Amniotic immune biomarkers as risk factors in women with different symptoms of threatened late miscarriage

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Abstract
Problem: To investigate risk factors that can help identify the possibility of pregnancy loss in threatened late miscarriage (TLM) patients with and without spontaneous uterine contractions.

Method of study: Amniotic immune biomarkers (IL2βR, IL6, IL8, IL10, IL1β, and TNFα) were assayed, and “sludge” was assessed. Patients without intrauterine infections were treated and followed up until delivery, and pregnancy outcomes were recorded. The two groups were compared for the differences in biomarker levels and “sludge,” and the independent associations of biomarkers, “sludge,” and other maternal factors with late miscarriage were investigated.

Results: The amniotic levels of IL2βR, IL8, and TNFα were higher in the group with contractions (P < .05). When considered alone, each of the six biomarkers was significantly associated with late miscarriage in the no-contractions group and four of these (IL8, IL10, IL1β, and TNFα) in the contractions group (P < .05). Biomarker levels were correlated, and in multivariate Cox regression analysis, there was an independent effect only for IL8 in the no-contractions group (HR = 18.16, 95% CI: 5.75-57.43) and TNFα in the contractions group (HR = 4.11, 95% CI: 1.68-10.08). For patients with contractions, IL10, IL8, and IL1β were different in those with and without “sludge,” but no such difference was seen in the no-contractions group.

Conclusion: For TLM patients without intrauterine infections, amniotic immune biomarkers differ between patients with different symptoms, not only for their levels but also for the impact of these biomarkers on the risk of late miscarriage. These findings suggest that the symptoms of TLM should be considered in the study of miscarriage risk.

KEYWORDS
amniotic fluid sludge, IL6, IL8, independent risk factors, threatened late miscarriage, transvaginal ultrasound
1 | INTRODUCTION

Late miscarriage, also called second-trimester or mid-trimester loss, usually refers to a miscarriage that happens when a baby dies after 14 weeks and before 22 or 24 weeks of pregnancy (depending on the country). However, the highest gestational age of late miscarriage is defined as 28 weeks in China, and so “Threatened late miscarriage (TLM)” is defined by the shortening or opening of the cervix with or without uterine contractions at or after 14-week gestation and before 28 weeks. Although there is no consensus on the definition and diagnosis of "cervical insufficiency (CI)" in published studies, "TLM without uterine contractions" is commonly considered as CI. So the CI patients in this study are TLM patients without uterine contractions. If not treated properly, TLM may progressively lead to late miscarriage or preterm birth, presenting a very difficult clinical situation for both patients and doctors. A better understanding of risk factors for adverse pregnancy outcomes in these two groups of patients can contribute to the understanding of the mechanisms leading to miscarriage and ultimately the identification and management of high-risk pregnancies.

It is widely accepted that the act of parturition is the final step of a proinflammatory cascade that is coordinated by an intrauterine environment connected with hormonal signals and maternal peripheral or amniotic immune biomarkers have for several decades been the focus of many published papers investigating various adverse pregnancy outcomes. As early as 2008, a study of the association of IL-6, IL-8, and TNF-alpha with PROM found that only one of IL-6 or IL-8 was necessary. An investigation of only IL-6 in an African American population in 2011 found this cytokine to be strongly associated with preterm birth, and more recently, a study of three cytokines (IL-6, IL-2R, TNF-alpha) found moderate associations with preterm birth. Since then, the cytokines IL-6 and TNF-alpha have been found to be strongly associated with fetal inflammatory response and to have significantly higher levels in women with preterm delivery. To our knowledge, there are no studies examining the joint effect of all common cytokines for their independent contribution to risk. The presence of sludge in amniotic fluid has also been shown to be associated with the risk of preterm delivery, but these studies did not adjust for the potential contribution of amniotic cytokines.

Given the accumulation of evidence, we hypothesized that some of the amniotic biomarkers that are routinely available from intrauterine inflammation screening are independently associated with TLM, with possibly different effects for patients with and without uterine contractions. Separate analysis of these groups is rare in the scientific literature, and furthermore, most studies present results from univariate analyses and many do not explicitly state if they include/exclude pregnancies with intrauterine infection (itself a risk factor for late miscarriage). We restricted our study population to TLM patients without intrauterine infection. The aim of this study is to compare the amniotic fluid in TLM pregnancies with and without uterine contractions for the levels of inflammatory markers and presence of sludge, and to investigate the effect of the biomarkers on risk of late miscarriage in the two groups.

2 | MATERIALS AND METHODS

2.1 | Enrollment of study cohort

The study participants were identified according to the Chinese Guideline for Diagnosis and Therapy of Preterm Birth from women who attended the obstetrics and gynecology department of Sun Yat-Sen memorial hospital from January 1, 2016, to December 30, 2017. Women determined to have a high risk of late miscarriage were enrolled in the study and classified by their clinical symptoms into two groups based on whether or not they had uterine contractions (Figure 1). These high-risk pregnant women were enrolled between 16±0 and 27+6 gestational weeks if they met all of the inclusion criteria and none of the exclusion criteria, resulting in 76 pregnant patients in the no-contractions group and 33 patients in the contractions group. The inclusion criterion for the no-contractions group was an intravaginal cervical length < 15 mm or cervical os open (<4 cm) without uterine contractions. The inclusion criterion for the no-contractions group was a normal cervix (cervical length ≥ 25 mm) with regular contractions.

FIGURE 1 Flowchart of the cohort study
at first visit to the emergency department, but shortening to
0-20 mm during the observation period. Women under observa-
tion in the emergency department are monitored at least every
4 hours and up to a maximum of 24-48 hours, according to the pa-
tient’s condition. Women were excluded if the cervical length was
≥ 20 mm after observation in the emergency department or the cer-
vical os opened during the observation period. Women were
excluded from the study if they had intrauterine infections. In
addition, the following six exclusion criteria were used: serious
heart, liver, or kidney disorders; fetal death; placenta previa with
uncontrolled vaginal bleeding; placenta accrete; preterm labor;
acute genital tract inflammation, especially chorioamnionitis; ma-
ternal temperature of ≥38.0°C and two or more of the following:
(a) uterine tenderness; (b) malodorous vaginal discharge; (c) mater-
nal leukocytosis (WBC ≥ 15 000 cells/mm³, or neutrophils > 90%); (d)
maternal tachycardia (>100 beats/min); (e) fetal tachycardia
(≥160 beats/min). All patients signed informed consent to have an
amniocentesis performed. For multiple gestation, the specimen of
amniotic fluid was obtained from the lower amniotic sac (ie, closest
to the cervix).

2.2 | Amniotic immune biomarker
measurements and "sludge" assessment

All patients were screened for intrauterine inflammation using a
standard set of inflammation biomarkers that are widely used in
clinical practice and research. A 20-milliliter specimen of amniotic
fluid was transported to the pediatric laboratory within two hours
after amniocentesis and analyzed by chemiluminescence using the
Siemens Immulite 1000 equipment, which provided levels of IL2βR,
IL6, IL8, IL10, IL1β, and TNFα. The amniotic WBC was tested by
Bayer AD, VIA2120 automatic blood cell instrument; The presence of
amniotic microorganisms (bacteria, mycoplasma, and candida
albicans) was assessed by culture. Meanwhile, a 4-milliliter sample
of maternal peripheral blood was sent to the general laboratory for
testing a standard set of markers (IL2βR, IL6, IL8, IL10, and IL1β) on
the same day, using the same method and the same type of ana-
lyzer. For imaging, we used transvaginal ultrasound (Voluson E8;
GE Healthcare, probe: RIC6, 12, D, 22Hz). Observations of cervical
dynamic changes were performed for several minutes. The cervical
length measurements were repeated after pressure on the pubic
symphysis or cough, and the shortest length recorded after three
repeated measurements. "Sludge" was defined as the presence of
dense aggregates of particulate matter in the proximity of the inter-
cervical os. Diagnosis of "sludge" was checked by two ultrasound
specialists.

2.3 | Treatment and follow-up of patients

A flowchart of the screening procedures is presented in Figure S1.
Cervical length and "sludge" were assessed every 2-4 weeks until
34-weeks gestation. Once "sludge" was definitively diagnosed,
the assessment interval was decreased to 1-2 weeks. If cervical
length was not continuously shortened for two consecutive as-
sessions, the screening interval was prolonged to the original
2-4 weeks. For the "no-contractions" patients, stressed McDonald
cerclage was performed if the cervix was short, and emergency
McDonald cerclage was performed if the cervical os was open
(<4 cm). The cerclage was performed on such patients at no more
than 27+6 weeks (the highest gestational age in this group of our
cohort was 27+4 weeks). For patients with uterine contractions,
tocolytic therapy was given after amniocentesis. Phloroglucin was
prescribed for patients with a gestational age <20 weeks, and the
highest dose was 200 mg/d. The first-line tocolytic medicine after
20 gestational weeks was ritodrine hydrochloride. Ritodrine hy-
drochloride was considered suitable for women with a singleton
pregnancy without vaginal bleeding or other contraindications
(including overactive thyroid gland, extreme loss of body water,
high blood pressure, increased pressure of pulmonary circulation,
abnormal heart rhythm, or diabetes). Atosiban was commonly
considered for patients whose gestational age ≥24 weeks, especially
with multiple gestation, diabetes or gestational diabetes, cardio-
vascular disease, or hyperthyroidism. Atosiban was also offered
to patients at less than 24-week gestation who had contraindications
of ritodrine. Tocolytic therapy of no more than seven continuous
days was continued until no regular uterine contractions were
detected by external tocography (AN24™: maternal-fetal holter,
Beijing) or abdominal palpation. If the first-line medicine failed
to control the contractions, nifedipine or indomethacin was used.
Tocolytic agents were not given after 34+6 gestational weeks.
Prophylactic tocolytic agents were also given after cerclage for
women without contractions for up to 3 days.

Cervical length was assessed 14 days after cerclage in the
no-contractions group, and 7 days after tocolytic therapy in the
contractions group. Meanwhile, transabdominal ultrasound was
performed to estimate the fetal growth and development every
4-8 weeks. Patients remained in the hospital until delivery if the cer-
vical length was shorter than 10 mm after cerclage or if uterine con-
tractions were not well inhibited; otherwise, patients were followed
up in the out-patient department. Maternal leukocytosis, CRP, and
high vaginal swab for culture were tested every 2-4 weeks.

All patients were prescribed vaginal progesterone 200-400 mg
until delivery or week 36. Other treatments used on indication were
(Figure S1): dexamethasone, which was used after 26+0 weeks and
before 34+6 weeks with a dose of 5 mg every 12 hours for four treat-
ments, and a repeated cycle of four treatments was considered if
the gestational age was ≤34 weeks and it was at least 2 weeks since
the first cycle; antibiotics, with indications being acute genital tract
inflammation or other infections according to the patient’s clinical
symptoms and laboratory results.

The onset of premature labor unresponsive to tocolytics and/or
strong suspicion of sepsis were indications for emergency re-
moval of the cerclage suture. Otherwise, the suture was removed
between weeks 36 and 37. In cases of premature preterm rupture.
of membranes (PPROM), the suture was maintained after antenatal corticosteroid therapy if there was no sign of intrauterine infection or any other infections.

2.4 | Statistical analysis

Biomarkers were inspected for their pairwise correlation using a scatter plot matrix, and the Spearman rank correlation coefficient was used to assess the degree of associations between gestational age (days) and the biomarker levels. Box plots and violin plots were used to present amniotic immune biomarker levels in the two patient groups, where high dispersion for some biomarkers was accommodated by a broken scale. To compare biomarker levels in the contractions and no-contractions groups, Student’s t tests or Mann-Whitney U tests were used for continuous variables and chi-squared or Fisher exact tests for categorical variables. For formal comparisons between different pregnancy outcomes, biomarker levels with extreme skew were converted to a logarithmic scale. Where there was evidence of limits of detection in the assay, we categorized the biomarker levels using the median, except where there were too few observations among the late miscarriage cases or non-cases, when we used the upper or lower quartile. The effects of these categorized biomarker levels on late miscarriage were analyzed using univariate logistic regression and multivariate logistic regression to estimate crude and adjusted ORs, where risk factors with a $P$-value < .20 in the univariate regression analysis were considered in the multivariate model using forward stepwise.

### TABLE 1

Reproductive history and immune biomarker levels in both maternal peripheral blood and amniotic fluid of threatened late miscarriage patients with and without uterine contractions

|                      | No-contractions (n = 76) | Constructions (n = 33) | $P$ value |
|----------------------|--------------------------|------------------------|-----------|
| Pregnancy characteristics and reproductive history |                         |                        |           |
| Age (y, Mean ± SD)   | 31.7 ± 5.3               | 31.8 ± 3.7             | .635      |
| Primigravida (n, %)  | 22 (28.9%)               | 7 (21.2%)              | .403      |
| Assisted reproduction (n, %) | 31 (40.8%)       | 14 (42.4%)             | .873      |
| Multiple gestation (n, %) | 25 (32.9%)         | 9 (27.3%)              | .560      |
| History of early spontaneous abortion (n, %) | 37 (48.7%) | 19 (57.6%) | .514 |
| History of late abortion (n, %) | 12 (13.2%) | 4 (12.1%) | .619 |
| History of preterm birth (n, %) | 4 (5.3%) | 1 (3.0%) | .678 |
| History of cesarean (n, %) | 10 (11.8%) | 2 (6.1%) | .301 |
| Gestational age at amniocentesis (d, mean ± SD) | 168 ± 14.5 | 159 ± 18.4 | .052 |

| Maternal peripheral immune biomarkers |                         |                        |           |
| IL2βR (u/mL) | 306.5 (283.5, 337.0) | 370.0 (253.5, 388.8) | .149      |
| IL6 (pg/mL)  | 2.8 (2.0, 4.2)        | 2.9 (2.4, 8.8)         | .239      |
| IL10 (pg/mL) | 5.0 (5.0, 5.0)        | 5.0 (5.0, 5.0)         | .998      |
| IL8 (pg/mL)  | 12.5 (8.2, 12.0)      | 13.3 (7.7, 39.2)       | .149      |
| IL1β (pg/mL) | 5.0 (5.0, 5.0)        | 5.0 (5.0, 5.0)         | .999      |

| Amniotic immune biomarkers |                         |                        |           |
| IL2βR (u/mL) | 420.5 (347.5, 550.0) | 518.0 (362.0, 713.0) | .034      |
| IL6 (pg/mL)  | 1000.0 (362.0, 1000.0) | 1000.0 (913.0, 1000.0) | .061      |
| IL10 (pg/mL) | 5.0 (5.0, 17.2)       | 6.4 (5.0, 89.1)        | .297      |
| IL8 (pg/mL)  | 1235.5 (323.8, 5269.5) | 7500.0 (1327.5, 7500.0) | .005      |
| IL1β (pg/mL) | 5.0 (5.0, 53.6)       | 22.7 (5.0, 593.5)      | .102      |
| TNFα (pg/mL) | 14.9 (11.3, 33.6)     | 35.9 (10.5, 196.0)     | .045      |
| Amniotic fluid sludge (n, %) | 11 (13.9%) | 6 (17.1%) | .410 |
| Amniotic WBC ($10^9$/L) | 0.12 (0.09, 0.23) | 0.13 (0.10, 0.37) | .662 |

Note: Immune biomarker levels are presented as medians, with lower and upper quartiles in parentheses.
selection. Since an earlier miscarriage is a reflection of the severity of risk, we used a Cox regression analysis of the time to late miscarriage (censoring at 196 gestational days) to estimate hazard ratios for the biomarker levels: Using the time scale also enabled us to overcome the limited power of the small sample size to obtain more stable estimates and better precision. Kaplan-Meier curves were used to compare the time from enrollment to delivery of the no-contractions group with and without “sludge.” Statistical significance was defined as \( P < .05 \). All data management and analysis were performed using SPSS 22.0 (IBM Corp).

### 3 | RESULTS

#### 3.1 | Amniotic IL2βR, IL8, and TNFα levels were higher in the group with contractions

Reproductive characteristics of patients with and without uterine contractions were compared and no significant differences were found (Table 1, top panel). Women with contractions were enrolled at slightly earlier gestational age (159 vs 168 days, \( P = .052 \)). There were no statistically significant differences in any of the biomarker levels in maternal peripheral blood between the two groups (Table 1, second panel). Amniotic biomarker levels did not differ significantly with gestational age at sampling, so biomarker levels were analyzed without considering gestational age. Amniotic IL2βR, IL8, and TNFα levels were higher in the contractions group than the no-contractions group (\( P < .05 \) for all comparisons), and there was a borderline significance of higher IL6 (\( P = .061 \)).

#### 3.2 | Fewer late miscarriages and PPROMs in the no-contractions group

Table 2 presents the main outcome (late miscarriage) together with other pregnancy outcomes, including gestational age at delivery, PPROMs, and clinical chorioamnionitis. Gestational age was longer for the no-contractions group (mean 220.9 vs 201.4 days, \( P = .014 \)), with fewer late miscarriages (22.4% vs 42.4%, \( P = .039 \)) and lower PPROM rate (15.8% vs 33.3%, \( P = .045 \)) than the contractions group.

#### 3.3 | Each of the amniotic immune biomarkers was significantly associated with late miscarriage in the no-contractions group and four of these (IL8, IL10, IL1β, and TNFα) in the contractions group

Box plots of amniotic immune biomarker levels in the two groups are presented in Figure 2, demonstrating wide variations and some limits of detection. Odds ratios for the univariate comparisons of maternal characteristics and amniotic immune biomarkers are presented for the two groups in Table 3 (with details of immune biomarker levels in Table S1). In the contractions group, late miscarriage was significantly associated with maternal age (\( P = .029 \)) and primigravida (\( P = .025 \)) and there was also a borderline association with multiple gestation (\( P = .095 \)), but none of these factors were significantly associated with late miscarriage in the no-contractions group. All of the amniotic immune biomarkers had significant crude associations with late miscarriage in the no-contractions group and four (IL10, IL8, IL1β, and TNFα) in the contractions group, where there was also a trend for an association with IL6 (\( P = .091 \)). “Sludge” was found to be borderline significant in the no-contractions group (\( P = .057 \)). Amniotic immune biomarker levels were also compared for patients with and without “sludge” stratified by uterine contractions (Table S2), with no significant differences found in the no-contractions group, but higher IL10, IL8, and IL1β in the contractions group. Comparisons using the log scale identified the same significant differences (data not shown).

#### 3.4 | IL8 had an independent effect for late miscarriage in the no-contractions group and TNFα for the contractions group

Figure 3 presents plots of immune biomarker levels for late miscarriage and live birth stratified by uterine contractions. Limits of detection were apparent for IL10, IL8, and IL1β, so we categorized these variables for further analysis. For the no-contractions group, when the transformed immune biomarker variables were entered into a multivariate logistic regression model together with “sludge,” only IL8 was found to be an independent risk factor for late miscarriage (Table 4), explaining almost half of the variation in pregnancy outcome (Nagelkerke \( R^2 = .473 \)). This was a pair which was unsurprising as there was a strong correlation between pairs of immune biomarkers: for example, IL10 and IL1β (\( R^2 = .884 \) in the no-contractions

### Table 2: Pregnancy outcomes of threatened late miscarriage patients with and without uterine contractions

| Outcome                  | No-contractions (n = 76) | Contractions (n = 33) | \( P \) value |
|--------------------------|--------------------------|-----------------------|---------------|
| Late miscarriage (n, %)  | 17 (22.4%)               | 14 (42.4%)            | .039          |
| Live born\(^a\) (n, %)   | 59 (77.6%)               | 19 (57.6%)            |               |
| 26\(^s\)–33\(^t\) wk     | 32 (42.1%)               | 10 (30.3%)            |               |
| 34\(^o\)–36\(^o\) wk     | 15 (19.7%)               | 2 (6.1%)              |               |
| ≥37 wk                   | 12 (15.8%)               | 7 (21.2%)             |               |
| Gestational age (d, mean ± SD) | 220.9 ± 34.1          | 201.4 ± 44.7          | .014          |
| PPROM (n, %)              | 12 (15.8%)               | 11 (33.3%)            | .045          |
| Clinical Chorioamnionitis (n, %) | 7 (9.2%)                | 5 (15.2%)             | .363          |

\(^a\) Four infants in the no-contractions group and four in the contractions group were live born before 28 wk, the lowest gestational age was 26\(^s\) wk.
Cox regression analysis also identified IL8 as the only independent risk factor for the time from enrollment to late miscarriage. Although “sludge” was not found to be an independent risk factor, a Kaplan-Meier plot of the no-contractions group illustrated that there was a clear pattern of earlier pregnancy loss in women with “sludge” (Figure S2). For the analysis of the contractions group, amniotic IL10, IL8, and IL1β had too few observations in one or other of the outcome categories to allow for a joint model. In a multivariate logistic model of IL6, TNFα and potential confounders (age, assisted reproduction, and multiple gestation), only TNFα was an independent risk factor for late miscarriage (OR: per factor of 10 increase 19.48; 95% CI: 2.40-158.04), but this one biomarker was strongly predictive (Nagelkerke $R^2 = .587$).

**FIGURE 2** Box plots of amniotic immune biomarker levels in patients with and without uterine contractions

4 | DISCUSSION

In this study, we found differences in TLM patients with and without contractions not only for their amniotic immune biomarker levels and “sludge,” but also for the impact of these biomarkers on the pregnancy outcomes. The contractions group had more late miscarriages and PPROM, and higher levels of amniotic IL2βR, IL8, and TNFα than the no-contractions group. We also found that amniotic immune biomarker levels were only different for patients with and without “sludge” in the contractions group. When considered individually, all six amniotic immune biomarkers had an association with late miscarriage in the no-contractions group but only IL10, IL8, IL1β, and TNFα in the contractions group. Only IL8 had an independent association in the patients without contractions and only TNFα in patients with contractions.

For pregnancies with and without contractions, TLM is an early manifestation of a (later) miscarriage, but the pathophysiology in these two groups is different: TLM without contractions has its clinical manifestations in the cervix, while TLM with contractions is identified from the myometrium. While additional characteristics of the myometrium (for patients with contractions) and cervix (for patients without contractions) may provide useful prognostic information in prospective cohort studies, our findings indicate that such studies would also need to include cytokine levels. Thus, we have identified some important differences that are readily available in clinical practice and can make a valuable contribution to understanding the pathogenesis of TLM and late miscarriage. However, the exact pathogenesis of TLM is still unknown, and we lack knowledge about whether abnormal amniotic immune biomarkers play a role in the entire pathogenesis of late miscarriage. Immune cells and epithelial cells of the uterus can produce many kinds of cytokines or chemokines, which maintain a normal pregnancy.$^{16,17}$ However, an imbalance in these cell populations and their immune markers can activate the innate immune response and a complex cascade of events that can lead to cervical ripening and uterine contractions, and ultimately the onset of delivery. Although one of the leading causes of late miscarriage or preterm birth is intrauterine infection, studies have also found that spontaneous preterm labor can occur in the absence of identifiable
microorganisms, suggesting that sterile inflammation may also lead to preterm births.\(^{18}\) IL-8 is considered an important mediator of the innate immune system response.\(^{19}\) A study in 2004 found IL-8 to be involved in cervical dilatation but not in cervical ripening.\(^{20}\) However, that study compared the IL-8 levels of the cervical tissue in term pregnancies women who accepted cesarean section and women who had vaginal deliveries. These are a different patient population than late miscarriage patients. We hypothesise that in TLM without contractions, an abnormal level of secreted IL-8 may contribute to early ripening of the cervix and initiate the procedure of giving birth. An investigation of cell-signaling pathways in female reproduction found that TNF\(\alpha\) can be

| TABLE 3 | Risk factors related to late miscarriage in patients with and without uterine contractions: univariate analysis |
|----------|--------------------------------------------------|
|          | No-contractions (76)                              | Contractions (33)                             |
|          | OR  | 95% CI      | P value | OR  | 95% CI      | P value |
| Age      | 1.08 | 0.97-1.10  | .143    | 0.75 | 0.58-0.97  | .029   |
| Primigravida | 1.43 | 0.41-4.98 | .577    | 13.50 | 1.39-131.32 | .025\(^a\) |
| Assisted reproduction | 1.39 | 0.47-4.12 | .551    | 2.90 | 0.69-12.12 | .147   |
| Multiple gestation | 1.15 | 0.37-3.57 | .811    | 4.00 | 0.79-20.32 | .095   |
| Previous miscarriage | 1.55 | 0.51-4.74 | .445    | 3.75 | 0.82-17.12 | .089\(^b\) |
| Previous preterm | 1.17 | 0.11-12.00 | .897    | 0.27 | 0.06-1.22  | .301   |
| Gestational age at amniocentesis (d) | 0.98 | 0.93-1.02 | .244    | 0.97 | 0.93-1.03  | .301   |

Amniotic immune biomarkers

|          | IL2\(\beta\)R | 1.00 | 1.00-1.003 | .360 |
|          | IL6\(^d\)   | 2.99 | 0.84-10.67 | .091 |
|          | IL10\(^d\) | 6.08 | 1.71-21.62 | .005 |
|          | IL\(\beta\) | 1.00 | 1.001-1.006 | .19 |
|          | IL8 | 15.20 | 1.58-146.49 | .002 |
|          | WBC | 13.76 | 2.51-75.53 | .003 |

\(^a\)Only one primigravid patient delivered a live born.
\(^b\)Three first-time pregnancies ended in late miscarriage.
\(^c\)Only one patient had previous preterm birth, and she had term birth this time.
\(^d\)Analysis conducted on the log scale.

FIGURE 3 Violin plots of amniotic immune biomarker levels in patients with and without uterine contractions, stratified by pregnancy outcomes. *Indicates log transform.
Cox regression analysis

late miscarriage when adjusted for cytokine levels, possibly due to different mechanisms leading to their different pregnancy outcomes, including late miscarriage. Furthermore, the association of sludge with some amniotic immune biomarkers in the contractions group can partly explain why it was not found to be an independent risk factor.

The strengths of this study are that we analyzed two different clinical phenotypes of TLM and investigated the value of immune biomarkers in the absence of intrauterine infection. Although many immune biomarkers have been reported as risk factors for late miscarriage and preterm birth, there is still no consensus and few studies have focused on TLM patients with contractions. Since amniocentesis was conducted prior to tocolytic therapy or cerclage to rule out intrauterine infections, we could exclude pregnancies with infection and thus add to the evidence from previous reports of worse perinatal outcomes for pregnancies with abnormal amniotic fluid markers indicative of inflammation or subclinical intra-amniotic-infection. By conducting the analysis in each group separately, we ensured that patients were similar with respect to treatment; all patients in the no-contractions group had cerclage, and no patient in the contractions groups had this procedure. In addition, the exclusion of patients with intrauterine infections reduces the potential bias from differential treatment.

Despite the similarities between patients in the two groups, a potential limitation of the study is that different tocolytic drugs may be used. However, amniotic immune biomarkers are not part of this decision, so the choice of drug is unlikely to be a mediator of the biomarker’s effect on pregnancy outcome. Factors associated with the tocolytic medicine include gestational age and multiple gestation, both of which have been adjusted in our analysis. Other factors that impact on the choice of drug are cardiovascular diseases, diabetes, thyroid diseases, and other medical conditions:

A novel aspect of our work is the investigation of immune biomarker levels and “sludge” in patients stratified by different symptoms. Other authors have studied amniotic immune biomarkers as risk factors in cervical insufficiency (CI) patients, who are comparable to the “no-contractions” patients in our study: one retrospective study found IL1β, IL6, IL7, IL17α, TNFα (but not IL8) and cervical dilation to be independently associated with very early preterm birth (<32 weeks of gestation). Another strength of our study is that, in contrast to many previous studies, we did not choose a subset of the cytokines, but examined all those available from a comprehensive laboratory package in order to determine their independent effect.

“Sludge" has also been reported by others as an independent risk factor for preterm birth in “short cervix” patients. In our study, we conducted a joint analysis of cytokines and “sludge" and failed to see an independent effect of “sludge" on our primary outcome (late miscarriage) when adjusted for cytokine levels, possibly due to low statistical power. However, there was a clear pattern of earlier “pregnancy loss" in women with “sludge" in the no-contractions group (Figure S2). Interestingly, three patients in the no-contractions group whose “sludge" disappeared during follow-up all had good pregnancy outcomes, delivering live-born infants at 35-36 weeks, 37+1 weeks, and 38+3 weeks.

For the patients with contractions, we failed to build a reasonable model to analyze the independent contributions of the risk factors, partly due to the extreme distribution of the amniotic immune biomarker levels but also the small sample size. Our limited model found TNFα to be associated with pregnancy loss, but a larger study is needed to verify if IL8 is an independent risk factor for these patients. We also failed to find a significant effect of “sludge" on late miscarriage in this group, although other researchers have identified it as an independent risk factor for preterm birth and one study reported a much higher prevalence of “sludge" in women who delivered preterm. Considering we had only 6 patients with “sludge" in the contractions group, larger prospective studies are needed.

Several studies have focused on the association between amniotic immune biomarkers and “sludge." One study reported that the presence of “sludge" was related to intra-amniotic inflammation with or without microorganisms. In our study, we found that amniotic immune biomarker levels were not different between patients with and without “sludge" in the no-contractions group, but IL10, IL8, and IL1β were different in the contractions group, suggesting potentially different mechanisms leading to their different pregnancy outcomes, including late miscarriage. Furthermore, the association of sludge with some amniotic immune biomarkers in the contractions group can partly explain why it was not found to be an independent risk factor.

The strengths of this study are that we analyzed two different clinical phenotypes of TLM and investigated the value of immune biomarkers in the absence of intrauterine infection. Although many immune biomarkers have been reported as risk factors for late miscarriage and preterm birth, there is still no consensus and few studies have focused on TLM patients with contractions. Since amniocentesis was conducted prior to tocolytic therapy or cerclage to rule out intrauterine infections, we could exclude pregnancies with infection and thus add to the evidence from previous reports of worse perinatal outcomes for pregnancies with abnormal amniotic fluid markers indicative of inflammation or subclinical intra-amniotic-infection. By conducting the analysis in each group separately, we ensured that patients were similar with respect to treatment; all patients in the no-contractions group had cerclage, and no patient in the contractions groups had this procedure. In addition, the exclusion of patients with intrauterine infections reduces the potential bias from differential treatment.

Despite the similarities between patients in the two groups, a potential limitation of the study is that different tocolytic drugs may be used. However, amniotic immune biomarkers are not part of this decision, so the choice of drug is unlikely to be a mediator of the biomarker’s effect on pregnancy outcome. Factors associated with the tocolytic medicine include gestational age and multiple gestation, both of which have been adjusted in our analysis. Other factors that impact on the choice of drug are cardiovascular diseases, diabetes, thyroid diseases, and other medical conditions:

### TABLE 4 Risk factors related to late miscarriage in patients with and without uterine contractions, from multiple logistic regression and Cox regression analysis

| Multiple logistic regression | Cox regression analysis |
|-----------------------------|-------------------------|
| Ref | OR | 95% CI | Ref | HR | 95% CI |
| IL8<sup>a</sup> | ≤5269.5 | 27.63 | 6.79-112.48 | 18.16 | 5.75-57.43 |
| **B. Contractions** | | | | | |
| Age | ~ | 0.68 | 0.46-1.02 | ~ | ~ |
| TNFα<sup>b</sup> | ~ | 19.48 | 2.40-158.04 | 4.11 | 1.68-10.08 |

<sup>a</sup>IL8 > 5269.5 (upper quartile) was considered high.
<sup>b</sup>Analysis conducted on the log scale.
we did not have access to this information for our cohort, but since these conditions are rare in our study population, they are unlikely to have confounded the effect of the biomarkers. There remains the possibility that during the follow-up period, different patients may have required different treatment strategies which could mediate the effect of the biomarkers on the pregnancy outcome. Thus, our estimates are of the total effects of amniotic immune biomarkers, and a more in-depth clinical study would be needed to answer the question regarding direct effects. Furthermore, since this is a relatively small study in a single hospital, further work is needed to validate the role of the amniotic immune biomarkers, and instead of simple categorization of high and low levels based on cohort-specific medians and quartiles, to identify clinical cut-offs that might be "transported" to other populations.

In summary, our work demonstrates that high levels of amniotic immune biomarkers present different characteristics of TLM pregnancies with and without contractions, in the absence of intrauterine infections. These findings can improve our understanding of the underlying biological mechanisms of miscarriage and help in the identification of at-risk pregnancies.

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CONFLICT OF INTEREST

We have no conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

Hui Chen and Lili Meng conceived and designed the study, Lili Meng, Marie Reilly, and Shengxin Liu analyzed the data. Lili Meng and Zhenhua Wang drafted the first version of the manuscript. Xiuli Liu and Dijin Lin did the laboratory testing. Yinglin Liu, Shuning Zhang, and Jianping Zhang enrolled patients in the study. Lili Meng, Zhenhua Wang, Marie Reilly, Xiuli Liu, Shuning Zhang, Dijin Lin, Shengxin Liu, Yinglin Liu, Jianping Zhang, and Chen Hui edited the manuscript, and read and approved the final version. Each author has confirmed compliance with the journal’s requirements for authorship.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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