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Microbial burden and inflammasome activation in amniotic fluid of patients with preterm prelabor rupture of membranes

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Abstract

Background: Intra-amniotic inflammation, which is associated with adverse pregnancy outcomes, can occur in the presence or absence of detectable microorganisms, and involves activation of the inflammasome. Intra-amniotic inflammasome activation has been reported in clinical chorioamnionitis at term and preterm labor with intact membranes, but it has not yet been investigated in women with preterm prelabor rupture of membranes (preterm PROM) in the presence/absence of detectable microorganisms. The aim of this study was to determine whether, among women with preterm PROM, there is an association between detectable microorganisms in amniotic fluid and intra-amniotic

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inflammation, and whether intra-amniotic inflammasome activation correlates with microbial burden.
Methods: Amniotic fluids from 59 cases of preterm PROM were examined for the presence/absence of microorganisms through culture and 16S ribosomal RNA (rRNA) gene quantitative real-time polymerase chain reaction (qPCR), and concentrations of interleukin-6 (IL-6) and ASC [apoptosis-associated spec-like protein containing a caspase recruitment domain (CARD)], an indicator of inflammasome activation, were determined.
Results: qPCR identified more microbe-positive amniotic fluids than culture. Greater than 50% of patients with a negative culture and high IL-6 concentration in amniotic fluid yielded a positive qPCR signal. ASC concentrations were greatest in patients with high qPCR signals and elevated IL-6 concentrations in amniotic fluid (i.e. intra-amniotic infection). ASC concentrations tended to increase in patients without detectable microorganisms but yet with elevated IL-6 concentrations (i.e. sterile intra-amniotic inflammation) compared to those without intra-amniotic inflammation.
Conclusion: qPCR is a valuable complement to microbiological culture for the detection of microorganisms in the amniotic cavity in women with preterm PROM, and microbial burden is associated with the severity of intra-amniotic inflammatory response, including inflammasome activation.

Keywords: culture; microbial invasion of the amniotic cavity; PPROM (preterm prelabor rupture of membranes); quantitative real-time PCR (qPCR); sterile intra-amniotic inflammation.

Introduction

Microbial invasion of the amniotic cavity can lead to intra-amniotic inflammation (i.e. intra-amniotic infection) [1–25] when bacteria from the lower genital tract gain access to this compartment [6, 26–28]. Yet, in some cases, intra-amniotic inflammation can occur in the absence of detectable microorganisms, a clinical condition referred to as sterile intra-amniotic inflammation [23, 24, 29–32]. Intra-amniotic infection is more common than sterile intra-amniotic inflammation in women with clinical chorioamnionitis at term [24] and preterm prelabor rupture of membranes (preterm PROM) [31, 32]. These clinical conditions are associated with adverse maternal outcomes and increased risk for neonatal sequelae [16, 33–70].

Preterm PROM occurs in approximately 30% of all preterm deliveries [53, 71] and thus represents a major contributing factor to adverse perinatal outcomes associated with prematurity [16, 33–35, 55, 56, 60, 72]. Given that the prevalence of intra-amniotic infection is increased in laboring women with preterm PROM [32, 73], it is tempting to suggest that the process of labor facilitates the ascension of microorganisms into the amniotic cavity [18, 74–77]. In line with this concept, several reports have shown that approximately 40% of women with preterm PROM have intra-amniotic infection [32, 62, 78–80]. Importantly, molecular microbiology is capable of detecting 50% more cases of microbial invasion of the amniotic cavity than conventional microbiological cultures [32]. These results suggest that the syndrome of preterm PROM is a heterogeneous condition that requires further investigation.

The mechanisms that lead to preterm birth following intra-amniotic infection involve a localized inflammatory response, which is partially mediated by the NLRP3 [also known as cryopyrin or NLR (nucleotide-binding domain and leucine-rich repeat) family pyrin domain-containing protein 3] inflammasome [81–85]. Inflammasomes are cytoplasmic multiprotein complexes composed of a sensor molecule, the adapter protein ASC [apoptosis-associated spec-like protein containing a caspase recruitment domain (CARD)], and inactive caspase-1 [86–101]. The assembly of inflammasomes promotes the activation of caspase-1, which subsequently cleaves the immature forms of the pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 into their bioactive forms [102–111]. Upon inflammasome activation, ASC proteins are released into the extracellular space where they can serve as a readout of inflammasome activation *in vivo* [112–114]. Indeed, we have previously shown that increased concentrations of extracellular ASC are observed in amniotic fluid from women with spontaneous labor at term [115] and those with clinical chorioamnionitis at term [116] or with preterm labor with intact membranes [82]. However, whether amniotic fluid concentrations of extracellular ASC can provide a readout of the intra-amniotic inflammatory response in women with preterm PROM has not been investigated.

Herein, in women with preterm PROM, we investigated: (1) the relationship between conventional microbiological cultures and 16S ribosomal RNA (rRNA) gene quantitative real-time polymerase chain reaction (qPCR) signals in amniotic fluid, (2) the association between detection of microbes in amniotic fluid by microbiological cultures and/or 16S rRNA gene qPCR and intra-amniotic inflammation (IL-6 >2.6 ng/mL [14]), and (3) whether

intra-amniotic inflammasome activation (amniotic fluid concentrations of extracellular ASC) correlates with microbial burden (defined as a positive microbiological culture and/or positive 16S rRNA gene signal).

Materials and methods

Study design and population

This retrospective cross-sectional study was conducted by searching our clinical database and bank of biological samples. The collection of samples was approved by the Institutional Review Boards of the Detroit Medical Center (Detroit, MI, USA), Wayne State University, and the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services. All women provided written informed consent prior to the collection of amniotic fluid.

This study included 59 amniotic fluid samples collected from patients with preterm PROM that were initially classified into the following groups (Table 1): (i) preterm PROM with a negative microbiological culture and low amniotic fluid concentrations of IL-6 (<2.6 ng/mL), (ii) preterm PROM with a negative microbiological culture and high amniotic fluid IL-6 (>2.6 ng/mL), (iii) preterm PROM with a positive microbiological culture and high amniotic fluid IL-6, and (iv) preterm PROM with a positive microbiological culture and low amniotic fluid concentrations of IL-6 (see Clinical definitions).

Clinical definitions

Gestational age was determined by the date of the last menstrual period and confirmed by ultrasound examination. The gestational age derived from sonographic fetal biometry was used if the estimation was inconsistent with menstrual dating. Preterm PROM was

Table 1: Clinical and demographic characteristics of patients with preterm PROM.

	Negative culture with low IL-6 (n=18)	Negative culture with high IL-6 (n=19)	Positive culture with high IL-6 (n=8)	Positive culture with low IL-6 (n=14)	P-value
Maternal age, years, median (IQR)[a]	25.5 (22–31)	29 (22.5–32)	21.5 (20.8–26.8)	26.5 (23.3–32)	0.5
Body mass index, kg/m^2, median (IQR)[a]	23.6 (21.4–29)[c]	24 (21–28)[d]	21.8 (19.4–30.9)[c]	21.1 (18.8–25.7)	0.6
Primiparity[b]	22.2% (4/18)	15.8% (3/19)	12.5% (1/8)	14.3% (2/14)	0.9
Race/ethnicity[b]					0.5
African American	100% (18/18)	84.2% (16/19)	87.5% (7/8)	85.7% (12/14)	
White	0% (0/18)	10.5% (2/19)	12.5% (1/8)	7.1% (1/14)	
Hispanic	0% (0/18)	0% (0/19)	0% (0/8)	7.1% (1/14)	
Other	0% (0/18)	5.3% (1/19)	0% (0/8)	0% (0/14)	
Gestational age at membrane rupture, weeks, median (IQR)[a]	31.9 (29.2–32.6)	27.8 (22.8–31.2)[c]	29.5 (27.6–30.4)	31.6 (29.5–33.3)	0.01
Gestational age at amniocentesis, weeks, median (IQR)[a]	32.2 (29.4–32.6)	27.7 (22.4–30.8)	29.5 (27.6–30.4)	31.6 (29.5–33.3)	0.007
IL-6, ng/mL, median (IQR)[a]	0.9 (0.5–1.2)	34.1 (6–161.3)	33.3 (19.9–46)	1 (0.4–1.9)	<0.001
Gestational age at delivery, weeks, median (IQR)[a]	33 (31.1–33.7)	28.7 (23.1–32.6)	30.2 (29–30.9)	31.9 (29.7–33.6)	0.02
Cesarean section[b]	11.1% (2/18)	26.3% (5/19)	25% (2/8)	28.6% (4/14)	0.5
Birthweight, g[a]	1902.5 (1756.3–2115)	1185 (490–1927.5)	1257.5 (1135–1506.3)	1767.5 (1402.5–2095)	0.02
Acute maternal inflammatory response					
Stage 1 (early acute subchorionitis or chorionitis)[b]	17.6% (3/17)[c]	6.3% (1/16)[e]	0% (0/8)	7.1% (1/14)	0.6
Stage 2 (acute chorioamnionitis)[b]	23.5% (4/17)[c]	25% (4/16)[e]	87.5% (7/8)	42.9% (6/14)	0.01
Stage 3 (necrotizing chorioamnionitis)[b]	0% (0/17)[c]	37.5% (6/16)[e]	12.5% (1/8)	14.3% (2/14)	0.02
Acute fetal inflammatory response					
Stage 1 (chronic vasculitis or umbilical phlebitis)[b]	23.5% (4/17)[c]	18.8% (3/16)[e]	25% (2/8)	14.3% (2/14)	0.9
Stage 2 (umbilical arteritis)[b]	17.6% (3/17)[c]	18.8% (3/16)[e]	62.5% (5/8)	28.6% (4/14)	0.1
Stage 3 (necrotizing funisitis)[b]	0% (0/17)[c]	18.8% (3/16)[e]	0% (0/8)	14.3% (2/14)	0.1

Data are given as median (interquartile range, IQR) and percentage (n/N). [a]Kruskal-Wallis test. [b]Fisher's exact test. [c]One missing datum. [d]Two missing data. [e]Three missing data.

defined as amniorrhexis confirmed by vaginal pooling, ferning, or a positive nitrazine test prior to the onset of labor before 37 weeks of gestation [117–120]. Intra-amniotic inflammation was defined using an established cutoff for amniotic fluid concentrations of IL-6 [14], where concentrations >2.6 ng/mL indicate intra-amniotic inflammation and concentrations <2.6 ng/mL are considered as no inflammation.

Amniotic fluid sample collection

Amniotic fluid samples were obtained by transabdominal amniocentesis under antiseptic conditions and ultrasound guidance to evaluate the microbial and inflammatory status of the amniotic cavity. Samples of amniotic fluid were transported to the laboratory in a sterile capped syringe. Clinical tests included culture of aerobic/anaerobic bacteria and genital mycoplasmas [7, 121], white blood cell count [122], Gram stain [123], glucose concentration [124], and IL-6 concentration [14]. The rest of the sample was utilized for research purposes.

Determination of IL-6 concentration in amniotic fluid

Amniotic fluid concentrations of IL-6 were determined as previously established [14] using a sensitive and specific enzyme immunoassay obtained from R&D systems (Minneapolis, MN, USA). The IL-6 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 sensitivity of the IL-6 assay was 0.09 pg/mL.

Placental histopathological examination

Sampling of the placentas was conducted according to protocols established by the Perinatology Research Branch. A minimum of five full-thickness sections of chorionic plate, three sections of umbilical cord, and three chorioamniotic membrane rolls from each case were examined by placental pathologists who were blinded to the clinical histories and additional testing results. Acute inflammatory lesions of the placenta (maternal inflammatory response and fetal inflammatory response) were diagnosed according to established criteria, including staging and grading [125].

DNA extraction from amniotic fluid

Samples of amniotic fluid (250 μL) were processed inside a biological safety cabinet by personnel equipped with sterile surgical gowns, hoods, surgical masks, and powder-free exam gloves (Kimberly-Clark, Roswell, GA, USA). DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (Cat# 12855, Qiagen, Germantown, MD, USA) with minor modifications to the manufacturer's protocol. Briefly, amniotic fluid samples were mixed with 400 μL of bead solution and 200 μL of phenol:chloroform:isoamyl alcohol (pH 7–8) in the supplied bead tube. Next, 60 μL of solution C1 was added, and microbial cells were lysed by mechanical disruption using a bead beater (BioSpec, Bartlesville, OK, USA) for 30 s. Afterward, the bead tubes were centrifuged at 10,000 × *g* for 1 min and the resulting supernatants were transferred to new tubes. Next, 100 μL of solution C2, 100 μL of solution C3 and 1 μL of RNase A enzyme were added to the sample tubes and incubated at 4°C for 5 min. Steps involving solutions C2 and C3 were combined to maximize DNA yield. The sample tubes were centrifuged at 10,000 × *g* for 1 min and the supernatants were transferred to new tubes containing 650 μL of solution C4 and 650 μL of 100% ethanol. Each amniotic fluid lysate was then loaded onto a filter column, centrifuged at 10,000 × *g* for 1 min, and the flow-through was discarded. Next, 500 μL of solution C5 was added to the filter columns and centrifuged at 10,000 × *g* for 1 min, after which the flow-through was discarded and the tube was centrifuged again for an additional 3 min as a dry-spin. Finally, 60 μL of solution C6 was placed on the filter column and incubated for 5 min before centrifuging at 10,000 × *g* for 30 s to elute the extracted DNA. For each set of extractions, at least one blank DNA extraction kit was processed as a background negative control (n = 8). Positive amniotic fluid control samples (n = 6) included DNA extractions performed on amniotic fluid supernatants from six different patients whose amniotic fluid yielded a bacterial isolate by culture. Negative amniotic fluid control samples (n = 7) included seven separate DNA extractions performed on an amniotic fluid sample from a patient previously determined not to have intra-amniotic infection by qPCR. These technical replicates are provided only for perspective and were not included in any statistical analyses in this study. Purified DNA was stored at −80°C.

Establishment of a quantitative real-time PCR for the 16S rRNA gene to determine microbial burden in amniotic fluid

Prior to the performance of qPCR in study samples, a preliminary test was conducted to investigate the existence of DNA amplification inhibition for amniotic fluid [126, 127]. For this test, 3 μL of purified DNA from amniotic fluid samples was serially diluted with solution C6 elution buffer by a factor of 1:3 (i.e. 1:0, 1:3, 1:9). Each qPCR reaction was then spiked with 3 μL of purified *Escherichia coli* ATCC 25922 (GenBank accession: CP009072) genomic DNA (10 pg/μL) containing seven 16S rDNA copies per genome. Genomic DNA was quantified using a Qubit 3.0 fluorometer with a Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Spiked reactions contained approximately 3.989×10^4 *E. coli* 16S rDNA copies, and there was evidence of DNA amplification inhibition (Figure S1). All amniotic fluid samples were subsequently diluted with solution C6 elution buffer by a factor of 1:3 prior to further analyses.

Total bacterial DNA abundance within samples was measured via amplification of the V1–V2 region of the 16S rRNA gene according to the protocol of Dickson et al. [128] with minor modifications as previously described [126, 129]. These modifications included the use of a degenerative forward primer (27f-CM: 5′-AGA GTT TGA TCM TGG CTC AG-3′) [130] and a degenerative probe containing locked nucleic acids (+) (BSR65/17: 5′-56FAM-TAA + YA + C ATG +CA + A GT + C GA-BHQ1-3′). Each 20 μL reaction contained 0.6 μM of 27f-CM primer, 0.6 μM of 357R primer (5′-CTG CTG CCT YCC GTA G-3′), 0.25 μM of BSR65/17 probe, 10 μL of 2X TaqMan Environmental Master Mix 2.0 (Life Technologies), and 3 μL of either purified DNA, elution buffer, or nuclease-free water. The total bacterial DNA qPCR was performed using the following conditions: 95°C for 10 min, followed by 45 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s. Duplicate reactions were run for all samples. All samples were run across a total of four runs. Raw DNA amplification

data were normalized to the ROX passive reference dye and analyzed using the on-line platform Thermo Fisher Cloud (Thermo Fisher Scientific, Waltham, MA, USA): Standard Curve (SR) 3.3.0-SR2-build15 using automatic threshold and baseline settings. Cycle of quantification (Cq) values were calculated for each sample based on the mean number of qPCR cycles required to observe an exponential increase in the normalized fluorescence signal.

DNA derived from *E. coli* ATCC 25922 (described earlier) and a *Ureaplasma parvum* isolate previously obtained from an amniotic fluid sample (using the same DNA extraction protocol) was quantified with the use of a Qubit 3.0 fluorometer with a Qubit dsDNA HS Assay kit and used for the generation of standard curves. The *E. coli* standard curve ranged from 1.99×10^{7} to 1.99×10^{1} copies. For the *U. parvum* isolate, it was estimated that its genome has a mass of 4.78×10^{5} kDa and contains two 16S rRNA gene copies. Therefore, the *U. parvum* standard curve ranged from 3.40×10^{6} to 3.40×10^{1} copies. Independently diluted standard curves containing 10-fold serial dilutions (three replicates each) were included in each of the four qPCR runs. The standard curves were used to evaluate the performance of the qPCR assay by estimation of its efficiency based on the slope of regression lines [131]. Analysis of Cq values generated for the standard curves indicated that the average amplification efficiency of the *E. coli* and *U. parvum* assays was 90.32 ± 1.47% [standard deviation (SD)] and 91.31 ± 0.93% (SD), respectively, with similar diagnostic sensitivity as previously observed for the qPCR assay [126, 129]. The regression curves were linear over the entire range of dilutions for both standard curves (Figure S2).

Determination of extracellular ASC in amniotic fluid

Concentrations of extracellular ASC in the amniotic fluid were determined as previously established [82, 115, 116] by using a sensitive and specific enzyme-linked immunosorbent assay (ELISA) kit obtained from LifeSpan Biosciences (Seattle, WA, USA). Amniotic fluid concentrations of ASC were obtained by interpolation from the standard curve. The inter- and intra-assay coefficients of variation were 5.0% and 8.6%, respectively. The sensitivity of the ASC assay was 0.131 ng/mL.

Statistical analysis

Differences in Cq values among samples were evaluated using the Kruskal-Wallis and Mann-Whitney *U* tests. Sequential Bonferroni corrections were applied to all *post hoc* pairwise comparisons. The strength of correlation between ASC concentrations and Cq values in amniotic fluid was evaluated using Spearman's rank-order correlation test. Graphical and statistical analyses were performed in PAST v3.25 (https://folk.uio.no/ohammer/past/).

Results

Characteristics of the study population

Clinical and demographic characteristics of the study population (n = 59) are described in Table 1. There were no differences in maternal age, body mass index, rate of primiparity, race, or the rate of cesarean section among the initial study groups (Table 1). Birthweights, as well as gestational ages at membrane rupture, amniocentesis, and delivery, were significantly different (Table 1). Acute maternal inflammatory responses (stage 2 and stage 3), but not fetal inflammatory responses, were significantly different among the study groups.

The relationship between microbiological culture and 16S rRNA gene qPCR signal

Microbial culture is regarded as the gold standard technique to identify microorganisms in amniotic fluid [6, 9, 23, 73, 78, 132–138]. Therefore, we first assessed whether microbial burden detected by 16S rRNA gene qPCR was capable of detecting bacterial signals in amniotic fluid samples that had a negative or positive microbiological culture. Microbial burden is reported using Cq, which represents the average number of qPCR cycles required to observe an exponential increase in the detected 16S rRNA gene signal. Thus, a lower Cq number is indicative of a greater microbial burden.

Amniotic fluid samples with a positive culture often had a greater microbial burden than those with a negative culture; however, this difference did not reach statistical significance ($P = 0.068$) (Figure 1). This result could potentially be explained by the presence of difficult-to-culture (i.e. fastidious) bacteria in amniotic fluid of women with a negative culture [78, 139–154]. Nonetheless, amniotic fluid samples, regardless of the microbiological culture result, had higher median 16S rRNA gene signals than the kit/extraction controls ($P < 0.0001$) (Figure 1). It is worth mentioning that several samples with a negative culture had similar signals of the 16S rRNA gene compared to kit/extraction controls (samples included in the dotted blue square), indicating that not all samples from cases of preterm PROM have detectable bacteria. Additionally, one sample with a positive amniotic fluid culture had a similar 16S rRNA gene signal compared to kit/extraction controls (sample included in the dotted red square), which could represent a possible laboratory contaminant isolated during microbiological culture (Figure 1). Taken together, these results indicate that increased microbial burden, as determined by 16S rRNA gene signal, is in general associated with a positive microbiological culture result. Yet, 16S rRNA gene qPCR can detect bacterial signals in samples that did not yield bacterial cultivars, thus demonstrating a higher sensitivity of qPCR than culture for detecting microbial invasion of the amniotic cavity.

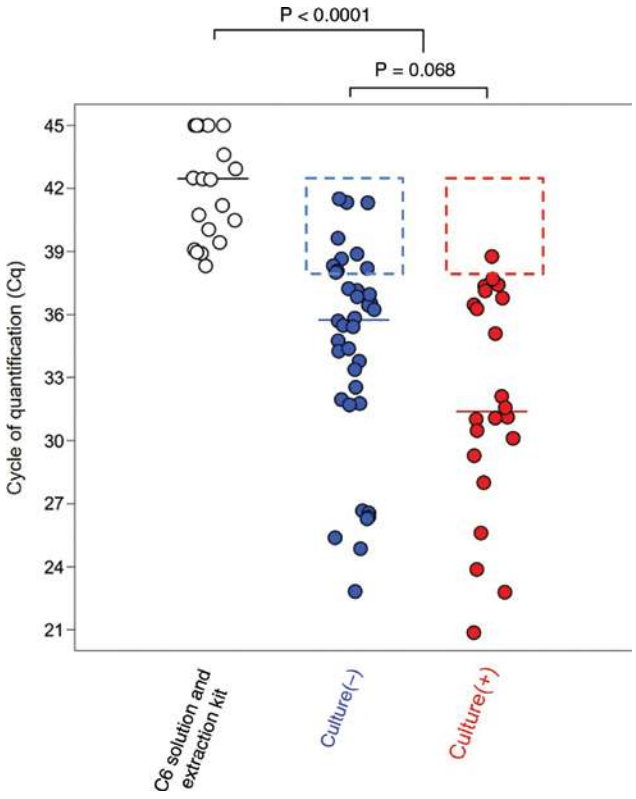

Figure 1: Association between microbiological culture and 16s rRNA gene qPCR in amniotic fluid.
Cycle of quantification (Cq) of background technical controls and amniotic fluid samples from women with preterm PROM based on the presence/absence of a positive microbial culture. Median values are indicated. Statistical results are from the Mann-Whitney *U* tests. Dotted squares represent the absence of microbial detection by 16S rRNA gene qPCR in culture positive/negative amniotic fluid samples. n = 20–37 per group.

The association between microbial detection and intra-amniotic inflammation

We next investigated whether the detection of microorganisms is associated with the IL-6 inflammatory response in amniotic fluid. The amniotic fluid concentration of IL-6 was used as the diagnostic criteria for intra-amniotic inflammation as previously described [14, 155–158]. According to amniotic fluid culture and IL-6 concentrations, we classified the patients into four groups: (1) negative culture with low IL-6 [culture(–)/IL-6(–)]; (2) negative culture with high IL-6 [culture(–)/IL-6(+)]; (3) positive culture with high IL-6 [culture(+)/IL-6(+)]; and (4) positive culture with low IL-6 [culture(+)/IL-6(–)]. We used amniotic fluid samples from a mid-trimester amniocentesis of a patient without preterm PROM or intra-amniotic inflammation as a negative control in addition to water and kit/extraction controls. Amniotic fluid samples from women with positive microbiological cultures but without preterm PROM were used as positive controls. The cutoff for a positive 16S rRNA gene signal was defined as a Cq value less than 34.66, which was determined based on the lowest Cq value among the water and kit/extraction controls (Figure 2).

As described earlier, the overall 16S rRNA gene signal in preterm PROM patients was significantly higher than in kit/extraction controls ($P < 0.001$) (Figure 2). Most of the culture(–)/IL-6(–) patients had a negative (Cq value >34.66) 16S rRNA gene signal (14/18: 77.8%). More than half of the culture(–)/IL-6(+) patients had a positive (Cq value <34.66) 16S rRNA gene signal (11/19: 57.9%) (Figure 2), indicating that non-culturable or fastidious

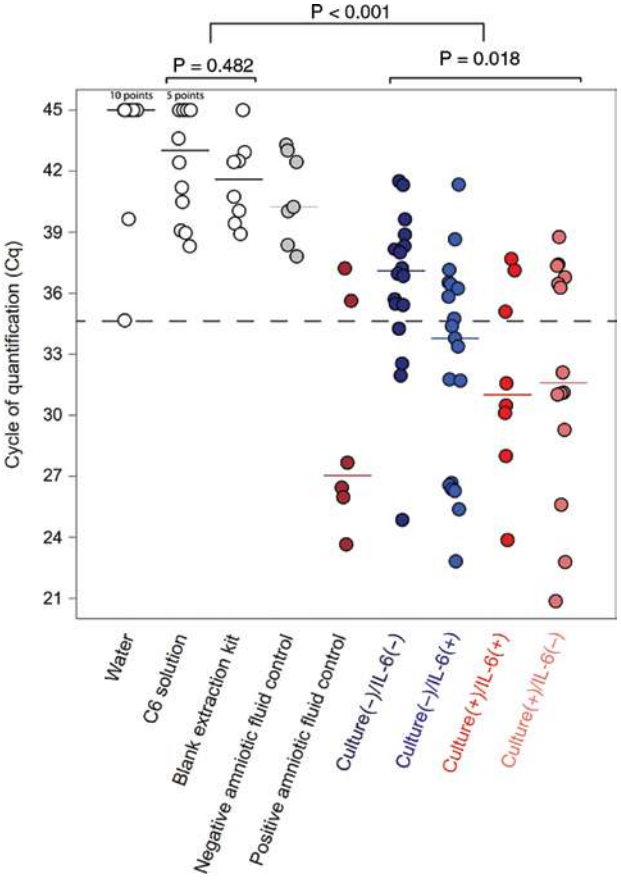

Figure 2: Microbial burden and intra-amniotic inflammation.
Cycle of quantification (Cq) of background technical controls and amniotic fluid samples based on the presence/absence of a positive microbiological culture and intra-amniotic inflammation (IL-6 concentrations >2.6 ng/mL). Median values are indicated. Statistical results are from the Mann-Whitney *U* and Kruskal-Wallis tests. Sequential Bonferroni corrections were applied to all *post hoc* pairwise comparisons. The dashed line indicates the lowest Cq value of any background technical control, which was used to define the cutoff for a positive 16S signal (Cq value <34.66 cycles). n = 12–19 per group.

microorganisms may induce an inflammatory response in the amniotic cavity. Notably, nearly 60% of the patients with a positive culture also had a positive (Cq value <34.66) 16S rRNA gene signal, regardless of the presence of intra-amniotic inflammation [culture(+)/IL-6(+): 5/8, 62.5%; culture(+)/IL-6(−): 8/14, 57.1%] (Figure 2). Indeed, the median 16S rRNA gene signal between patients with a positive culture, regardless of the presence of intra-amniotic inflammation, was similar (Figure 2). These results show that high levels of IL-6 (>2.6 ng/mL) are associated with the detection of microbes by culture or 16S rRNA gene qPCR; however, a subset of preterm PROM patients with detectable microorganisms do not present with intra-amniotic inflammation.

Based on the results of 16S rRNA gene qPCR, we re-stratified our patients into new study groups (Table 2). The first group included cases with both a negative amniotic fluid culture and negative 16S rRNA gene qPCR as well as low IL-6 (n = 14/59, 23.7%), and represented preterm PROM patients with neither detectable bacteria nor intra-amniotic inflammation. The second group included patients with both a negative amniotic fluid culture and negative 16S rRNA gene signal but high IL-6 (n = 8/59, 13.6%), a condition which has been termed sterile intra-amniotic inflammation [23, 29, 30, 32]. The third group included all patients with a positive amniotic fluid culture and/or positive 16S rRNA gene signal together with high IL-6 (n = 19/59, 32.2%), referred to as microbial-associated intra-amniotic inflammation or intra-amniotic infection [23, 29, 30, 32]. Finally, the fourth group included those patients with a positive amniotic fluid culture and/or positive 16S rRNA gene signal with low IL-6 (n = 18/59, 30.5%). These reassigned patient groups are utilized in Figures 3 and 4. These results confirm that preterm PROM is a heterogeneous condition that includes different subsets of patients [31, 32].

The correlation between microbial burden and extracellular ASC concentrations in amniotic fluid (i.e. intra-amniotic inflammasome activation)

Subsequently, we investigated the intra-amniotic inflammatory response in women with preterm PROM by measuring amniotic fluid concentrations of extracellular ASC (i.e. intra-amniotic inflammasome activation). A significant correlation was observed between amniotic fluid ASC concentrations and microbial burden, i.e. low 16S rRNA gene Cq values ($P = 0.013$, $\rho = -0.322$) (Figure 3). Hence, most of the samples with detectable microorganisms (red dots) had elevated concentrations of extracellular ASC, whereas most of the samples without detectable microorganisms (blue dots) had low concentrations of extracellular ASC (Figure 3).

The correlation between microbial burden and extracellular ASC (Figure 3) appeared to be related to the severity of the intra-amniotic inflammatory response, as amniotic fluid ASC concentrations were significantly elevated in patients with intra-amniotic infection [microbe(+)/IL-6(+)] compared to those with a positive bacterial culture/16S rRNA gene signal without intra-amniotic inflammation [microbe(+)/IL-6(−)] or those with neither detectable microorganisms nor intra-amniotic inflammation [microbe(−)/IL-6(−)] (Figure 4). This association was partially independent of the detection of microorganisms given that patients with sterile intra-amniotic inflammation [microbe(−)/IL-6(+)] tended to display higher ASC concentrations in amniotic fluid compared to those with detectable microorganisms without intra-amniotic inflammation [microbe(+)/IL-6(−), non-significant] or those with neither detectable microorganisms nor intra-amniotic inflammation [microbe(−)/IL-6(−), non-significant] (Figure

Table 2: Amniotic fluid sample categorization in patients with preterm PROM.

Category	Bacterial culture	Bacterial qPCR	IL-6 concentration	Number of cases	Percentages
Negative microbial detection and low IL-6	Negative	Negative	Negative	14	23.7%
Negative microbial detection and high IL-6	Negative	Negative	Positive	8	13.6%
Positive microbial detection and high IL-6	Positive	Negative	Positive	3	32.2%
	Negative	Positive	Positive	11	
	Positive	Positive	Positive	5	
Positive microbial detection and low IL-6	Positive	Negative	Negative	6	30.5%
	Negative	Positive	Negative	4	
	Positive	Positive	Negative	8	

Categorization of amniotic fluid samples based on the presence/absence of a positive microbiological culture, positive bacterial 16S rRNA gene quantitative real-time PCR (qPCR), and intra-amniotic inflammation (IL-6 concentrations >2.6 ng/mL). A positive bacterial qPCR result was defined as having a Cq value <34.66 cycles (the lowest Cq value among the negative technical controls).

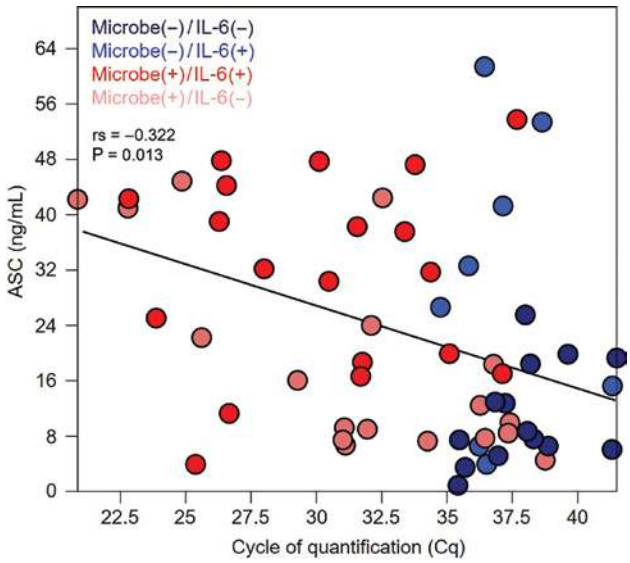

Figure 3: Correlation between microbial burden and extracellular ASC in amniotic fluid.
Extracellular ASC concentration in relation to the cycle of quantification (Cq) of amniotic fluid samples. Categorization of amniotic fluid samples is based on the presence/absence of a positive microbiological culture and/or positive bacterial 16S rRNA gene qPCR, and intra-amniotic inflammation (IL-6 concentrations >2.6 ng/mL). The statistical result is from the Spearman's rank-order correlation test. The regression line is indicated. n = 8–19 per group.

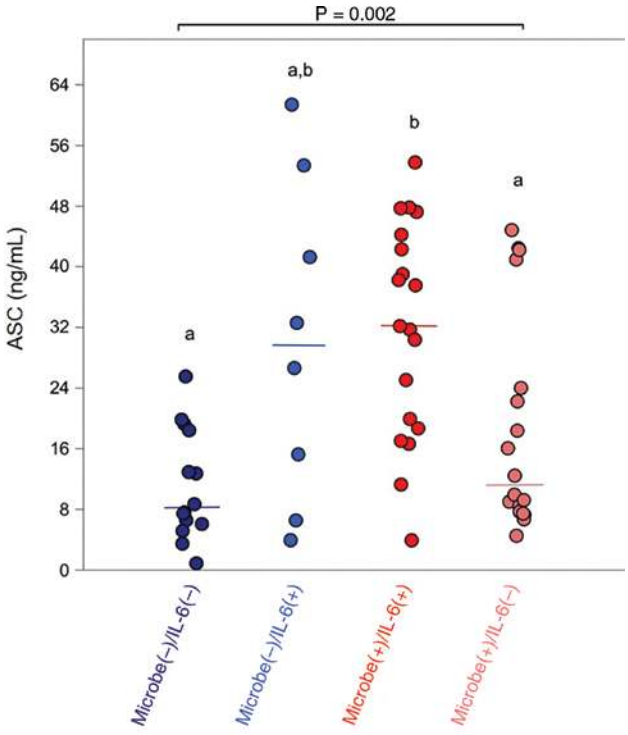

Figure 4: Extracellular ASC concentrations in amniotic fluid of women with preterm PROM in the presence/absence of positive microbial detection and intra-amniotic inflammation.
Extracellular ASC concentrations of amniotic fluid samples based on the presence/absence of a positive microbial culture and/or positive bacterial 16S rRNA gene qPCR, and intra-amniotic inflammation (IL-6 concentrations >2.6 ng/mL). Median values are indicated. Statistical results are from the Kruskal-Wallis and Mann-Whitney *U* tests. Amniotic fluid categories marked by different letters were statistically different after sequential Bonferroni corrections were applied. n = 8–19 per group.

4). These data suggest that the intra-amniotic inflammatory response in patients with preterm PROM involves inflammasome activation, which partially depends on the detection of microbes in the amniotic cavity.

Discussion

Principal findings

In the current study, we report that in patients with preterm PROM: (1) a positive amniotic fluid microbiological culture result was associated with high 16S rRNA gene signal; (2) 16S rRNA gene qPCR can identify a greater number of microbe-positive amniotic fluids than conventional culture; (3) over 50% of patients with a negative culture and high IL-6 in amniotic fluid yielded a positive 16S rRNA gene signal; (4) 16S rRNA gene signal was positively correlated with amniotic fluid concentrations of extracellular ASC; (5) ASC concentrations were greatest in patients with a high positive 16S rRNA gene signal and elevated IL-6 concentrations in amniotic fluid (i.e. intra-amniotic infection); and (6) ASC concentrations tended to increase in patients without detectable microorganisms but yet with elevated IL-6 concentrations in amniotic fluid (i.e. sterile intra-amniotic inflammation) compared to those without intra-amniotic inflammation. Collectively, these results indicate that 16S rRNA gene qPCR can be an effective and valuable complement to microbiological culture for the detection of microbial invasion of the amniotic cavity in women with preterm PROM, and that microbial burden is associated with intra-amniotic inflammation, including the activation of the inflammasome.

Detection of microbial burden in amniotic fluid by 16S rRNA gene qPCR compared to conventional microbiological cultures

Conventional microbiological culture has been widely used to diagnose microbial invasion of the amniotic cavity [2, 3, 5, 6, 9, 73, 122, 123, 132–138, 145, 146, 159–165]. However,

this method has several limitations, most notably the length of time required to obtain results and the variety of microorganisms that can be detected [23, 24, 78, 152, 166, 167]. Clinically, this delay in obtaining culture results has led to the standard practice of administering broad-spectrum antibiotics to patients presenting with inflammation without knowing the specific microorganisms present [168–171]. Recently, the use of advanced molecular microbiological PCR-based techniques was proposed as a solution to these problems [23], as such methods can identify a greater number of microorganisms, including those which may be difficult to culture [78, 139–145, 147–154, 172–174], and the results can be rapidly obtained [23, 175–181]. Molecular microbiological techniques can also rule out false positives obtained by conventional culture likely due to contamination [126]. In line with these previous studies, we report that patients with preterm PROM and a positive amniotic fluid culture have a higher 16S rRNA gene signal than those with a negative culture. More importantly, several of the patients with a negative culture also displayed a high 16S rRNA gene signal, providing further confirmation that molecular microbiological techniques can detect microorganisms in amniotic fluid that are not found using conventional clinical methods.

Microbial detection and intra-amniotic infection

In the current study, we found that preterm PROM patients with a positive culture and intra-amniotic inflammation (diagnosed as the elevated amniotic fluid concentration of IL-6 >2.6 ng/mL) have elevated bacterial burden using 16S rRNA gene qPCR. These results are consistent with previous studies in which women with preterm labor [29, 82], clinical chorioamnionitis at term [116] or preterm PROM [31, 32] and proven intra-amniotic infection display higher levels of IL-6 than those with intra-amniotic inflammation without detectable microorganisms. Patients with a positive culture do not seem to display differences in the intensity of the intra-amniotic inflammatory response, as evidenced by amniotic fluid IL-6 concentrations. This suggests that further investigation of the identities of the different cultured, as well as uncultured, microorganisms in these amniotic fluids, using deep sequencing, is warranted.

In the current study, we also found that some patients with a negative culture had an elevated amniotic fluid IL-6 concentration and detectable 16S rRNA gene signal, suggesting that microorganisms that were not cultured from amniotic fluid, yet still present, may also initiate an intra-amniotic inflammatory response. There are several microorganisms associated with intra-amniotic infection that are difficult to culture in a clinical laboratory setting, namely mycoplasmas (e.g. *Ureaplasma urealyticum*) [23, 32, 66, 79, 143, 182–184], *Sneathia* spp. [23, 32, 66, 152, 184], *Neisseria* spp. [32, 152], and *Fusobacterium nucleatum* [23, 66, 184, 185]. In addition, there are several fastidious species that are known to exist in a viable but non-culturable state [186]. These non-culturable microorganisms can be notably different from their viable counterparts with respect to their metabolic, adhesive, and virulence capacities, as well the biochemical composition of their cell walls and membranes [187–209]. Therefore, it is likely that both the viability and culturable state of the microorganisms in amniotic fluid may affect the severity of the inflammatory response.

The mechanisms whereby microbes invading the amniotic cavity induce high concentrations of IL-6 involve the activation of the nuclear factor-κB (NF-κB) pathway [210–213]. Indeed, *in vitro* studies have shown that incubation of the chorioamniotic membranes with microbial products such as lipopolysaccharide (LPS) triggers the activation of such a pathway [214]. Another potential cellular source of IL-6 in amniotic fluid is the immune cells present in this compartment, particularly monocytes/macrophages [215–218]. Nonetheless, further research is needed to investigate whether viable yet non-culturable microorganisms are sensed by different pattern recognition receptors than culturable microorganisms, leading to distinct inflammatory responses.

Microbial burden correlated with intra-amniotic inflammasome activation

Herein, we showed that there is a significant correlation between 16S rRNA gene signal, microbial burden, and extracellular ASC concentrations in amniotic fluid of patients with preterm PROM. Extracellular ASC has been previously utilized as an *in vivo* indicator of inflammasome activation in amniotic fluid [82, 115, 116]. Indeed, we have recently demonstrated that the concentrations of this protein are increased in the amniotic cavity during the sterile physiological process of spontaneous labor at term [115], as well as in pathological processes such as clinical chorioamnionitis at term [116] and preterm labor/birth [82]. These observations are in line with previous studies showing that inflammasome-related molecules such as caspase-1 [219] and IL-1β are increased in amniotic fluid of women who underwent preterm labor with intact membranes [220–223] or preterm PROM [220]. There are several possible sources for extracellular ASC and other

inflammasome components in the amniotic cavity. First, the chorioamniotic membranes from women in preterm labor with intra-amniotic inflammation/infection express increased levels of inflammasome sensor molecules and the active forms of both caspase-1 and IL-1β, as well as greater numbers of ASC/caspase-1 protein complexes (i.e. enhanced inflammasome assembly) [81]. Second, amniotic fluid of women with intra-amniotic infection contains large numbers of immune cells such as neutrophils and monocytes/macrophages [215–217], which may undergo inflammasome-mediated inflammatory cell death (i.e. pyroptosis) [224]. Together, these data indicate that women with a high microbial burden in the amniotic cavity – intra-amniotic infection – display inflammasome activation. Yet, the sole presence of microorganisms in this compartment may not always result in intra-amniotic inflammasome activation.

In this study, we report that a subset of women with preterm PROM had elevated concentrations of IL-6 and ASC (i.e. intra-amniotic inflammasome activation) in the absence of detectable microorganisms, which has been termed "sterile intra-amniotic inflammation" [23, 29, 30, 32]. This is consistent with previous reports showing that there is evidence of *in vivo* activation of the inflammasome in women with sterile intra-amniotic inflammation and preterm labor/birth [82] or clinical chorioamnionitis at term [116]. The mechanisms leading to sterile intra-amniotic inflammation involve the activation of the NLRP3 inflammasome in the chorioamniotic membranes [81, 82, 85]. Indeed, animal experimentation has shown that alarmins (molecules that trigger sterile inflammation [225–227]) are capable of activating the NLRP3 inflammasome in the fetal membranes [228]. Importantly, these studies have generated promising data showing that, by tackling the activation of the NLRP3 inflammasome, sterile intra-amniotic inflammation can be treated and preterm birth prevented.

It is worth mentioning that, regardless of the nature of the stimuli (microbes and/or alarmins), the single determination of ASC in amniotic fluid does not allow for the identification of the canonical and non-canonical activation of the NLRP3 inflammasome. Yet, *in vivo* concentrations of extracellular ASC provide an overall readout of inflammasome activation in the amniotic cavity.

Microbial detection in amniotic fluid does not always correspond with intra-amniotic infection

A subset of patients with preterm PROM had detectable microbes either by culture or 16S rRNA gene qPCR but had low concentrations of IL-6 and extracellular ASC. This subset of patients are considered to have microbial invasion of the amniotic cavity in the absence of an inflammatory response [23, 24, 29–32, 229–231]. A possible explanation for the lack of inflammation in these patients is that the amniotic fluid sample was collected before the initiation of the inflammatory cascade. However, the detection of microorganisms in the absence of intra-amniotic inflammation may also represent downstream contamination of the amniotic fluid sample. Additional research is required to investigate the clinical significance of the detection of microbes in the amniotic cavity in the absence of an inflammatory response.

Conclusion

In summary, the data presented herein provide evidence that preterm PROM is a heterogeneous condition that can be categorized based on the microbial burden and/or presence of intra-amniotic inflammation. The intra-amniotic inflammatory response is characterized by elevated concentrations of IL-6 as well as enhanced inflammasome activation (i.e. extracellular ASC) in amniotic fluid. These results provide insight into the biological processes occurring in the amniotic cavity of women with preterm PROM, and show that molecular microbiological techniques are valuable complements of conventional microbiological culture for the detection of microbial invasion of the amniotic cavity.

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