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The earlier the gestational age, the greater the intensity of the intra-amniotic inflammatory response in women with preterm premature rupture of membranes and amniotic fluid infection by *Ureaplasma* species

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Abstract

Objectives: To determine the relationship between the intensity of the intra-amniotic inflammatory response and the gestational age at the time of diagnosis in cases with preterm premature rupture of membranes (PROM) and intra-amniotic infection caused by *Ureaplasma* spp.

Methods: A retrospective cohort study was conducted which included 71 women with preterm PROM and a positive amniotic fluid culture with *Ureaplasma* spp. Women with mixed intra-amniotic infections were excluded. The study population was classified into three groups according to gestational age: group 1, <26 weeks (extreme preterm PROM, n = 17); group 2, 26.0–33.9 weeks (moderate preterm PROM, n = 39); group 3, 34.0–36.9 weeks (late preterm PROM, n = 15). The intensity of the intra-amniotic and maternal inflammatory response was compared among the three groups. The intensity of the intra-amniotic inflammatory response was assessed by the concentration of amniotic fluid matrix metalloproteinase-8 (MMP-8) and white blood cell (WBC) count. The maternal inflammatory response was assessed by the concentration of C-reactive protein (CRP) and WBC count in maternal blood at the time of amniocentesis.

Results: (1) The median values of amniotic fluid MMP-8 concentration and WBC count were the highest in the extreme preterm PROM group and the lowest in the late preterm PROM group ($P < 0.001$ and $P = 0.01$, respectively); (2) the intensity of the maternal inflammatory response measured by maternal blood WBC count and CRP concentration was not significantly associated with gestational age at the time of diagnosis.

Conclusion: The earlier the gestational age at the time of PROM, the higher the intensity of the intra-amniotic inflammatory response in women with preterm PROM and intra-amniotic infection caused by *Ureaplasma* spp.

Keywords: chorioamnionitis; intra-amniotic infection; intra-amniotic inflammation; matrix metalloproteinase-8; microbial invasion of amniotic cavity; prematurity; preterm labor; preterm premature rupture of membranes; *Ureaplasma*.

Introduction

Ureaplasma spp. are the most common microorganisms isolated from the amniotic fluid of women with preterm premature rupture of membranes (PROM) [1–14], preterm labor with intact membranes [15–17], acute cervical insufficiency [18–20], clinical chorioamnionitis at preterm [21] and term [22], PROM at term [23] and idiopathic vaginal bleeding [24].

A strong body of evidence supports that intra-amniotic infection with *Ureaplasma* spp. is associated with a robust host response in the fetal, amniotic and maternal compartments [4, 6, 9, 16, 25–27] and that these organisms are associated with neonatal morbidity including neonatal bacteremia [28, 29], intraventricular hemorrhage [30, 31], chronic lung disease [32–36], meningitis [37] and adverse neuromotor development [38].

Ureaplasma spp. are frequently detected in the amniotic fluid of asymptomatic women at early mid-trimester [39–41], which are often associated with preterm delivery, but not in all cases [40, 41]. These findings suggest that not only microbial invasion of the amniotic cavity but also the host response to microorganisms may be an important factor in determining the outcome of pregnancy.

Previous studies showed that the lower the gestational age, the higher the frequency of intra-amniotic infection

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and intra-amniotic inflammation in symptomatic women with preterm PROM [42–44] and preterm labor [45, 46]. We hypothesized that the earlier the gestational age of PROM, the more intense the host inflammatory response in women with intra-amniotic infection by *Ureaplasma* spp.

The purpose of this study was to determine the relationship between the intensity of intra-amniotic inflammatory response and the gestational age in women with preterm PROM and intra-amniotic infection caused by *Ureaplasma* spp.

Materials and methods

Study design

This study population consisted of 71 consecutive patients admitted to Seoul National University Hospital between 1993 and 2012 with the diagnosis of preterm PROM (<37 weeks of gestation) who met the following criteria: (1) singleton pregnancy; (2) amniotic fluid obtained for microbiologic studies by transabdominal amniocentesis or at the time of cesarean delivery; and (3) proven intra-amniotic infection by *Ureaplasma* spp. using cultivation techniques. Women with a mixed intra-amniotic infection (*Ureaplasma* spp. and other microorganisms) were excluded.

Patients were divided into three groups according to the gestational age at amniocentesis: group 1, gestational age <26 weeks (extreme preterm, n = 17); group 2, gestational age between 26.0 and 33.9 weeks (moderate preterm, n = 39); group 3, gestational age between 34.0 and 36.9 weeks (late preterm, n = 15). The intensity of the intra-amniotic inflammatory response was determined by the amniotic fluid matrix metalloproteinase-8 (MMP-8) concentration and white blood cell (WBC) count, and that of the maternal inflammatory response was determined by C-reactive protein (CRP) and WBC count in maternal blood at the time of amniocentesis. Amniocentesis is routinely offered to all patients who are admitted with the diagnosis of preterm PROM for microbiologic studies and/or assessment of fetal lung maturity. Retrieval of amniotic fluid and maternal blood was performed after written informed consent was obtained.

We followed the ethical standards for human experimentation established in the Declaration of Helsinki. The Institutional Review Board of Seoul National University Hospital approved the collection and use of these samples and information for research purposes. The University has a Federal Wide Assurance with the Office for Human Research Protection (OHRP) of the Department of Health and Human Services (DHHS) of the United States.

Amniotic fluid analysis

Amniotic fluid was retrieved by transabdominal amniocentesis or at the time of cesarean delivery. The fluid was collected and transferred into commercially available culture media and transported immediately to the Department of Laboratory Medicine of our hospital and cultured for genital mycoplasmas (*Ureaplasma* spp. and *Mycoplasma hominis*) as well as aerobic and anaerobic bacteria. An aliquot of amniotic fluid was examined in a hemocytometer chamber to determine the WBC count. Fluid not used for clinical purposes was centrifuged and stored in polypropylene tubes at –70°C. The stored amniotic fluid was analyzed for MMP-8, which was measured using a commercially available enzyme-linked immunosorbent assay (R&D Systems, Inc., Minneapolis, MN, USA). Each measurement was performed in duplicate. Intra- and inter-assay coefficients were <10% each. Intra-amniotic inflammation was defined as an elevated concentration of MMP-8 (>23 ng/mL), as previously reported [19, 42, 47–54].

Diagnosis of chorioamnionitis and neonatal morbidity

Acute histologic chorioamnionitis was defined as the presence of acute inflammatory changes in the choriodecidua and amnion, respectively; acute funisitis was diagnosed by the presence of neutrophil infiltration into umbilical vessel walls or Wharton's jelly using previously published criteria [55–57]. Clinical chorioamnionitis was diagnosed in the presence of a maternal temperature of ≥37.8°C and ≥2 of the following criteria: (1) uterine tenderness; (2) malodorous vaginal discharge; (3) maternal leukocytosis (WBC count of >15,000 cells/mm^3); (4) maternal tachycardia (>100 beats/min); and (5) fetal tachycardia (>160 beats/min) [22, 58–67]. Significant neonatal morbidity was defined as the presence of any of the following conditions: respiratory distress syndrome, bronchopulmonary dysplasia, intraventricular hemorrhage (grade ≥II), proven congenital neonatal sepsis and necrotizing enterocolitis. These conditions were diagnosed according to definitions previously described in detail [55].

Statistical analysis

Non-parametric analyses were used. Proportions were compared using Fisher's exact test. The Kruskal-Wallis test and Jonckheere-Terpstra test were used for comparison of continuous variables and linear-by-linear association was used for comparison of the proportions among the three groups. A P-value <0.05 was considered as significant. SPSS 22.0 for Windows (IBM, Armonk, NY, USA) was used for statistical analyses.

Results

A total of 73 patients met the inclusion criteria. Amniotic fluid was not available for MMP-8 determination in two cases; therefore, these patients were excluded from further analysis. Seventeen women comprised group 1 (extreme preterm PROM group, gestational age <26 weeks), 39 comprised group 2 (moderate preterm PROM group, gestational age from 26.0 weeks to 33.9 weeks) and 15 comprised group 3 (late preterm PROM group, gestational age from 34.0 weeks to 36.9 weeks). Table 1 displays the clinical characteristics and pregnancy outcomes according to the gestational age at amniocentesis. There were

Table 1: Clinical characteristics and pregnancy outcomes according to gestational age at amniocentesis.

Characteristics	Gestational age at amniocentesis			P-value
	–25.9 weeks (n=17)	26–33.9 weeks (n=39)	34–36.9 weeks (n=15)	
Maternal age, years	31.5±4.3	30.3±3.6	29.1±5.2	0.390
Nulliparity	65% (6/17)	51% (20/39)	40% (6/15)	0.492
Gestational age at amniocentesis, weeks	23.3±2.9	30.3±2.4	35.4±0.6	<0.001
Cervical dilatation, cm	0.5±0.9	0.7±0.8	2.2±2.2	0.017
Gestational age at delivery, weeks	25.0±3.6	31.6±2.3	35.5±0.7	<0.001
Male newborns[a]	67% (5/15)	62% (23/37)	43% (6/14)	0.363
Cesarean delivery[b]	31% (5/16)	42% (16/38)	20% (3/15)	0.297
Antenatal corticosteroids use	71% (12/17)	69% (27/39)	20% (3/15)	0.002
Antenatal antibiotics use	100% (17/17)	97% (38/39)	87% (13/15)	0.129
Clinical chorioamnionitis	12% (2/17)	5% (2/39)	0% (0/15)	0.347

GA, gestational age. Data are presented as mean±standard deviation or % (n/N). [a]Five cases with unavailable data were excluded from the analysis. [b]Two cases with unavailable data were excluded from the analysis.

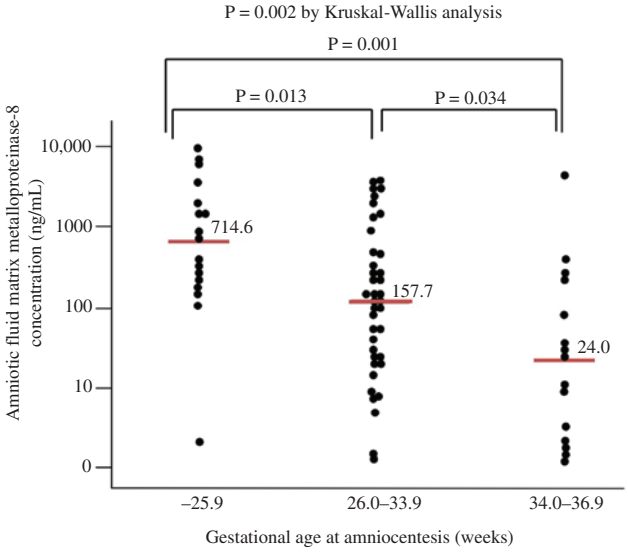

Figure 1: Amniotic fluid matrix metalloproteinase-8 concentrations according to gestational age at amniocentesis (i.e. –25.9 weeks, 26.0–33.9 weeks and 34.0–36.9 weeks) in women with intra-amniotic infection by *Ureaplasma* species (median: 714.6 ng/mL, interquartile range (IQR): 165.8–2681.2 ng/mL vs. median: 157.7 ng/mL, IQR: 24.9–870.8 ng/mL vs. median: 24.0 ng/mL, IQR: 1.8–272.3 ng/mL; P=0.002 by Kruskal-Wallis analysis).

no significant differences in maternal age, parity, rate of cesarean delivery, antibiotics use and neonatal gender among the three groups. Gestational age at delivery and use of antenatal corticosteroids were significantly different among the groups. The prevalence of clinical chorioamnionitis was highest in the extreme preterm PROM group [12% (2/17)], followed by the moderate preterm PROM group [5% (2/39)] and by the late preterm PROM group [0% (0/15)]. However, the difference was not statistically significant (P>0.1).

Figure 1 shows the intensity of the intra-amniotic inflammatory response assessed by the amniotic fluid MMP-8 concentrations according to the gestational age at amniocentesis. The median amniotic fluid MMP-8 concentration in the extreme preterm PROM group was 714.6 ng/mL [interquartile range (IQR): 165.8–2681.2 ng/mL], which was significantly higher than that of the moderate preterm PROM group (median: 157.7 ng/mL, IQR: 24.9–870.8 ng/mL) and that of the late preterm PROM group (median: 24.0 ng/mL, IQR: 1.8–272.3 ng/mL) (P<0.05, respectively).

Table 2 shows that the amniotic fluid MMP-8 concentrations and the WBC counts decrease as a function of advancing gestational age at the time of amniocentesis

Table 2: The amniotic fluid matrix metalloproteinase-8 concentration and amniotic white blood cell count according to gestational age at amniocentesis.

Gestational age at amniocentesis, weeks	Amniotic fluid matrix metalloproteinase-8, ng/mL	P-value[a]	Amniotic fluid white blood cell, cells/mm^3	P-value[a]
–25.9 (n=17)	714.6 (165.8–2681.2)	<0.001	>1000 (384–>1000)	0.010
26.0-33.9 (n=39)	157.7 (24.9–870.8)		372 (22–>1000)	
34.0-36.9 (n=15)	24.0 (1.8–272.3)		101 (3–>1000)	

[a]Jonckheere-Terpstra test. Values are given as median (interquartile range).

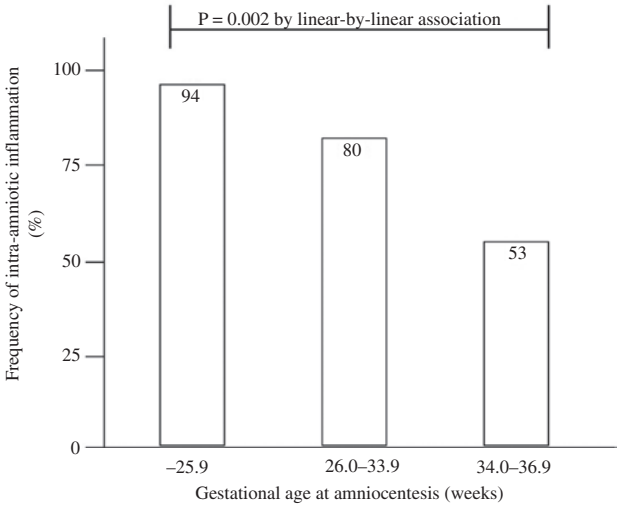

Figure 2: The frequency of intra-amniotic inflammation (defined as an amniotic fluid matrix metalloproteinase-8 concentration >23 ng/mL) according to the gestational age (P = 0.002 by linear-by-linear association.

(P < 0.001 for amniotic fluid MMP-8 concentrations and P = 0.010 for amniotic fluid WBC count by the Jonckheere-Terpstra test). Figure 2 shows the rate of intra-amniotic inflammation defined as an amniotic fluid MMP-8 concentration of >23 ng/mL according to the gestational age at amniocentesis. The rate of intra-amniotic inflammation was 94% (16/17) in the extreme preterm PROM group, 80% (31/39) in the moderate preterm PROM group and 53% (8/15) in the late preterm PROM group. It decreases with advancing gestational age (P = 0.002 by linear-by-linear association).

Table 3 displays the maternal WBC counts and the CRP concentrations according to the gestational age at the time of amniocentesis. There were no significant differences in these maternal inflammatory markers among the groups (P = 0.186 for maternal blood WBC count and P = 0.146 for serum CRP concentrations by the Jonckheere-Terpstra test).

Table 4 presents the correlation between markers of amniotic fluid and a systemic maternal inflammatory response, and gestational age at amniocentesis. The intensity of the amniotic fluid inflammatory response markers was inversely correlated with gestational age (P < 0.001 for amniotic fluid MMP-8 and P = 0.003 for amniotic fluid WBC count). However, the maternal inflammatory markers were not significantly associated with gestational age at the time of amniocentesis (P = 0.103 for maternal blood CRP and P = 0.055 for maternal blood WBC count).

Table 4: Correlation between amniotic fluid and maternal blood inflammatory markers and gestational age at amniocentesis.

	Correlation coefficients in Spearman's rank correlation test	P-value
Amniotic fluid		
Matrix metalloproteinase-8	−0.467	<0.001
White blood cell count	−0.355	0.003
Maternal blood		
C-reactive protein	−0.204	0.103
White blood cell count	−0.247	0.055

Table 5 shows the neonatal outcome according to the gestational age at amniocentesis. The lower the gestational age, the higher the frequency of low Apgar scores of <7 for 1 and 5 min, neonatal death and significant morbidity.

Discussion

Principal findings of this study

(1) The lower the gestational age at the time of PROM, the higher the intensity of the intra-amniotic inflammatory response in women with preterm PROM and intra-amniotic infection caused by *Ureaplasma* spp.; (2) the frequency of intra-amniotic inflammation was 94% (16/17) in the extreme preterm PROM group (gestational age <26 weeks), 80% (31/39) in the moderate preterm PROM group (gestational age between 26.0 and 33.9 weeks) and

Table 3: The maternal blood white blood cell count and serum C-reactive protein concentration according to gestational age at amniocentesis.

Gestational age at amniocentesis, weeks	Maternal blood white blood cell, cells/mm³	P-value[a]	Maternal serum C-reactive protein, mg/dL	P-value[a]
−25.9 (n = 17)	13,730 (9845–17,890)	0.186	1.3 (0.7–3.1)	0.146
26.0–33.9 (n = 39)	11,495 (9318–14,043)		0.5 (0.2–1.6)	
34.0–36.9 (n = 15)	12,335 (8363–15,200)		0.9 (0.2–2.0)	

[a]Jonckheere-Terpstra test. Values are given as median (interquartile range).

Table 5: Neonatal outcome according to gestational age at amniocentesis.

Characteristics	–25.9 weeks (n=16)[a]	26–33.9 weeks (n=37)[b]	34–36.9 weeks (n=15)	P-value
Birth weight	742±430	1645±513	2441±303	<0.001
Apgar score at 1 min <7	100% (16/16)	64.9% (24/37)	20.0% (3/15)	<0.001
Apgar score at 5 min <7	81.3% (13/16)	16.2% (6/37)	0% (0/15)	<0.001
Neonatal death	37.5% (6/16)	13.5% (5/37)	0% (0/15)	0.015
Significant morbidity[c]	91.7% (11/12)	62.2% (23/37)	26.7% (4/15)	0.003
Respiratory distress syndrome	41.7% (5/12)	18.9% (7/37)	0% (0/15)	0.022
Bronchopulmonary dysplasia	72.7% (8/11)	18.8% (6/32)	0% (0/15)	<0.001
Intraventricular hemorrhage	25.0% (3/12)	32.4% (12/37)	14.3% (2/14)	0.422
Necrotizing enterocolitis	25.0% (3/12)	2.7% (1/37)	0% (0/15)	0.011
Proven early neonatal sepsis	8.3% (1/12)	0% (0/37)	6.7% (1/15)	0.236

[a]One case with unavailable data was excluded from the analysis. [b]Two cases with unavailable data were excluded from the analysis. [c]Four neonates who died *in utero* or immediately after birth because of extreme prematurity and thus could not be evaluated with respect to the presence or absence of complications were excluded from the analysis.

53% (8/15) in the late preterm PROM group (gestational age between 34.0 and 36.9 weeks); (3) the intensity of the maternal inflammatory response was not significantly associated with gestational age at amniocentesis.

Intra-amniotic infection by *Ureaplasma* spp.

Ureaplasma spp. are the most frequent isolates from amniotic fluid in patients with preterm labor [15–17, 68–70], preterm PROM [1–11], clinical chorioamnionitis [21, 22], acute cervical insufficiency [18, 20] and a short cervix [71]. Even in asymptomatic mid-trimester patients, isolation of *Ureaplasma* spp. is linked to early preterm delivery [40, 41, 72]. In a primate model of intra-amniotic infection, inoculation of *Ureaplasma* spp. in the amniotic fluid results in initiation of intra-amniotic pro-inflammatory cascade and spontaneous preterm birth [73, 74]. Previous studies showed that intra-amniotic infection caused by *Ureaplasma* spp. has a similar or more intense inflammatory response compared to that caused by other microorganisms [4, 9, 75].

Several studies based on culture methods showed that cervicovaginal colonization of these organisms is associated with preterm birth [76–81]. However, cervicovaginal colonization of these organisms is found in 30–60% of pregnant women [76, 77, 79, 82] and there is no evidence that detection of *Ureaplasma* spp. in cervicovaginal fluid can be helpful in preventing preterm birth. In our previous study [83], bacterial vaginosis but not a positive culture for genital mycoplasmas is a risk factor for spontaneous preterm birth. This is consistent with previous studies [79, 82]. Interestingly, about a half of *Trichomonas vaginalis* isolates from vaginal samples of patients with purulent vaginitis harbor intra-cellular genital *Mycoplasmas* including *M. hominis* and *Ureaplasma* spp. [84].

It is unclear why most intra-amniotic infections are caused by *Ureaplasma* spp. [7, 26]. In this process, it is thought that the host defense system, such as the cervical canal and cervical mucus, plays an important role [85–88]. A solid body of evidence indicates that a short cervical length is associated with intra-amniotic infection/inflammation [68, 71, 89–91] and development of spontaneous preterm birth [68, 90, 92–112]. A recent microbiome study has found considerable greater complexity in the placental membrane community [113–115]. Virulent microorganisms including *Escherichia coli* and *Enterobacter* spp. are frequently found and similar in abundance between term and preterm subjects with and without chorioamnionitis [114]. Of interest is that preterm subjects with severe chorioamnionitis had high abundances of *Ureaplasma* spp. and *Fusobacterium nucleatum* [114, 116]. In our previous study which was directed at twins, *Ureaplasma* spp. were found in both amniotic cavities in all cases with intra-amniotic infections with *Ureaplasma* spp. Contrariwise, most of other microorganisms were found only in the presenting amniotic cavity [117]. Collectively, these findings suggest that *Ureaplasma* spp. could more easily cross the chorioamniotic membrane, thereby reaching the amniotic fluid than did other bacteria.

Host inflammatory response to intra-amniotic infection caused by *Ureaplasma* spp.

In the current study, the intensity of the intra-amniotic inflammatory response was different in cases with intra-amniotic infections with *Ureaplasma* spp. as a function of gestational age. This may be due to a difference in

inoculum size, the host's immunological response, timing and duration of infection, co-infected microorganisms and the presence or absence of any other inflammatory modifiers such as prior immune priming or viral exposure [118]. Previous studies showed that the inoculum size plays a role in determining the intensity of the inflammatory response [2, 119, 120]. Therefore, the differences in the host inflammatory response may largely depend on the burden of these organisms. Another reason may be due to different host-microbe interactions. Some investigators have reported that host genetic background and racial disparity impact the disease outcome during intrauterine infection with *Ureaplasma* spp. [121, 122]. However, these findings cannot explain the result of the current study that the earlier the gestational age, the greater the intensity of the intra-amniotic inflammatory response in women with preterm PROM and intra-amniotic infections with *Ureaplasma* spp.

It is well known that oxytocin plays a central role in spontaneous human labor. Progesterone and estradiol are thought as the primary regulators of oxytocin receptor expression [123, 124]. However, expression of the oxytocin receptor is also regulated by interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF) in uterine smooth muscle cells [125–127]. Other investigators showed that IL-1β and TNF upregulate inflammation-related rapid genes [128], which regulate oxytocin receptor [129, 130]. The level of oxytocin receptor messenger RNA (mRNA) and oxytocin receptor in human myometrium at term is greater than that found in preterm myometrium [131]. Therefore, a more intense inflammatory response may be required to induce labor at early gestation. Considering the survival of newborn, it may be more advantageous to maintain pregnancy in early preterm cases with mild infection/inflammation.

The maternal systemic and intrauterine local inflammation

A strong body of evidence suggests that preterm birth is associated with maternal systemic inflammatory conditions such as appendicitis [132–135], acute pyelonephritis [136–138], influenza [139–141] and sepsis [142]. The rates of spontaneous preterm birth in such conditions are 5.1% in appendicitis [133], 10.3% in acute pyelonephritis [138] and 5–30% in influenza [139]. Of note, among pregnancies with appendicectomy, the spontaneous preterm birth rate is associated with the gestational age at appendicectomy (4.4% with gestational age <24 weeks, 7.5% with gestational age between 24 and 28 weeks and 9.1% with gestational age between 29 and 36 weeks) [133]. This finding supports the view that a more intense inflammatory response is required to induce spontaneous preterm labor at earlier gestational ages.

Most pregnant women deliver at term despite maternal infections such as appendicitis, acute pyelonephritis and influenza. By contrast, most patients with intra-amniotic infection and/or inflammation deliver preterm [2–4, 15, 17, 42, 45, 46, 143–145]. One of the unexpected findings in the current study is that the intensity of the maternal systemic inflammatory response was not associated with the gestational age at PROM. These findings suggest that the local inflammatory response has a more important role in spontaneous preterm birth than did the maternal systemic inflammatory response.

Previous studies have shown that the analysis of maternal inflammatory markers may help predict intra-amniotic infection. However, this does not appear to have clinical utility compared to the assessment of amniotic cavity [146–148]. In a series of studies, the maternal concentration of inflammatory markers including IL-6 and CRP had a sensitivity of 56–79% and a specificity of 59–77% in the identification of intra-amniotic infection [146–148]. Clinical chorioamnionitis based on maternal fever also has only 20–30% sensitivity to detect intra-amniotic infection [4, 147]. In the current study, only 25% of patients had an elevated maternal blood WBC count of >15,000 cells/mm^3. Therefore, it is unlikely that analysis of maternal systemic inflammatory response is able to replace the evaluation of intra-amniotic infection and inflammatory response in women with preterm PROM [149].

Strengths and limitations of the study

The strength of this study is that the relationship between the intensity of intra-amniotic inflammation and gestational age was analyzed in a relatively large population with intra-amniotic infections with *Ureaplasma* spp. A limitation of this study is that the intra-amniotic infection was determined by cultivation techniques. Therefore, some of the cases may have infections with other microorganisms not detected using cultivation techniques [17, 150].

Conclusion

The most important clinical implication of our study is that the intra-amniotic inflammatory response varies as a function of gestational age at the time of PROM in women

with intra-amniotic infection caused by *Ureaplasma* spp. Specifically, the earlier the gestational age at which PROM occurs, the more intense the intra-amniotic inflammatory response.

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