG (n=3)**
Age, years	29.0 (22.0-34.0)	27.50 (21.0-35.0)	27.0 (20.50-35.0)	32.0 (23.0-38.50)	30.0 (28.0-30.0)
Delivery, WoG*	38.30±1.02	33.50±3.56*****	35.20±1.10	31.0±0.24	22.10±0.52*****
Gestations, n (%)	Gestations, n (%)	Gestations, n (%)	Gestations, n (%)	Gestations, n (%)	Gestations, n (%)
1	33 (34.40)	16 (33.30)	10 (28.60)	4 (40.0)	2 (66.70)
[?]2	63 (65.60)	32 (66.70)	25 (71.40)	6 (60.0)	1 (33.30)
History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)	History of PTB, n (%)
Negative*	91 (94.80)	40 (83.0)*****	29 (82.90)	8 (80.0)	3 (100.0)
Positive*	5 (5.21)	8 (16.70)*****	6 (17.10)	2 (20.0)	NA

Data are presented as either mean±SD or median (IQR) according to variable distributions. WoG, weeks of gestation; PTB, preterm birth. * P<0.05, Chi-squared test or Fisher exact test for categorical variables, Kruskal-Wallis rank-sum test or Welch's ANOVA and Mann-Whitney U or Welch's t test for continuous variables. NA, "not available", insufficient subjects.

Table 3 . Optimal cut-point of IL-6 for identification of spontaneous labor (Stage 4).

Area under the ROC curve (AUC) = 0.862 (95% CI 0.758-0.967)

Parameter	**Estimate**	**95% CI**
Cutoff	34.60 pg/mL	
Sensitivity	91.67%	73.00-98.97
Specificity	79.17%	70.80-86.04
Positive predictive value	46.80%	35.95-88.53
Negative predictive value	97.93%	92.11-98.71
Positive diagnostic likelihood ratio	4.40	3.04-6.36
Negative diagnostic likelihood ratio	0.10	0.02-0.39
False positive	25	
False negative	2	

CI, confidence interval. Criterion: Youden Index. N=144.

Table 4. Time-varying diagnostic performance and predictive accuracy measures of IL-6 using time-dependent ROC curves.

Time (t)	Cases	Controls	Censored	AUC (95% CI)	%Se (SE)	%Sp (SE)	%PPV (SE)	%NPV (SE
0	0	113	31	-	-	80.53 (±3.74)	-	100.0 (±0)
12	42	100	2	0.785 (0.693-0.877)	64.29 (±7.42)	81.0 (±3.94)	58.70 (±7.29)	84.38 (±3.
33	71	70	3	0.686 (0.596-0.776)	45.07 (±5.93)	81.43 (±4.66)	71.11 (±6.78)	59.38 (±5.
54.1	101	43	0	0.658 (0.566-0.750)	37.62 (±4.84)	81.40 (±5.96)	82.61 (±5.61)	35.71 (±4.
66	115	29	0	0.557 (0.453-0.660)	33.91 (±4.43)	75.86 (±7.97)	84.78 (±5.31)	22.45 (±4.

t, time point (days); AUC, area under the ROC Curve; CI, confidence interval; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value; SE, standard error.