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Sterile and Microbial-associated Intra-amniotic Inflammation in Preterm Prelabor Rupture of Membranes

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Abstract

Objective—The objectives of this study were to: 1) determine the amniotic fluid (AF) microbiology of patients with preterm prelabor rupture of membranes (PROM); and 2) examine the relationship between intra-amniotic inflammation with and without microorganisms (sterile inflammation) and adverse pregnancy outcomes in patients with preterm PROM.

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Declaration of interest:

The authors report no conflicts of interest.

Methods—AF samples obtained from 59 women with preterm PROM were analyzed using cultivation techniques (for aerobic and anaerobic bacteria as well as genital mycoplasmas) and with broad-range polymerase chain reaction coupled with electrospray ionization mass spectrometry (PCR/ESI-MS). AF concentration of interleukin-6 (IL-6) was determined using ELISA. Results of both tests were correlated with AF IL-6 concentrations, and the occurrence of adverse obstetrical/perinatal outcomes.

Results—1) PCR/ESI-MS, AF culture, and the combination of these two tests, each identified microorganisms in 36% (21/59), 24% (14/59) and 41% (24/59) of women with preterm PROM, respectively; 2) the most frequent microorganisms found in the amniotic cavity were *Sneathia species* and *Ureaplasma urealyticum*; 3) the frequency of microbial-associated and sterile intra-amniotic inflammation was overall similar [29% (17/59)]: - however, the prevalence of each differed according to the gestational age when PROM occurred; 4) the earlier the gestational age at preterm PROM, the higher the frequency of both microbial-associated and sterile intra-amniotic inflammation; 5) the intensity of the intra-amniotic inflammatory response against microorganisms is stronger when preterm PROM occurs early in pregnancy; and 6) the frequency of acute placental inflammation (histologic chorioamnionitis and/or funisitis) was significantly higher in patients with microbial-associated intra-amniotic inflammation than in those without intra-amniotic inflammation [93.3% (14/15) vs. 38% (6/16); $p=0.001$].

Conclusions—1) The frequency of microorganisms in preterm PROM is 40% using both cultivation and PCR/ESI-MS; 2) PCR/ESI-MS identified microorganisms in the AF of 50% more women with preterm PROM than did AF culture; and 3) sterile intra-amniotic inflammation was present in 29% of these patients, and it was as or more common than microbial-associated intra-amniotic inflammation among those presenting after, but not before, 24 weeks of gestation.

Keywords

infection; polymerase chain reaction with electrospray ionization mass spectrometry; pregnancy; prematurity; preterm delivery; *Sneathia* sp

Introduction

Prelabor rupture of membranes (PROM) is defined as the spontaneous rupture of the chorioamniotic membranes occurring before the onset of labor [1–14]. When the rupture takes place before 37 weeks of gestation, the condition is known as preterm PROM, which affects approximately 2% of all pregnancies [1,8,9,11,15,16]. The main consequence of preterm PROM is the onset of premature labor and delivery [17–19]. Indeed, preterm PROM occurs in 40% of all spontaneous preterm deliveries, representing a significant contributor to perinatal morbidity and mortality worldwide [2,4,12,20–23].

The clinical management of preterm PROM relies on balancing the benefits of prolonging gestation to reduce adverse events related to prematurity against the risk of intra-amniotic infection and its potential consequences for both mother and infant. The frequency of intra-amniotic infection in patients with preterm PROM in the absence of labor is 20 – 40% [7,24–34]. In contrast, when amniocentesis is performed at the time of the onset of labor, prevalence of intra-amniotic infection as high as 75% has been reported [7]. The

identification of microorganisms in the amniotic fluid (AF) presents a major diagnostic challenge, since the results of culture require several days to obtain, which is too long to inform clinical care.

Here, we describe a recently-developed method (PCR-ESI-MS) which combines broad-range real-time PCR with electrospray ionization mass spectrometry (ESI-MS) for detection and characterization of amplified DNA from bacteria and viruses in AF. The PCR/ESI-MS assay detects and identifies 3400 bacteria and over 40 *Candida* species within 8 hours [35–57]. The ability to early detection of microorganisms in the AF of patients with preterm PROM would allow for timely intervention in order to reduce the risk of maternal infection and perinatal complications. The objectives of this study were to: 1) determine the AF microbiology of patients with preterm PROM; and 2) examine the relationship between intra-amniotic inflammation with and without microorganisms and adverse pregnancy outcomes in patients with preterm PROM using both cultivation and PCR/ESI-MS.

Methods

Study population

This was a retrospective cohort study of women with singleton pregnancies who had the diagnosis of preterm PROM. Patients were identified by searching the clinical database and Bank of Biological Samples of Wayne State University, the Detroit Medical Center, and the Perinatology Research Branch of the *Eunice Kennedy Shriver* National Institutes of Child Health and Human Development (NICHD) (Detroit, MI). The inclusion criteria were: 1) singleton gestation; 2) amniocentesis (trans-abdominal amniocentesis) between 20 and 35 weeks performed for microbiological studies; 3) available AF for the performance of molecular microbiologic studies; and 4) neonatal outcomes were known. Patients were excluded from the study if they had: 1) a chromosomal or structural fetal anomaly; or, 2) placenta previa.

Patients with the diagnosis of preterm PROM were counseled by their treating physicians about the potential value of identifying microorganisms in amniotic fluid. Women who agreed to undergo an amniocentesis were asked to donate additional AF other than that required for clinical studies and allow collection of clinical information for research purposes. Further management of these patients was at the discretion of the attending physician. All patients provided written informed consent and the use of biological specimens and clinical data for research purposes were approved by the Institutional Review Boards of NICHD and Wayne State University.

Clinical definitions

Gestational age was determined by the last menstrual period and confirmed by ultrasound examination, or by ultrasound examination alone if the sonographic determination of gestational age was not consistent with menstrual dating. Preterm prelabor rupture of membranes was diagnosed with a sterile speculum examination with documentation of pooling of amniotic fluid in the vagina in association with a positive nitrazine test and/or and positive ferning tests when necessary. Clinical chorioamnionitis was diagnosed when

maternal temperature was elevated to 37.8° C and two or more of the following criteria were present: uterine tenderness, malodorous vaginal discharge, maternal leukocytosis ($>15,000$ cells/mm³), maternal tachycardia (>100 beats/min), and fetal tachycardia (>160 beats/min) [58,59].

The presence of microorganisms in the amniotic cavity was defined according to the results of AF culture and PCR/ESI-MS (Ibis® Technology - Athogen, Carlsbad, CA) [60–63]. Intra-amniotic inflammation was diagnosed when AF interleukin (IL)-6 concentration was ≥ 2.6 ng/mL [64,65]. Based on the results of AF culture, PCR/ESI-MS and AF concentration of IL-6, patients were classified as: 1) no intra-amniotic inflammation/infection (either using AF culture or PCR/ESI-MS); 2) microbial invasion of the amniotic cavity (MIAC) (identification of microorganisms by either AF cultures or PCR/ESI-MS without intra-amniotic inflammation); 3) microbial-associated intra-amniotic inflammation (combination of MIAC and intra-amniotic inflammation); or 4) sterile intra-amniotic inflammation (an elevated AF IL-6 concentration without evidence of microorganisms using cultivation or molecular methods). Acute histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes [66,67], and acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or Wharton's jelly, using criteria previously described [66–68]. For all newborns, data records regarding morbidity and mortality were reviewed. Neonatal outcome was assessed by measuring composite neonatal morbidity and mortality, defined as the presence of one or more of the following: bronchopulmonary dysplasia, respiratory distress syndrome, necrotizing enterocolitis, intraventricular hemorrhage grade III, and respiratory failure requiring mechanical ventilation. Perinatal mortality (stillbirth and neonatal death) were documented separately.

Sample collection

Patients with the diagnosis of preterm PROM who underwent transabdominal ultrasound-guided amniocentesis for evaluation of intra-amniotic infection were eligible for the study. AF was transported in a capped sterile syringe to the clinical laboratory and cultured for aerobic and anaerobic bacteria, as well as genital mycoplasmas. Evaluation of white blood cell (WBC) count, AF glucose concentration and Gram stain of AF were also performed shortly after collection. AF not required for clinical assessment was centrifuged for 10 minutes at 4°C shortly after the amniocentesis, and the supernatant was aliquoted and stored at -70°C until analysis. Following delivery, the placenta, umbilical cord, and chorioamniotic membranes were collected and the presence or absence of acute histologic chorioamnionitis and/or funisitis was determined.

Detection of microorganisms with cultivation and molecular methods

AF was analyzed using cultivation techniques (for aerobic and anaerobic bacteria as well as genital mycoplasmas) and with PCR/ESI-MS (Ibis® Technology - Athogen, Carlsbad, CA). Briefly, DNA was extracted from 300 μL of AF using a method that combines bead-beating cell lysis with a magnetic-bead based extraction method [69,70]. The extracted DNA was amplified on the bacterial artificial chromosome (BAC) spectrum assay according to the manufacturer's instructions. PCR/ESI-MS can identify 3400 bacteria and 40 *Candida spp.*

which are represented in the platform's signature database [43,50,71]. A total of 200 μ L of extract was used per sample.

For viral detection, the nucleic acids were extracted from 300 μ L of AF using a method that combines chemical lysis with a magnetic-bead based extraction method. The extracted RNA/DNA was amplified on the broad viral assay according to the manufacturer's instructions. In the 8 wells, there were fourteen primer pairs used to detect the following viruses: *Herpes simplex virus 1 (HHV-1)*, *Herpes simplex virus 2 (HHV-2)*, *Varicella-zoster virus (HHV-3)*, *Epstein-Barr virus (HHV-4)*, *Cytomegalovirus (HHV-5)*, *Kaposi's sarcoma-associated herpes virus (HHV-8)*, *Human adenoviruses*, *Human enteroviruses*, *BK polyomavirus*, *JC polyomavirus* and *Parvovirus B19* [71].

After PCR amplification, 30- μ L aliquots of each PCR product were desalted and analyzed via Electrospray-Ionization Time-of-Flight Mass Spectrometer (ESI-MS) as previously described [39,43]. The presence of microorganisms was determined by signal processing and triangulation analysis of all base composition signatures obtained from each sample and compared to a database. Along with organism identification, the ESI-MS analysis includes a Q-score and level of detection (LOD). The Q-score, a rating between 0 (low) and 1 (high), represents a relative measure of the strength of the data supporting identification; only Q-scores ≥ 0.90 were reported for the BAC Spectrum assay [51]. The LOD describes the amount of amplified DNA present in the sample: this is calculated with reference to an internal calibrant, as previously described,[38] and is reported herein as genome equivalents per PCR reaction well (GE/well). The sensitivity (LOD) of the Ibis assay for the detection of bacteria in blood is in average 100 CFU/mL (95% CI, 6 – 600 CFU/mL) [50]. A comparison of detection limits between blood and amniotic fluid showed that the assays have comparable detection limits (100 CFU/mL). The sensitivity (LOD) for the broad viral in plasma ranges from 400 copies/mL to 6600 copies/mL [72]. Detection limits in AF were similar to plasma, ranging from \sim 800 to 1600 copies/mL (depending upon the specific microorganism).

Determination of IL-6 in amniotic fluid

AF concentrations of IL-6 were determined to assess the magnitude of the intra-amniotic inflammatory response. We used a sensitive and specific enzyme immunoassay obtained from R&D Systems (Minneapolis, MN). Briefly, the immunoassay utilized was the quantitative sandwich enzyme immunoassay technique, and the concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The detection limit of the IL-6 assay was 0.09 pg/mL. AF IL-6 concentrations were determined for research purposes, and such results were not used in patient management. We have previously reported the use of IL-6 for the assessment of intra-amniotic inflammation [30,54,60,62,64,73–88].

Statistical analysis

The Kolmogorov-Smirnov test and visual plot inspection were used to assess the normality of continuous data distributions. Patients were stratified by the gestational age at which the ruptured of the membranes (ROM) occurred and according to the presence of intra-amniotic

inflammation with or without detectable microorganisms. Spearman's non-parametric correlation coefficients were calculated. Between-group comparisons were performed using the Kruskal-Wallis and Mann Whitney U tests to examine the differences in arithmetic variable distributions. The X^2 or Fischer's exact test was used to test for differences in proportions, as appropriate. A two-tailed p-value of <0.05 was considered statistically significant. The statistical package used was SPSS v.15.0 (SPSS, Chicago, IL).

Results

Characteristics of the study population

Fifty-nine patients with the diagnosis of preterm PROM were identified. Demographic and clinical characteristics of the study population are displayed in Table I. The median (IQR) gestational age at amniocentesis was 28 (25 – 31) weeks. The distribution of patients according to the gestational age at diagnosis was 23.7% (14/59) before 25 weeks of gestation, 66.1% (39/59) between 25 and <33 weeks, and 10.2% (6/59) between 33 and 35 weeks of gestation (Table I).

Upon admission, 37.3% (22/59) of the patients delivered within 48 hours of membrane rupture. The remaining 37 (62.7%) patients had a latent phase of greater than 48 hours, with 19 patients (32.2%) having a latent phase of more than 14 days. Labor began spontaneously in 28.8% (17/59) of the women and was induced in 50.8% (30/59) of the patients. The route of delivery was vaginal in 56% (33/59) of women and 44% (26/59) of the patients were delivered by cesarean section..

Microbial prevalence and diversity using PCR/ESI-MS and cultivation techniques

Among the study population, 24% (14/59) had a positive microbial culture in AF, and in 36% (21/59) of the cases, PCR/ESI-MS detected genomic material from bacteria or viruses. Microorganisms in AF were identified in 40.6% (24/59) of the patients when combining the results from the two techniques. Figure 1 shows that both PCR/ESI-MS and AF cultures were positive in 18.6% (11/59) of patients, whereas three culture-positive samples (5.1%) were negative by PCR/ESI-MS, and ten (17%) PCR/ESI-MS positive samples were negative by AF culture.

Table II shows the microorganisms identified, the microbial burden (GE/well) reported by PCR/ESI-MS, concentrations of inflammatory markers in AF and pregnancy outcomes for each patient with a positive AF culture and/or PCR/ESI-MS. The most frequent microorganism identified in AF by PCR/ESI-MS was *Sneathia spp.* [28.5% (6/21)], followed by *Ureaplasma parvum* [14.3% (3/21)] and *Ureaplasma urealyticum* [14.3% (3/21)]; the latter was the most common microorganism identified by AF culture (Table II). Among the 24 patients whose AF tested positive by AF culture or PCR/ESI-MS, 15 bacterial species, one fungus and one virus were identified. Of the 15 bacterial taxa identified, four were detected by both AF culture and PCR/ESI-MS (*Ureaplasma sp.*, *Streptococcus pneumoniae*, *Prevotella bivia*, *Haemophilus influenzae*), three were detected only by AF culture (*Bacteroides ureolyticus*, *Lactobacillus sp.*, *Saccharomyces cerevisiae*), and eight were detected by only PCR/ESI-MS (*Sneathia species*, *Ureaplasma parvum*, *Mycoplasma*

hominis, *Streptococcus mitis*, *Gardnerella vaginalis*, *Bacteroides fragilis*, *Rothia mucilaginosa*, *Neisseria gonorrhoeae*). Three cases had positive detection for *Candida albicans*, which was detected by both techniques. One patient had positive detection of *Human enterovirus* [1.7% (1/59)] which was also positive for *Mycoplasma hominis* (Table II).

The frequency of microbial-associated and sterile intra-amniotic inflammation in patients with preterm PROM

Intra-amniotic inflammation (defined as AF IL-6 ≥ 2.6 ng/mL) was identified in 57.6% (34/59) of the cases. When combining the results of AF culture, PCR/ESI-MS and AF IL-6 concentrations, 30.5% (18/59) of patients did not have either intra-amniotic inflammation or infection, 12% (7/59) had MIAC, and 29% (17/59) had microbial-associated intra-amniotic inflammation. Twenty-nine percent (17/59) of the patients had intra-amniotic inflammation without detection of bacteria or viruses using both PCR/ESI-MS and AF cultures, and were thus categorized as having sterile intra-amniotic inflammation.

The prevalence of microbial-associated and sterile intra-amniotic inflammation differed according to the gestational age in which the ruptures of the membranes (ROM) occurred (Figure 2). The earlier the gestational age at ROM occurred, the higher the prevalence of microbial-associated inflammation. Among patients with ROM at <25 weeks of gestations, the frequency of microbial-associated intra-amniotic inflammation was significantly higher than that of sterile intra-amniotic inflammation [64.3% (9/14) vs. 28.6 (4/14); $p=0.005$]. In contrast, microbial-associated intra-amniotic inflammation was present in only 18% (7/39) of patients when the ROM occurred between 25 and <33 weeks of gestation (Figure 2). Two-thirds (4/6) of patients who had ROM between 33 and 35 weeks of gestation did not have evidence of intra-amniotic inflammation (Figure 2).

Among patients with a positive PCR/ESI-MS, there was no correlation between the microbial load from bacteria or viruses [Genome copies per PCR well reaction (GE/well)] and the intensity of the intra-amniotic inflammatory response, (defined by AF concentration of IL-6 and AF WBC count) ($p=0.6$ and $p=0.7$, respectively). However, the median AF IL-6 concentration was significantly higher in patients who presented with preterm PROM at <25 weeks and either a positive AF culture or PCR/ESI-MS than among those who presented between 25 and <33 weeks of gestation [120 (21.5 – 241.8) vs. 3.6 (0.7 – 24) ng/mL; $p=0.008$] (Figure 3).

Pregnancy outcomes according to the results of cultivation and molecular techniques

To determine the clinical relevance of detecting microbial-associated or sterile intra-amniotic inflammation using AF IL-6 concentrations, PCR/ESI-MS and AF culture, pregnancy outcomes were compared among the groups according to the results of the tests. The median (IQR) AF IL-6 concentrations and WBC count in patients with sterile intra-amniotic inflammation were significantly higher than those of patients without intra-amniotic inflammation [AF IL-6: 12 (4.7 – 137) vs. 0.7 (0.5 – 1.1) ng/mL; $p<0.001$; and WBC count: 175 (21 – 395) vs. 1 (0 – 4) cells/mm³; $p<0.001$]. However, there were no significant differences in those parameters (AF IL-6 and WBC) between patients with sterile intra-

amniotic inflammation and those with microbial-associated intra-amniotic inflammation (IL-6; $p=0.1$ and WBC; $p=0.4$) (Table III). The median amniocentesis-to-delivery interval of women with microbial-associated intra-amniotic inflammation was significantly shorter than that of women without intra-amniotic inflammation [median, 3 IQR: 1 – 4 days vs. median, 12 IQR: 1 – 22 days; $p=0.02$].

Neonatal outcomes were known in 96.6% (57/59) of the patients. Neonatal morbid events (assessed by composite neonatal morbidity) were significantly more common in patients with microbial-associated intra-amniotic inflammation than in those without intra-amniotic inflammation [82.4% (14/17) vs. 43.8% (7/16); $p=0.02$] (Table III). Importantly, there was no significant difference in the prevalence of neonatal morbid events between neonates born to mothers with sterile intra-amniotic inflammation and those born to mothers with microbial-associated intra-amniotic inflammation [64.7% (11/17) vs. 82.4% (14/17); $p=0.2$]. Additionally, 17.5% (10/57) of the patients had a neonatal death – five, were periviable gestations, and all were born to mothers with microbial-associated intra-amniotic inflammation.

The relationship between detectable microorganisms in the amniotic fluid and acute histological chorioamnionitis

The extraplacental membranes and umbilical cord were examined in 91.5% (54/59) of the cases; 57.4% (31/54) had acute histologic chorioamnionitis and 42.6% (23/54) had funisitis. The prevalence of acute placental inflammation (histologic chorioamnionitis and/or funisitis) was significantly higher in patients with microbial-associated intra-amniotic inflammation than in patients with either sterile intra-amniotic inflammation or no intra-amniotic inflammation [93% (14/15) vs. 50% (8/16); $p=0.01$ and 93% (14/15) vs. 38% (6/16); $p=0.001$]. However, there were no significant differences in the frequency of acute placental inflammation between patients with sterile intra-amniotic inflammation and those without intra-amniotic inflammation ($p=0.5$; Table III).

Discussion

Principal findings of this study

1) PCR/ESI-MS, AF culture, and the combination of these two tests, each identified microorganisms in 36% (21/59), 24% (14/59) and 41% (24/59) of women presenting with preterm PROM, respectively; 2) the most frequent microorganisms found in the amniotic cavity were *Sneathia species*, *Ureaplasma parvum* and *Ureaplasma urealyticum*; 3) the frequency of microbial-associated and sterile intra-amniotic inflammation was overall similar [29% (17/59)]; - however, the prevalence of each differed according to the gestational age when PROM occurred; 4) the earlier the gestational age at rupture of the membranes, the higher the frequency of both microbial-associated and sterile intra-amniotic inflammation; 5) the intensity of the intra-amniotic inflammatory response (as measured by the AF concentration of IL-6) in the presence of microorganisms was stronger in patients in whom preterm PROM occurs at <25 weeks as opposed to at ≥25 weeks; and 6) the frequency of acute placental inflammatory lesions (histologic chorioamnionitis and/or funisitis) was significantly higher in patients with microbial-associated intra-amniotic

inflammation than in those without intra-amniotic inflammation [93.3% (14/15) vs. 38% (6/16); $p=0.001$]. A major finding of this study is that intra-amniotic inflammation without demonstrable bacteria (sterile inflammation) was frequently identified (31%) in patients with preterm PROM between 25–<33 weeks of gestation.

The importance of microbial invasion of the amniotic cavity in preterm PROM

Microorganisms may gain access to the amniotic cavity in patients with intact membranes, and induce an inflammatory response leading to the production of cytokines [73,75,76,81,89–107], chemokines [108–114], other inflammatory mediators [115–124], and thrombin [125–130]. Microorganisms and their products can also induce the production of matrix degrading enzymes [65,131–137], which have been implicated in the mechanisms responsible for membrane rupture. Matrix metalloproteinases, elastases, cathepsin, etc. can degrade the extracellular matrix, weakening the membranes [136,138–144]. Cytokines, which induce apoptosis such as members of the tumor necrosis factor α super family, may also participate in the mechanisms responsible for membrane rupture as they can induce programmed cell death (TNF α , TNF α soluble receptors [145], FAS and FAS ligand [145–147]). Why some patients with microbial invasion have preterm labor with intact membranes and others have preterm PROM, is not clear. It is possible that genetic factors controlling the composition and quality of extracellular matrix and/or the host inflammatory response (maternal and fetal) play a role. We have previously reported that AF and fetal plasma concentrations of MMP-9 are higher in fetuses with preterm PROM than in those with preterm labor with intact membranes [148]. We have also reported that polymorphisms in MMP-1 [149,150], MMP-8 [151], MMP-9 [152], and *SERPINE1* [153,154] are associated with preterm PROM.

Rupture of the chorioamniotic membranes can also favor secondary microbial invasion of the amniotic cavity [155–158]. Indeed, microorganisms are detected more frequently in the AF as the duration of the latency period lengthens [159]. Specifically, we have previously reported that the frequency of microbial invasion in patients with preterm PROM who were not in labor at admission was 25%; yet, when an amniocentesis was repeated when patients began contracting after a quiescent period, the frequency of positive cultures was close to 75% [7]. Thus, microbial invasion of the amniotic cavity in patients with preterm PROM may also lead to the onset of preterm labor. The long held belief that the initiation of preterm labor in patients with preterm PROM is a sign of infection is grounded in clinical and microbiologic studies. Intra-amniotic infection may also lead to fetal invasion; approximately 30% of babies with preterm PROM have evidence of fetal bacteremia determined by cordocentesis [160] or umbilical cord blood cultures [68,93]. In turn, these microorganisms may elicit a systemic fetal inflammatory response syndrome (FIRS) and place neonates at risk for short- and long-term adverse outcomes [68,93,161–205]. Collectively, the relationship between intra-amniotic infection, preterm labor, fetal infection, and puerperal complications justifies the systematic study of microorganisms in preterm PROM.

Molecular microbiologic techniques to detect microorganisms in the amniotic cavity

The introduction of molecular microbiologic techniques was expected to improve the detection of microorganisms in the amniotic cavity in patients with complications of pregnancy. We have previously reported that 50% of patients with preterm PROM have microbial invasion of the amniotic cavity using a combination of cultivation and molecular techniques [61]. However, such study was conducted using research techniques which are not available for clinical microbiology of the studies in a hospital setting. This is the first study to use PCR/ESI-MS to characterize microbial invasion in patients with preterm PROM. We found that it identified genomic material from bacteria, fungi and viruses in 36% (21/59) of the participants, whereas AF culture was positive for only 24% (14/59) of these women. Thus, these results indicate that the use of PCR/ESI-MS in the AF from patients with preterm PROM results in an increase in the detection of microorganisms in AF by 50%.

PCR/ESI-MS has the potential to reduce the time required to obtain results to 8 hours, compared to 48 – 72 hours for standard AF cultivation. Another advantage of the use of PCR/ESI-MS is the ease of detecting multiple organisms simultaneously, even compared to molecular techniques including specific or broad range PCR. Organisms identified in this study include common pathogens in intra-amniotic infection (*Sneathia amnii* spp., *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma hominis*), and uncommon microorganisms, such as *Rothia mucilaginosa*, *Prevotella bivia*. PCR/ESI-MS has also been used to identify infection in other body sites and fluids, such as blood in cases of bacterial endocarditis [51] or culture negative infections of the central nervous system, such as meningitis [48].

Sneathia amnii was the most commonly identified organism in our study, found in 28.5% of patients. *Sneathia*, a Gram-negative non-motile rod, was previously named “*Leptotrichia sanguinegens*” and is found in the lower genital tract of normal women and those with bacterial vaginosis. We have previously found this microorganism in the amniotic fluid of women in preterm labor [54,60], preterm PROM [61], preeclampsia [62], short cervix [56], and clinical chorioamnionitis at term [57]. Moreover, this microorganism has been isolated in postpartum bacteremia [206,207]. Harwich et al. reported the genomic sequence of *Sneathia*, its morphology, growth requirements, and antibiotic sensitivity [208]. *Sneathia* is sensitive to metronidazole and vancomycin (in contrast to other Gram-negative bacteria, which are resistant to vancomycin) [208]. Our observations highlight the importance of *Sneathia* in intra-amniotic infection.

Ureaplasma species were the second most common microorganism (14.3%) in the amniotic fluid. In previous studies, *Ureaplasma* spp. was the microorganism most frequently isolated from the amniotic fluid with standard microbiologic techniques in patients with preterm PROM [83,209–214], as well as other complications of pregnancy associated with intra-amniotic inflammation [215–219]. Isolation of *Ureaplasma* spp. in the midtrimester is associated with an increased risk of subsequent development of preterm PROM [159,220,221]. Yoon et al. reported that patients with preterm PROM and a positive PCR assay for *Ureaplasma urealyticum* but a negative AF culture had a worse pregnancy outcome and higher frequency of histological chorioamnionitis and funisitis than patients with a sterile culture and a negative PCR [211].

Candida species are common saprophytes in the genital tract, are present in up to 20–25% of pregnant women [222], and have been associated with intra-amniotic infection in patients with and without intrauterine devices [223–225]. In the current study, *Candida albicans* was detected in 5.1% (3/59) of patients with preterm PROM, and two of these three patients had acute histologic chorioamnionitis, which are consistent with our prior reports [61,226]. Fungal infections are important because they are well recognized pathogens implicated in fetal death and serious neonatal complications [227–237], and they require specific anti-microbial agents not generally used in the context of preterm PROM.

The role of viral infection in preterm PROM has not been extensively investigated. Previous studies using PCR-based methods have concluded that viruses are uncommon in the amniotic fluid of normal women in the midtrimester [238–242], as well as in women with preterm PROM [243,244]. In the study herein, PCR/ESI-MS detected one viral infection with an enterovirus. This patient also had a positive PCR in the amniotic fluid for *Mycoplasma hominis*. However, given that viral infection may predispose to bacterial infection [245–248], further investigations of the role of systemic or local viral infections during pregnancy are necessary.

Sterile intra-amniotic inflammation in preterm PROM

Sterile intra-amniotic inflammation, defined by the presence of acute inflammatory response (elevated IL-6) in the absence of detectable microorganisms, has been reported in the subset of patients with preterm labor with intact membranes [54], short cervix [56], and clinical chorioamnionitis at term [57]. Sterile intra-amniotic inflammation is a risk factor for preterm delivery in patients with an episode of preterm labor, and also among those with a short cervix [56]. In this study, patients with preterm PROM and sterile intra-amniotic inflammation presented at a more advanced gestational age than those with microbial associated intra-amniotic inflammation, but earlier than those without intra-amniotic inflammation. Further studies with a larger sample size would be required to determine the clinical significance of sterile intra-amniotic inflammation in preterm PROM. The present series includes only 17 patients, which is insufficient to draw inferences about neonatal outcome. It is noteworthy that sterile intra-amniotic inflammation was characterized by a normal amniotic fluid white blood cell count and glucose concentration in patients with preterm labor [54], short cervix [56], and clinical chorioamnionitis at term [57]. However, in patients with preterm PROM, the amniotic fluid white blood cell count was elevated (median 175) and the glucose was low (median 10 mg/dL), suggesting that there may be differences between sterile intra-amniotic inflammatory process in preterm PROM and other obstetrical syndromes.

The mechanisms responsible for the induction of sterile intra-amniotic inflammation in preterm PROM remain to be determined. We have previously proposed that “danger signals” resulting from cellular stress or necrotic cells may engage pattern recognition receptors (PAMPs) and stimulate an intra-amniotic inflammatory response [55]. The prototypic alarmin, high mobility group box-1 (HMGB-1), is higher in patients with sterile intra-amniotic inflammation and preterm labor, suggesting that alarmins may play a role in this condition [55]. Indeed, IL-1 alpha, an alarmin previously reported in amniotic fluid [91], can

induce labor in pregnant animals [249,250]. A role for the inflammasome in parturition and preterm labor has been recently proposed [251–254].

Insights into the origin of preterm PROM

Although preterm PROM is pragmatically considered a single entity, the data reported herein suggest clinical and pathogenic heterogeneity. The gestational age at preterm PROM was related to the frequency of intra-amniotic inflammation and the specific subtype. Before 25 weeks, intra-amniotic inflammation was present in 90% of patients with preterm PROM, and 64% of cases were due to microbial associated inflammation. However, between 25–<33 weeks of gestation, intra-amniotic inflammation was present in 50%, and infection accounted for only 18% of all cases. Importantly, after 33 weeks of gestation, most cases of preterm PROM was not related to intra-amniotic inflammation at the time of presentation (see Figure 3). These observations indicate that preterm PROM is a group of entities which can be classified according to gestational age, the presence of intra-amniotic inflammation and microbial invasion of the amniotic cavity. This has implications for the understanding of the mechanisms of disease in this important obstetrical complication.

Future directions

The assessment of patients with preterm PROM relies largely on maternal clinical signs and biophysical tests of fetal well-being [255–260]. Therapy is mainly aimed at inducing fetal maturation and antibiotic administration [2,16,261]. The data presented herein suggests that there are substantial differences in the mechanisms responsible [262] for preterm PROM. It is possible that the methods to monitor maternal and fetal health may differ according to the pathologic process operative in cases of preterm PROM with and without infection, and also those without intra-amniotic inflammation. For example, the administration of antibiotics to patients with preterm PROM results in prolongation of the latency period and a reduction in the rate of clinical chorioamnionitis and neonatal sepsis [263–273]. However, studies in which amniocentesis has been performed at the time of admission and after the administration of antibiotics show that antimicrobial agents do not eradicate intra-amniotic infection present at admission, nor prevent subsequent microbial invasion [274]. Whether treatment of patients with antimicrobial agents selected on knowledge of the identity of the microorganism is a more effective strategy remains to be determined. For example, several microorganisms found in the amniotic cavity in patients with preterm PROM are not adequately treated with antimicrobial agents currently administered in the clinical setting. This is also the case for treatment of the infected newborn. Genital mycoplasmas and fungi are not adequately covered by antimicrobial agents generally administered in the neonatal intensive care unit. An important developing area of investigation is unraveling the causes of sterile intra-amniotic inflammation in preterm PROM and also PROM in which there is neither infection nor inflammation.

The diagnosis of intra-amniotic infection and inflammation currently relies on analysis of amniotic fluid for Gram stain [24,25,27,28,275–277], and other rapid tests such as white blood cell count and glucose determination [30,209,277,278], as well as microbial cultures for aerobic and anaerobic bacteria including genital mycoplasmas. However, it is now clear that these tests are not adequate for the rapid diagnosis of microbial invasion of the amniotic

cavity or intra-amniotic inflammation [277–279]. Cultivation techniques are insensitive, and results are typically not available for clinical decision making. The amniotic fluid white blood cell count and glucose determination are not adequate for the diagnosis of sterile intra-amniotic inflammation. Therefore, determination of cytokines and chemokines appear to be necessary to define the presence of intra-amniotic inflammation, and molecular microbiologic techniques are needed for the rapid detection of bacteria or viruses. PCR/ESI-MS can identify bacteria at the species level in 8 hours, bringing state-of-the-art microbiology to clinical obstetrics.

Conclusions

The frequency of microorganisms in preterm PROM is 40% using both cultivation and PCR/ESI-MS. PCR/ESI-MS identified microorganisms in the AF of 50% more women with preterm PROM than did AF culture. Sterile intra-amniotic inflammation was present in 29% of these patients, and it was as or more common than microbial-associated intra-amniotic inflammation among those presenting after, but not before, 25 weeks of gestation.

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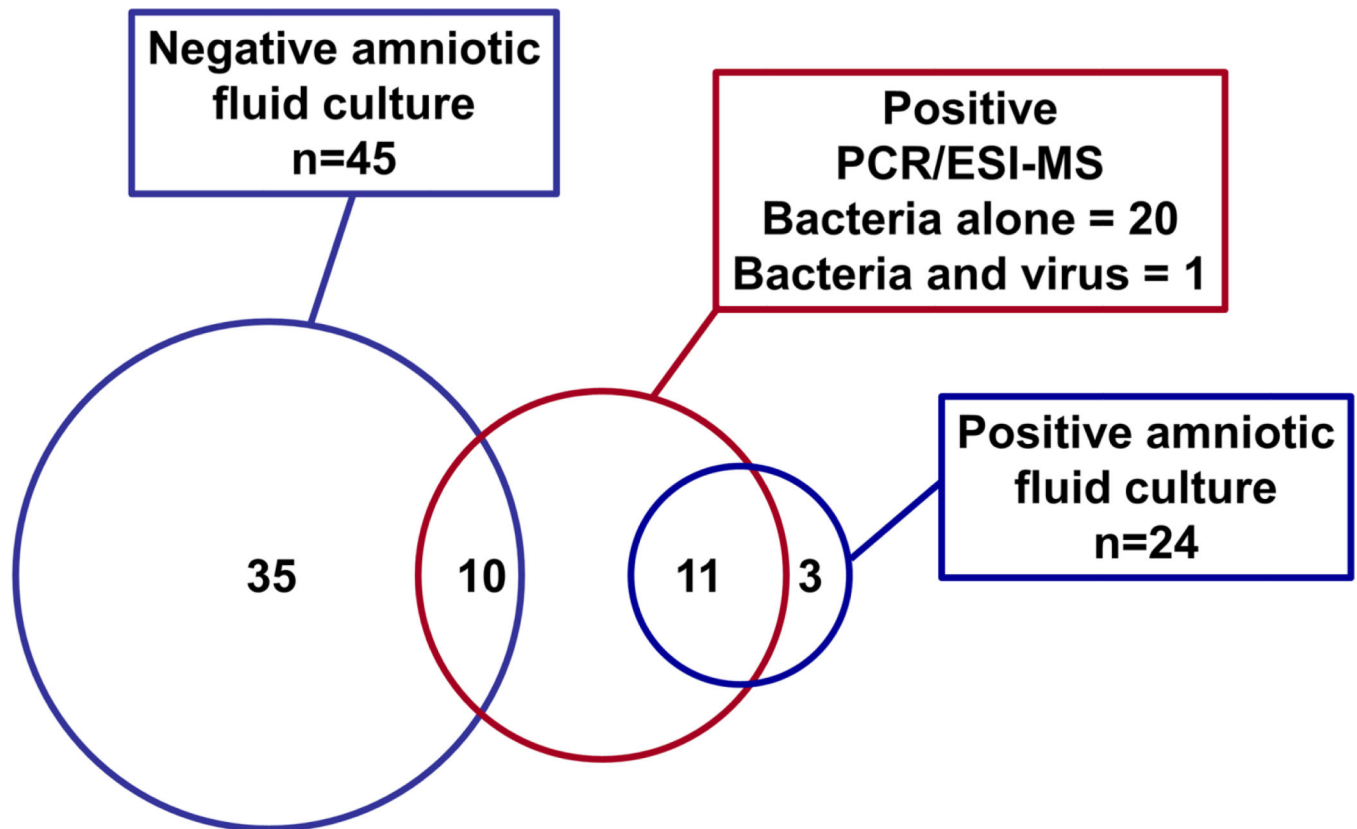


Figure 1.

Bacteria and viruses detected in amniotic fluid of patients with preterm prelabor rupture of membranes standard cultivation techniques vs. PCR/ESI-MS. Amniotic fluid culture includes routine cultivation techniques for bacteria (aerobes, anaerobes, and genital mycoplasmas). PCR/ESI-MS refers to broad range polymerase chain reaction (PCR) and electrospray-ionization mass spectrometry (ESI-MS).

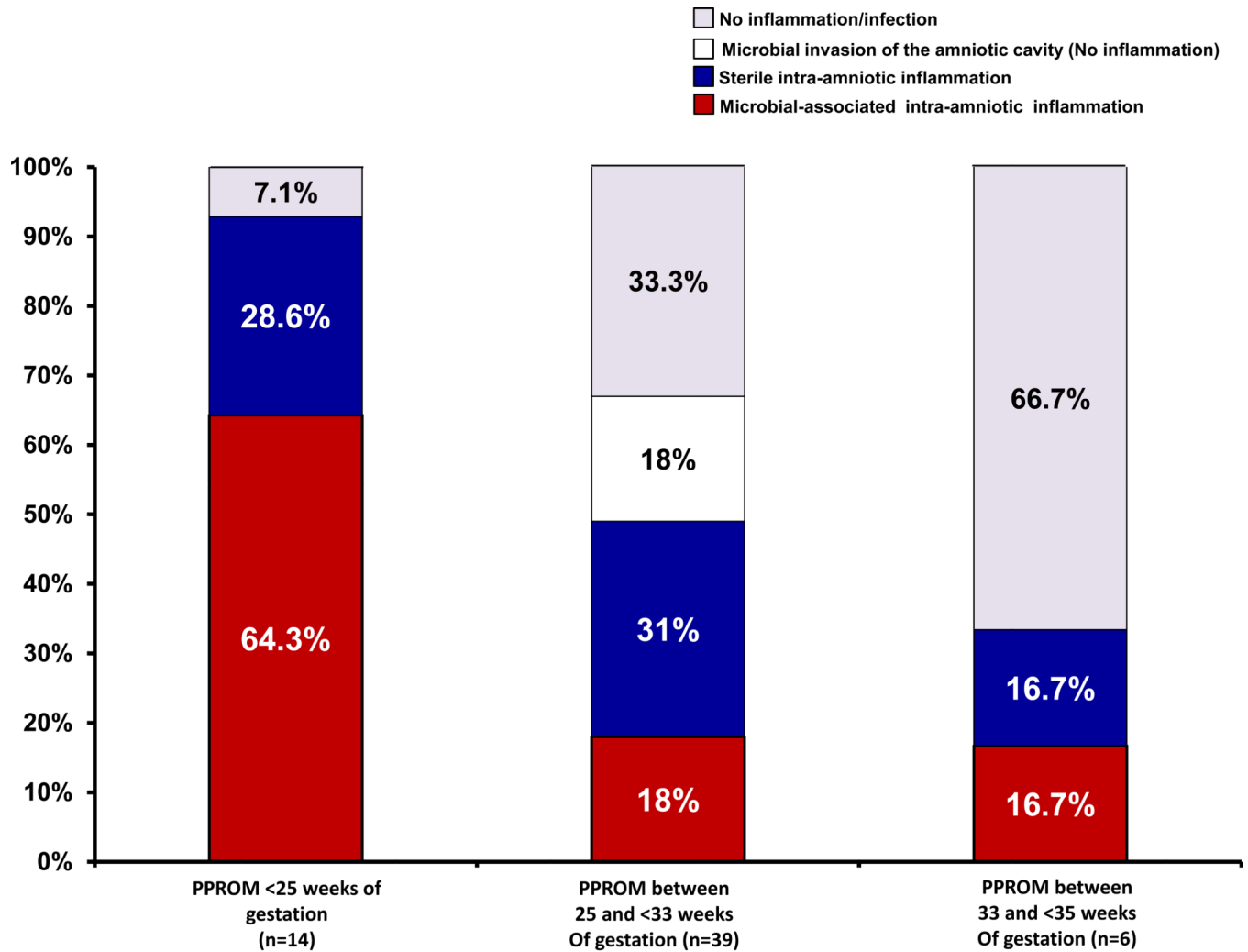


Figure 2.

Prevalence of microbial associated and sterile intra-amniotic inflammation in patients with preterm prelabor premature of membranes according to the gestational age at diagnosis. The earlier the gestational age the rupture of the membranes occurs, the higher the frequency of both microbial-associated and sterile intra-amniotic inflammation.

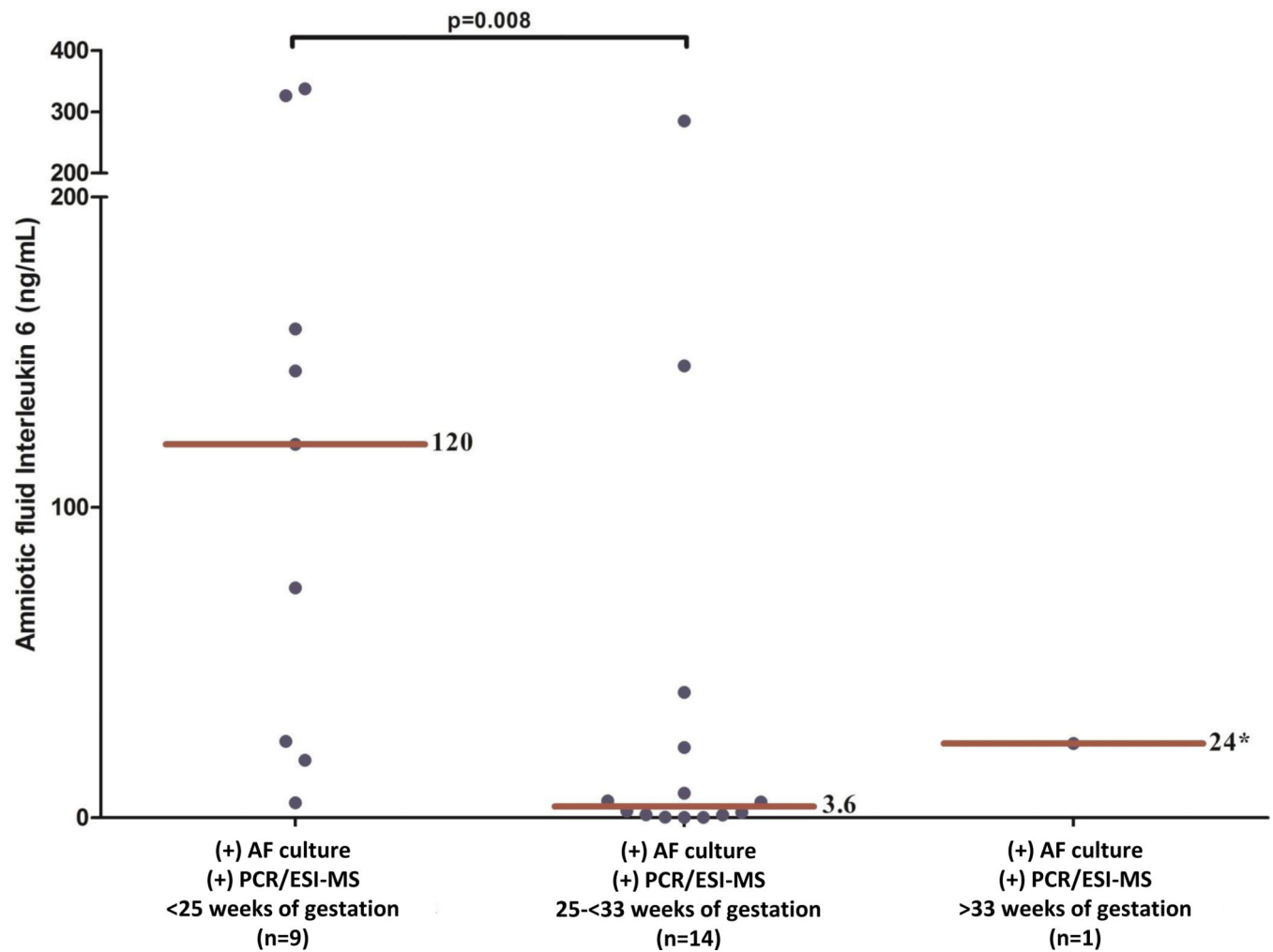


Figure 3.

Amniotic fluid concentrations of interleukin 6 in patients with a positive AF culture or PCR/ESI-MS according to the gestational age in which the rupture of the membranes occurred. Patients who presented with preterm PROM <25 weeks of gestation with a positive AF culture or PCR/ESI-MS had a significantly higher AF IL-6 concentrations than those who presented with a positive AF culture or PCR/ESI-MS between 25 and <33 weeks of gestation.

Table I

Maternal characteristics and demographic data of the study population

| | Median (IQR) or % (n/N) |
|---------------------------------------|------------------------------------|
| Maternal age (years) | 27 (22 – 32) |
| Body mass index (kg/m ²) | 25.1 (21 – 28) |
| Nulliparity | 29 (17/59) |
| Tobacco use during pregnancy | 34 (20/59) |
| Illicit drugs use | 27 (16/59) |
| Cervical dilatation at admission (cm) | 1 (0 – 1.5) |
| GA at amniocentesis (weeks) | 28 (25 – 31) |
| Route of delivery | |
| Vaginal | 56 (33) |
| Cesarean | 44 (26) |
| Clinical signs of chorioamnionitis | 7 (4/59) |

IQR: interquartile range; AF: amniotic fluid; GA: gestational age; PCR: polymerase chain reaction; ESI-MS: electrospray ionization mass spectrometry.

Table II

Amniotic fluid IL-6 concentrations, white blood cell count, placenta pathology results, pregnancy outcome, microorganisms and microbial burden detected in the amniotic fluid of patients with PPROM using cultivation techniques vs. PCR/ESI-MS.

| Group | Amniotic Fluid Culture | PCR/ESI-MS | (GE/well) | AF IL-6 (ng/mL) | AF WBC | GA at delivery | Acute placental inflammation |
|---|--------------------------|------------------------|-----------|-----------------|--------|----------------|------------------------------|
| Microbial-associated intra-amniotic inflammation (n=17) | Bacteroides ureolyticus | Sneathia species | 554 | 5.4 | 10 | 29.7 | Yes |
| | Negative | Sneathia species | 311 | 284.9 | 660 | 27.6 | Yes |
| | Negative | Sneathia species | 156 | 24.6 | 58 | 22.6 | Yes |
| | Ureaplasma spp. | Ureaplasma parvum | 664 | 23.9 | 159 | 33.1 | Yes |
| | Negative | Ureaplasma parvum | 651/194 | 144 | 210 | 23.6 | No information |
| | Negative | Ureaplasma parvum | 2470 | 74.1 | 28 | 23.1 | Yes |
| | Ureaplasma spp. | Ureaplasma urealyticum | 59 | 7.9 | 2 | 27.0 | Yes |
| | Negative | Mycoplasma hominis | 373/>1000 | 22.7 | 158 | 30.3 | Yes |
| | Prevotella bivia | Prevotella bivia | 32/83 | 145.6 | 765 | 27.6 | Yes |
| | Streptococcus pneumoniae | Streptococcus mitis | 217 | 120.4 | 723 | 22.9 | Yes |
| | Candida albicans | Candida albicans | 9 | 337.5 | 225 | 22.3 | Yes |
| | Candida albicans | Candida albicans | 52/62 | 18.5 | 43 | 25.1 | Yes |
| | Negative | Bacteroides fragilis | 207 | 40.4 | 15 | 33.0 | Yes |
| | Negative | Rothia mucilaginosa | 15 | 4.80 | 1 | 22.3 | No information |
| | Bacteroides Ureolyticus | Negative | N/A | 326.2 | 19 | 20.1 | Yes |
| | Streptococcus spp | Negative | N/A | 157.5 | 760 | 24.3 | Yes |
| | Candida albicans | Negative | N/A | 5.1 | 4 | 26.7 | 0 |
| Microbial invasion of the amniotic cavity (No inflammation) (n=7) | Negative | Sneathia species | 38/29 | 0.1 | 10 | 28.4 | No |
| | Negative | Sneathia species | 12 | 0.9 | 68 | 32.1 | Yes |
| | Negative | Sneathia species | 87 | 0.1 | 70 | 30.6 | Yes |
| | Lactobacillus | Ureaplasma urealyticum | 12 | 2.2 | 0 | 31.6 | No |
| | Ureaplasma spp. | Ureaplasma urealyticum | 522 | 0.9 | 20 | 29.6 | No |
| | Saccharomyces cerevisiae | Neisseria gonorrhoeae | 5 | 0.2 | 0 | 34.1 | No |
| | Haemophilus influenzae | Haemophilus influenzae | 173 | 1.8 | 0 | 32.1 | Yes |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

AF: amniotic fluid; GA: gestational age; PCR: polymerase chain reaction; ESI-MS: electrospray ionization mass spectrometry; GE: Genome copies per PCR well reaction; WBC: white blood cell count

Inflammatory markers in amniotic fluid, pregnancy outcome and placenta pathology results in patients with preterm prelabor rupture of membranes according to the results of amniotic fluid cultures and PCR/ESI-MS

Table III

| | No inflammation/infection (n=18) | * p value | Sterile intra-amniotic inflammation (n=17) | p [§] value | Microbial associated intra-amniotic inflammation (n=17) | p [†] value |
|--|--|-----------------|--|-------------------------|--|-------------------------|
| GA at amniocentesis | 31.5 (29.4 – 32.8) | 0.02 | 28.5 (25.1 – 31.7) | 0.04 | 24.8 (22.4 – 27.9) | <0.001 |
| GA at delivery | 33.7 (32.5 – 34.1) | 0.006 | 31.8 (28 – 33) | 0.003 | 25.1 (22.7 – 28.6) | <0.001 |
| AF white blood cell count (cells/mm ³) | 1 (0 – 4) | <0.001 | 175 (21 – 395) | 0.4 | 58 (12.5 – 442) | <0.001 |
| AF glucose (mg/dL) | 25 (13.5 – 31.7) | 0.005 | 10 (10 – 16) | 0.4 | 13 (10 – 22) | 0.02 |
| AF IL-6 (ng/mL) | 0.7 (0.5 – 1.1) | <0.001 | 12 (4.7 – 137) | 0.1 | 40.4 (13.2 – 151.5) | <0.001 |
| Interval amniocentesis to delivery | 12 (1 – 22) | 0.6 | 9 (1 – 22) | 0.06 | 3 (1 – 4) | 0.02 |
| Composite neonatal morbidity | 43.8 (7/16) | 0.2 | 64.7 (11/17) | 0.2 | 82.4 (14/17) | 0.02 |
| Acute placental inflammation | 37.5 (6/16) | 0.5 | 50 (8/16) | 0.01 | 93.3 (14/15) | 0.001 |
| Acute histologic chorioamnionitis | 37.5 (6/16) | 0.5 | 50 (8/16) | 0.01 | 93.3 (14/15) | 0.001 |
| Funisitis | 25 (4/16) | 0.4 | 37.5 (6/16) | 0.1 | 66.7 (10/15) | 0.02 |

IQR: interquartile range; AF: amniotic fluid; GA: gestational age; PCR: polymerase chain reaction; ESI-MS: electrospray ionization mass spectrometry

Data presented as median (interquartile) and percentage and (n); AF: amniotic fluid; IL: interleukin

* Comparison between no inflammation and sterile intra-amniotic inflammation

§ Comparison between patients with sterile intra-amniotic inflammation and microbial-associated intra-amniotic inflammation

† Comparison between patients with no inflammation/infection and microbial-associated intra-amniotic inflammation