OBSTETRICS

Antibiotic treatment reduces the intensity of intraamniotic inflammation in pregnancies with idiopathic vaginal bleeding in the second trimester of pregnancy

Ivana Musilova, MD, PhD; Jaroslav Stranik, MD, PhD; Bo Jacobsson, MD, PhD; Marian Kacerovsky, MD, PhD

BACKGROUND: Idiopathic bleeding in the second trimester of pregnancy complicates <1% of all pregnancies. This pregnancy complication can be caused by alterations in local hemostasis in the decidua due to infection/inflammation in the choriodecidual niche. This condition is associated with intraamniotic inflammatory complications. Antibiotic therapy effectively reduces the intensity of intraamniotic inflammation in certain pregnancy pathologies. However, whether antibiotic administration can reduce the intensity of the intraamniotic inflammatory response or eradicate microorganisms in patients with idiopathic bleeding during the second trimester of pregnancy remains unclear.

OBJECTIVE: This study primarily aimed to determine whether antimicrobial agents can reduce the magnitude of intraamniotic inflammation in patients with idiopathic bleeding in the second trimester of pregnancy by assessing the concentration of interleukin-6 in the amniotic fluid before and after 7 days of antibiotic treatment. The secondary aim was to determine whether treatment with a combination of antibiotics altered the microbial load of *Ureaplasma* species DNA in amniotic fluid.

STUDY DESIGN: This retrospective cohort study included singleton-gestation patients with idiopathic bleeding between 15+0 and 27+6 weeks who underwent transabdominal amniocentesis at the time of admission. Follow-up amniocentesis was performed in a subset of patients unless abortion or delivery occurred earlier. Concentrations of interleukin-6 were measured in the amniotic fluid samples, and the presence of microbial invasion of the amniotic cavity was assessed using culture and molecular microbiological methods. Intraamniotic inflammation was defined as an interleukin-6 concentration ≥3000 pg/mL in the amniotic fluid samples.

RESULTS: A total of 36 patients with idiopathic bleeding in the second trimester of pregnancy were included. All the patients underwent initial amniocentesis. Patients with intraamniotic inflammation (n=25) were treated using a combination of antibiotics consisting of intravenous ceftriaxone, intravenous metronidazole, and peroral clarithromycin. The patients without intraamniotic inflammation (n=11) were treated expectantly. In total, 25 patients delivered 7 days after admission. All patients with intraamniotic inflammation at the initial amniocentesis who delivered after 7 days underwent follow-up amniocentesis. Treatment with antibiotics decreased the interleukin-6 concentration in the amniotic fluid at follow-up amniocentesis compared with that at the initial amniocentesis in patients with intraamniotic inflammation (median [interquartile range]: 3457 pg/mL [2493—13,203] vs 19,812 pg/mL [11,973—34,518]; P=.0001). Amniotic fluid samples with *Ureaplasma* species DNA had a lower microbial load at the time of follow-up amniocentesis compared with the initial amniocentesis (median [interquartile range]: 1.5×10^5 copies DNA/mL [1.3×10^5—1.7×10^5] vs 8.0×10^7 copies DNA/mL [6.7×10^6—1.6×10^8]; P=.02).

CONCLUSION: Antibiotic therapy was associated with reduced intraamniotic inflammation in patients with idiopathic bleeding in the second trimester complicated by intraamniotic inflammation. Moreover, antibiotic treatment has been associated with a reduction in the microbial load of *Ureaplasma* species DNA in the amniotic fluid.

Key words: 16S ribosomal RNA, abortion, amniocentesis, amniotic fluid, bacteria, biomarker, ceftriaxone, chorioamnionitis, clarithromycin, funisitis, genital mycoplasma, inflammation, interleukin 6, intraamniotic infection, microbial invasion of the amniotic cavity, neonatal outcome, nucleic acid, polymerase chain reaction, pregnancy, prematurity, preterm birth, rapid point-of-care test, sterile intraamniotic inflammation, *Ureaplasma*

Introduction

Vaginal bleeding during pregnancy is a relatively common condition that manifests mainly in the first trimester and may affect up to one-fourth of all pregnancies.[1,2] In contrast, vaginal bleeding restricted to the second trimester of pregnancy is less frequent and complicates <1% of all pregnancies.[3] When all major identifiable causes of bleeding, such as placenta previa major, placental abruption, and local (bleeding originating from the introitus, vagina, or cervix) and systemic (coagulopathy) causes are excluded, the remaining cases of bleeding can be classified as idiopathic or unexplained.[4] This subset of cases is associated with an increased risk of adverse maternal and perinatal outcomes due to higher risks of pregnancy complications, such as preterm prelabor rupture of membranes,[4,5] preterm labor with intact membranes,[5,6] oligohydramnios,[3,4] fetal growth restriction,[3,4] small-for-gestational age,[3] and stillbirth.[5]

One possible explanation for idiopathic bleeding in the second trimester of pregnancy is an alteration in local hemostasis in the decidua due to infection/inflammation in the choriodecidual niche.[7] Its presence may also affect the neighboring compartment, the intraamniotic cavity, leading to elevated concentrations of inflammatory mediators in the amniotic fluid (intraamnion

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AJOG at a Glance

Why was this study performed?

This study aimed to assess whether treatment with ceftriaxone, metronidazole, and clarithromycin could attenuate or eradicate intraamniotic inflammation in a subset of patients with idiopathic bleeding in the second trimester complicated by intraamniotic infection or sterile intraamniotic inflammation.

Key findings

Intraamniotic infection and sterile intraamniotic inflammation complicate 36% and 33% of pregnancies with idiopathic bleeding during the second trimester of pregnancy, respectively. Antibiotic treatment was associated with the attenuation and resolution of intraamniotic inflammation in 100% (8/8) and 25% (2/8) of patients with idiopathic bleeding in the second trimester of pregnancy and intraamniotic infection who underwent follow-up amniocentesis, respectively. In 100% (8/8) of these patients, antibiotic treatment was associated with the reduction of amniotic fluid microorganism's DNA load. Antibiotic treatment was also associated with the attenuation and resolution of intraamniotic inflammation in 100% (6/6) and 86% (6/7) of women with idiopathic bleeding in the second trimester of pregnancy and sterile intraamniotic inflammation who underwent follow-up amniocentesis, respectively.

What does this add to what is known?

This study provides evidence that antibiotic treatment with ceftriaxone, metronidazole, and clarithromycin attenuates or resolves intraamniotic inflammation in patients with idiopathic bleeding during the second trimester of pregnancy.

inflammation)[8] and/or the presence of microorganisms in amniotic fluid (microbial invasion of the amniotic cavity [MIAC]). The observations of Gomez et al[7] support this finding. Microorganisms, mainly *Ureaplasma* species (spp), are present in the amniotic fluid of 25% of women with vaginal bleeding between gestational ages of 18 and 28 weeks.[7]

The association between idiopathic bleeding in the second trimester and intraamniotic infection/inflammation is of utmost importance because of accumulating evidence that antibiotic treatment consisting of ceftriaxone, clarithromycin, and metronidazole can be successfully used in pregnancies complicated by: (1) preterm prelabor rupture of membranes,[9–11] (2) spontaneous preterm birth with intact membranes,[11] (3) cervical insufficiency,[10,12] and (4) threatened miscarriage in the second trimester of the pregnancy[13] to reduce or resolve intraamniotic inflammatory complications. However, whether this antibiotic treatment effectively manages intraamniotic inflammation in patients with idiopathic bleeding during the second trimester of pregnancy remains unclear. Given the absence of a specific treatment for this pregnancy complication,[14] this issue is of paramount clinical importance.

Thus, the 2 main objectives of this study were: (1) to determine the effect of antibiotic therapy on the intensity of intraamniotic inflammation, measured by the concentration of IL (interleukin) 6 in the amniotic fluid, in patients with idiopathic bleeding in the second trimester of pregnancy complicated by intraamniotic infection and sterile intraamniotic inflammation, and (2) to evaluate the effect of antibiotic therapy on the microbial load of *Ureaplasma* spp DNA in the amniotic fluid of patients with idiopathic bleeding in the second trimester of pregnancy complicated by intraamniotic infection caused by *Ureaplasma* spp.

Materials and Methods

This retrospective cohort study assessed pregnant patients admitted with active vaginal bleeding in the second trimester of pregnancy to the Department of Obstetrics and Gynecology, University Hospital Hradec Králové, Czech Republic, between January 2020 and February 2023. The study enrolled eligible patients who met the following criteria: (1) age ≥18 years; (2) singleton pregnancy; (3) vaginal bleeding of uterine origin; (4) gestational ages from 15+0 to 27+6 weeks; and (5) written informed consent obtained for transabdominal amniocentesis at the time of admission.

Women were excluded if: (1) the fetus had signs of congenital structural or chromosomal abnormalities; (2) regular uterine activity was present; (3) leakage of amniotic fluid was identified; (4) placenta previa major was confirmed; (5) trauma of the introitus or the vagina was identified as a cause of the bleeding during speculum examination; (6) bleeding originating from the ectocervix observed on speculum examination was revealed as a cause of the bleeding; or (7) systemic disorders such as coagulopathy were present.

The gestational age was established using first-trimester fetal biometry. Vaginal bleeding was confirmed using a sterile speculum to verify the presence of blood flowing through the external os of the cervix. Ultrasound evaluation was performed upon admission to exclude placental localization and abruption. Patients presenting with idiopathic vaginal bleeding in the second trimester of pregnancy underwent amniocentesis to assess the intraamniotic environment (IL6 concentrations and MIAC) as part of standard clinical practice due to a previously published association between vaginal bleeding and inflammatory complications.[7,15] Ultrasound-guided transabdominal amniocentesis was performed at admission, and approximately 3 mL of amniotic fluid was aspirated. Amniotic fluid was immediately dispensed into 3 polypropylene tubes. Tubes containing uncentrifuged samples of amniotic fluid were immediately transported to the biochemistry, molecular biology, and microbiology laboratories for IL6 assessment via polymerase chain reaction (PCR) testing for *Ureaplasma* spp, *Mycoplasma hominis*, and *Chlamydia trachomatis*,

respectively, to evaluate 16S ribosomal RNA (rRNA) and cultivation of amniotic fluid.

Once the results of amniotic fluid IL6 were available (within an hour from amniocentesis) and intraamniotic inflammation was confirmed (a concentration of IL6 in amniotic fluid ≥3000 pg/mL), antibiotic treatment was initiated. Patients received 2-g ceftriaxone every 24 hours intravenously, 500-mg metronidazole every 8 hours intravenously, and 500-mg clarithromycin every 12 hours orally. After 7 days of treatment, follow-up amniocentesis was performed for the patients to evaluate the effect of antibiotic treatment. Treatment with metronidazole lasted for a maximum of 4 weeks unless abortion or delivery occurred. The duration of treatment with ceftriaxone and clarithromycin was based on the attending clinician's discretion, taking at least 4 (ceftriaxone) and 8 (clarithromycin) weeks unless abortion or delivery occurred. Once the results from culture or PCR were known, after consultation with a clinical microbiologist, the attending clinician administered individualized treatment to determine the optimal antibiotic therapy. Women without intraamniotic inflammation (a concentration of IL6 in amniotic fluid <3000 pg/mL) were treated expectantly. The concentration of C-reactive protein in maternal serum and full blood cell counts were determined at the time of admission and subsequently once a week until discharge from the hospital or abortion/delivery. In women admitted at ≥24+0 weeks of gestation, a vaginal-rectal swab is obtained to test for the presence of group B streptococcus. According to the discretion of the attending clinician, a decision was made regarding the initiation of a course of corticosteroid treatment (14-mg betamethasone intramuscularly, 24 hours apart). Women who were positive for the vaginal-rectal presence of group B streptococcus or did not have these results available received intravenous benzylpenicillin (clindamycin in case of penicillin allergy) during active labor.

Written informed consent was obtained from all participants before sampling. This retrospective study was approved by the Institutional Review Board of University Hospital Hradec Králové (March 25, 2023: No. 202306 P01). Women self-reported as White.

Assessment of the concentration of IL6 in amniotic fluid

The IL6 concentration in fresh uncentrifuged amniotic fluid was determined via an automated electrochemiluminescence immunoassay method using the cobas e602 immunoanalyzer, which is part of the cobas 8000 platform (Roche Diagnostics, Basel, Switzerland).[16] The measurement range was 1.5 to 5000 pg/mL, which could be extended to 50,000 pg/mL with a 10-fold sample dilution. This test is available for patient care in Europe and has been approved by regulatory agencies.[17–20]

Detection of *Ureaplasma* spp, *Mycoplasma hominis*, and *Chlamydia trachomatis*

DNA was isolated from the amniotic fluid using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions (using the protocol for isolating bacterial DNA from biological fluids). Real-time PCR was performed on a Rotor-Gene 6000 instrument (QIAGEN) using the commercial AmpliSens *C. trachomatis*/*Ureaplasma*/*M. hominis*-MULTIPRIME-FRT PCR kit (Federal Budget Institute of Science, Central Research Institute of Epidemiology, Moscow, Russia) to detect DNA from *Ureaplasma* spp, *M hominis*, and *C trachomatis* using common PCR tubes. As a control, a PCR run for *ACTB* (actin beta), a housekeeping gene, was performed to assess the presence of inhibitors of the PCR. The number of *Ureaplasma* spp DNA (copies/mL) was quantified using an external calibration curve. Plasmid DNA (pCR4, Invitrogen; Thermo Fisher Scientific, Waltham, MA) was used to prepare the calibration curve. The concentration of *Ureaplasma* spp DNA in copies/L was converted to copies/mL using the following formula: concentration of *Ureaplasma* spp DNA (copies/μL)×elution volume (μL)/input volume (mL).

Detection of nucleic acids of other bacteria in amniotic fluid

Bacterial DNA was identified by PCR targeting 16S rRNA using the following primers: 5-CCAGACTCCTACGGGAG GCAG-3 (V3 region) and 5-ACATTTCA CAACACGAGC-GACGA-3 (V6 region).[21] Each reaction contained 3 μL of target DNA, 500 nM forward and reverse primers, and Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) in a total volume of 25 μL. Amplification was performed using the 2720 Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific). The products were visualized on an agarose gel. Positive reactions yielded 950-bp products that were subsequently analyzed by sequencing. The 16S PCR products were purified and sequenced using the above-mentioned primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific). The bacteria were then typed using sequences obtained from BLAST and SepsiTest BLAST (Molzym GmbH & Co. KG, Bremen, Germany).[22] These tests are routinely offered clinically at our medical center and do not represent research tests.[23]

Aerobic and anaerobic cultures of amniotic fluid

Amniotic fluid samples were cultured on Columbia agar with sheep blood, *Gardnerella vaginalis*—selective medium, MacConkey agar, *Neisseria*-selective medium (modified Thayer—Martin medium), Sabouraud agar, and Schaedler anaerobe agar. The plates were cultured for 6 days and checked daily. Species were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany).

Diagnosis of short-term neonatal morbidity

Neonatal medical records were reviewed by 2 investigators (M.K. and I.M.), and information on short-term neonatal morbidity was recorded.

Clinical definitions

MIAC was defined as the presence of microorganisms detected in amniotic fluid

and/or microbial nucleic acids in the amniotic fluid. **Intraamniotic inflammation** was defined as a concentration of IL6 in the amniotic fluid of ≥3000 pg/mL. **Intraamniotic infection** was defined as the concomitant presence of MIAC and intraamniotic inflammation.[24–26] **Sterile intraamniotic inflammation** was defined as intraamniotic inflammation without MIAC.[24–26] **Negative amniotic fluid for infection/inflammation** was defined as the absence of MIAC and intraamniotic inflammation.[24–26] **Oligohydramnios** was defined as an amniotic fluid index ≤5.0 cm at admission.

Statistical analysis

Demographic characteristics were compared using the nonparametric Kruskal–Wallis or Mann–Whitney *U* tests, as appropriate, for continuous variables and were presented as medians (interquartile range [IQR]). Categorical variables were compared using the chi-square or Fisher exact test, as appropriate, and presented as numbers (percentages). The normality of the data was tested using the Anderson–Darling normality test. The concentrations of IL6 and microbial loads of *Ureaplasma* spp in the amniotic fluid between the initial and follow-up amniocenteses were compared using the Wilcoxon matched-pairs signed-rank test. Differences were considered statistically significant at $P<.05$. All *P* values were obtained from 2-sided tests, and all statistical analyses were performed using GraphPad Prism, Version 9.5.1 for Mac OS X (GraphPad Software, San Diego, CA) or the IBM SPSS Statistics, Version 19.0 statistical package for Mac OS X (IBM Corp., Armonk, NY).

Results

Thirty-six patients met the inclusion criteria, and initial amniocentesis was performed in all patients. The overall rates of MIAC and intraamniotic inflammation were 36% (13/36) and 69% (25/36), respectively. The bacterial species identified in the amniotic fluid during the initial amniocentesis were *Ureaplasma* spp (n=11), *Rothia mucilaginosa* (n=1), and *Streptococcus parasanguinis* (n=1).

Intraamniotic infection and sterile intraamniotic inflammation were diagnosed in 36% (13/36) and 33% (12/36) of the women, respectively. The demographic and clinical data of patients with intraamniotic infection, sterile intraamniotic inflammation, and no intraamniotic inflammation are shown in Table 1. Patients with intraamniotic infection had the highest rate of oligohydramnios at the time of admission, the shortest interval between admission and abortion/delivery, the lowest gestational age at abortion/delivery, the lowest abortion/birthweight, and the lowest rate of placental findings consistent with maternal vascular malperfusion. In contrast, women without intraamniotic inflammation had the highest rate of deliveries at term, the lowest rate of Apgar scores <7 at 5 and 10 minutes, and placental findings consistent with acute inflammatory lesions.

There were 74% (25/36) of patients who delivered >7 days after admission (60% [14/25] of patients with intraamniotic inflammation from the initial amniocentesis and 100% [11/11] without intraamniotic inflammation from the initial amniocentesis). All women with intraamniotic inflammation from the initial amniocentesis who delivered >7 days after admission underwent follow-up amniocentesis (14/14). A flowchart of the patient selection is shown in Figure 1. The demographical and clinical data of patients with intraamniotic infection and sterile intraamniotic inflammation from the initial amniocentesis who delivered/aborted ≤7 days and delivered >7 days after admission without or with follow-up amniocentesis are shown in the Supplemental Table.

The effect of antibiotic therapy on intraamniotic inflammation in a subset of patients with intraamniotic inflammation

In the subset of patients with intraamniotic inflammation who underwent follow-up amniocentesis and delivered >7 days after admission (n=14), the concentrations of IL6 in the amniotic fluid were lower in the samples from follow-up amniocentesis than in those from initial amniocentesis (initial: median [IQR], 19,812 pg/mL [11,973–34,518] vs follow-up: 3457 pg/mL [2493–13,203]; $P=.0001$) (Figure 2, A). Notably, diminishing concentrations of IL6 in the amniotic fluid and resolution of intraamniotic inflammation were observed in 100% (14/14) and 50% (7/14) of the patients, respectively.

The effect of antibiotic therapy on intraamniotic inflammation in a subset of patients with intraamniotic infection

In the subset of patients with intraamniotic infection from the initial amniocentesis who delivered >7 days after admission (n=8), concentrations of IL6 in the amniotic fluid were lower in the samples from the follow-up amniocentesis than in those from the initial amniocentesis (initial: median [IQR], 27,881 pg/mL [14,922–50,000] vs follow-up: 12,424 pg/mL [3801–29,627]; $P=.008$) (Figure 2, B). In this subset of patients, diminishing intensity of intraamniotic inflammation and resolution of intraamniotic inflammation after antibiotic treatment were observed in 100% (8/8) and 25% (2/8) of patients, respectively.

In this subset of patients, 88% (7/8) had proven *Ureaplasma* spp DNA in amniotic fluid samples from both initial and follow-up amniocenteses. After antibiotic treatment, the microbial load of *Ureaplasma* spp DNA in amniotic fluid was lower (initial: median [IQR], 8.0×10^7 copies DNA/mL [6.7×10^6–1.6×10^8] vs follow-up: 1.5×10^5 copies DNA/mL [1.3×10^5–1.7×10^5]; $P=.02$) (Figure 3). One patient had *S parasanguinis* in the amniotic fluid collected from the initial amniocentesis (proven by culture methods); however, the amniotic fluid from the follow-up amniocentesis after antibiotic therapy was negative (by both culture and PCR methods).

The effect of antibiotic therapy in a subset of patients with idiopathic bleeding with sterile intraamniotic inflammation

In the subset of patients with sterile intraamniotic inflammation from the initial amniocentesis who delivered >7 days after admission (n=6), concentrations of IL6 in the amniotic fluid were

TABLE 1
Maternal and clinical characteristics of pregnant patients with idiopathic bleeding in the second trimester of pregnancy according to presence of intraamniotic infection, sterile intraamniotic inflammation and absence of intraamniotic inflammation

Characteristic	Women with intraamniotic infection (n=13)	Women with sterile intraamniotic inflammation (n=12)	Women without intraamniotic inflammation (n=11)	*P* value
Maternal age (y), median (IQR)	33 (29–38)	33 (31–36)	33 (30–37)	.92
Primiparous, n (%)	3 (23%)	4 (33%)	6 (55%)	.27
Bleeding in the first trimester of pregnancy, n (%)	10 (77%)	12 (100%)	10 (91%)	.18
Prepregnancy body mass index (kg/m^2), median (IQR)	24.3 (21.7–27.1)	25.2 (20.2–29.1)	26.1 (19.1–30.1)	.95
Smoking, n (%)	1 (8%)	0 (0%)	0 (0%)	.40
Gestational age at admission (wk), median (IQR)	21+1 (19+6–26+4)	24+2 (21+5–26+0)	21+0 (19+4–26+6)	.75
Length of the cervix at admission (mm), median (IQR)	32 (25–42)	35 (22–44)	39 (30–40)	.51
Oligohydramnios at admission, n (%)	7 (54%)	2 (17%)	0 (0%)	.007[a]
Placenta previa minor at admission, n (%)	6 (46%)	3 (25%)	3 (27%)	.47
Subchorionic hematoma at admission, n (%)	6 (46%)	7 (58%)	5 (45%)	.78
Discolored (blood-stained, brown) amniotic fluid, n (%)	8 (62%)	8 (67%)	2 (18%)	.04[a]
Amniotic fluid IL6 levels at admission (pg/nL), median (IQR)	29,357 (15,959–50,000)	19,812 (7528–31,631)	1117 (348–1594)	<.0001[a]
CRP levels at admission (mg/L), median (IQR)	15.3 (4.4–28.0)	10.6 (5.9–16.6)	3.4 (2.3–16.5)	.16
WBC count at admission ($\times 10^9$ L), median (IQR)	11.9 (10.0–15.3)	12.5 (11.5–16.0)	10.5 (9.4–11.6)	.15
Latency between admission and delivery (d), median (IQR)	14 (3–41)	74 (5–113)	80 (57–146)	.03[a]
Gestational age at delivery (wk), median (IQR)	28+0 (21+2–30+6)	31+2 (24+4–38+5)	37+0 (30+6–40+3)	.007[a]
Subsequent abortion before gestational age of 22+0 wk, n (%)	3 (23%)	0 (0%)	0 (0%)	.06
Subsequent development of PPROM, n (%)	4 (31%)	4 (33%)	2 (18%)	.69
Subsequent development of PTL, n (%)	4 (31%)	4 (33%)	3 (27%)	.95
Subsequent delivery at term, n (%)	1 (8%)	4 (33%)	6 (55%)	.04[a]
Cesarean delivery, n (%)	4 (31%)	6 (50%)	1 (9%)	.10
Birthweight (g), median (IQR)	915 (465–1540)	1480 (680–3428)	3130 (1670–3540)	.01[a]
Apgar score <7; 5 min, n (%)	6 (46%)	3 (25%)	0 (0%)	.03[a]
Apgar score <7; 10 min, n (%)	4 (31%)	3 (25%)	0 (0%)	.13
Acute histologic chorioamnionitis, n (%)	12 (100%)[b]	8 (100%)[c]	2 (40%)[d]	.001[a]

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(continued)

TABLE 1
Maternal and clinical characteristics of pregnant patients with idiopathic bleeding in the second trimester of pregnancy according to presence of intraamniotic infection, sterile intraamniotic inflammation and absence of intraamniotic inflammation *(continued)*

Characteristic	Women with intraamniotic infection (n=13)	Women with sterile intraamniotic inflammation (n=12)	Women without intraamniotic inflammation (n=11)	*P* value
Funisitis, n (%)	8 (67%)[b]	4 (50%)[c]	0 (0%)[d]	.04[a]
Maternal vascular malperfusion, n (%)	3 (25%)[b]	6 (75%)[c]	4 (80%)[d]	.03[a]

Continuous variables were compared using a nonparametric Kruskal–Wallis test. Categorical variables were compared using the chi-square test. Continuous variables are presented as median (IQR) and categorical variables as number (percentage).

CRP, C-reactive protein; *IL6*, interleukin 6; *IQR*, interquartile range; *PPROM*, preterm prelabor rupture of membranes; *PTL*, preterm labor with intact membranes; *WBC*, white blood cells.

[a] Statistically significant results; [b] Data available for 92% (12/13) of patients; [c] Data available for 67% (8/12) of patients; [d] Data available for 45% (5/11) of patients.

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lower in the samples from the follow-up amniocentesis than in those from the initial amniocentesis (initial: median [IQR], 16,209 pg/mL [4033−21,551] vs follow-up: 2690 pg/mL [1868−5794]; *P*=.03) (Figure 2, C). In this subset of patients, diminishing intensity of intraamniotic inflammation and resolution of intraamniotic inflammation after antibiotic treatment were observed in 100% (6/6) and 86% (6/7) of patients, respectively.

Outcomes of patients with idiopathic bleeding with intraamniotic inflammation who underwent a follow-up amniocentesis

Table 2 shows the outcomes of 14 patients with idiopathic bleeding in the second trimester of pregnancy and intraamniotic inflammation who underwent follow-up amniocentesis. The median of gestational age at delivery was 34+5 (IQR, 30+0−39+3) weeks. The rates of preterm prelabor rupture of membranes, spontaneous preterm labor with intact membranes, and delivery at term were 50% (7/14), 14% (2/14), and 36% (5/14), respectively.

Outcomes of patients with idiopathic bleeding without intraamniotic inflammation

All patients without intraamniotic inflammation delivered >7 days after admission. Table 3 shows the outcomes of the 11 patients with idiopathic bleeding in the second trimester of pregnancy without intraamniotic inflammation. The median gestational age at delivery was 37+0 (IQR, 30+6−40+3) weeks. The rates of preterm prelabor rupture of membranes, spontaneous preterm labor with intact membranes, and delivery at term were 18% (2/11), 27% (3/11), and 55% (6/11), respectively.

Discussion

Principal findings

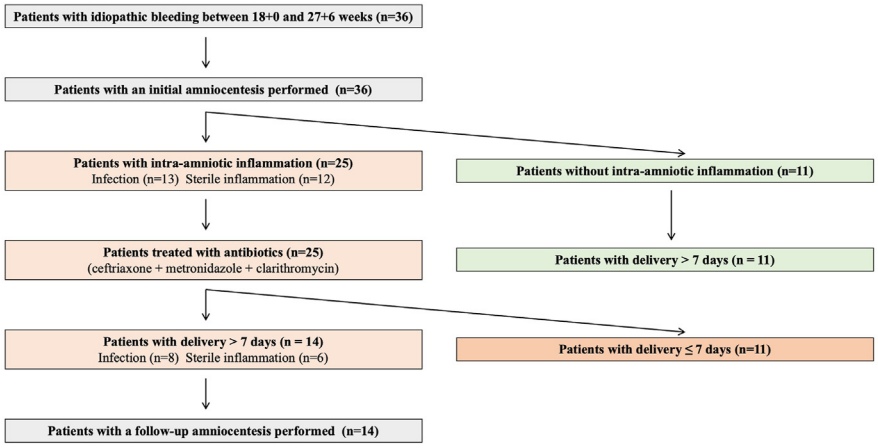
The main findings of this study were the following: (1) intraamniotic inflammation complicates approximately two-thirds of pregnancies with idiopathic bleeding in the second trimester of pregnancy, with intraamniotic infection and sterile intraamniotic inflammation found in 36% and 33% of patients, respectively; (2) in the presence of intraamniotic infection, antibiotic treatment was associated with attenuation of the intensity of intraamniotic inflammation and reduction of the load (measured by gene copy numbers) of amniotic fluid microorganisms in patients with idiopathic bleeding in the second trimester of pregnancy complicated by follow-up amniocentesis; and (3) in the presence of sterile intraamniotic inflammation, antibiotic treatment was associated with attenuation of the intensity of intraamniotic inflammation in patients with idiopathic bleeding in the second trimester of pregnancy who underwent follow-up amniocentesis.

Collectively, these observations provide objective evidence that treatment with ceftriaxone, metronidazole, and clarithromycin can diminish or eradicate intraamniotic inflammation and intraamniotic infection in patients with idiopathic bleeding in the second trimester of pregnancy.

Results in the context of what is known

The first evidence of an association between idiopathic bleeding during pregnancy and inflammation came from pioneering studies describing an association between vaginal bleeding and acute histologic chorioamnionitis.[27,28] Later, Gómez et al[7] published an important observation that a subset of cases with idiopathic bleeding in the second and third trimesters of pregnancy were complicated by the presence of microorganisms in the amniotic fluid and intraamniotic inflammation. They found the presence of microorganisms in amniotic fluid in 14% and 28% of patients with idiopathic bleeding at gestational ages between 18 and 35 weeks and between 18 and 28 weeks, respectively, with *Ureaplasma* spp being the most common bacteria implicated.[7] This observation agreed with the results of the present study, in which intraamniotic infection was observed in 36% of the patients. Except for 1 case of *S*

FIGURE 1
Flow of the patients throughout the study



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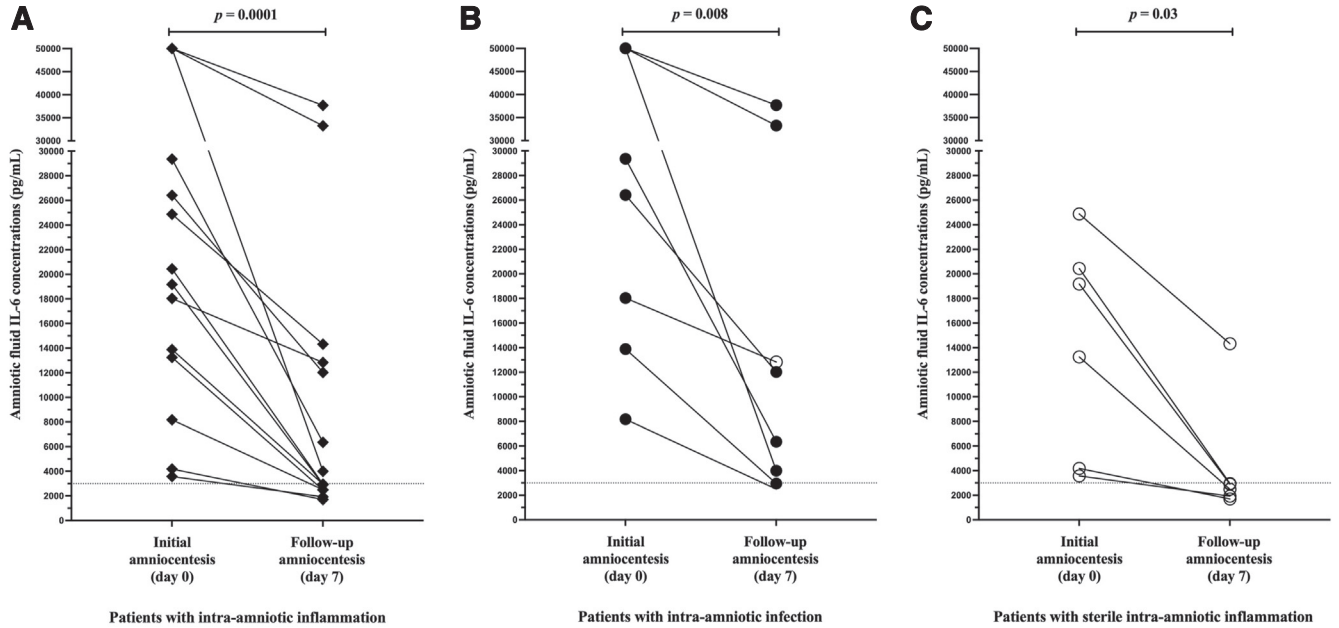
parasanguinis in the amniotic fluid, *Ureaplasma* spp was found in the amniotic fluid of all patients with intraamniotic infection in this study. In addition, the rates of acute histologic chorioamnionitis and funisitis among those with intraamniotic infection did not differ between studies (chorioamnionitis: 91% vs 100%; funisitis: 67% vs 67%).[7] It is important to mention that in the second trimester of pregnancy, not only vaginal bleeding but also uterine contractility can be associated with intraamniotic inflammation and infection.[13]

Interestingly, no data are available on the presence of sterile intraamniotic inflammation, a condition characterized by the presence of intraamniotic inflammation without the simultaneous presence of microorganisms and/or their nucleic acids in the amniotic fluid,[24–26] representing a second clinical phenotype of intraamniotic inflammation. This type of intraamniotic inflammation was previously reported in: (1) 26% of patients with preterm labor with intact membranes,[24] (2) 29% of patients with preterm prelabor rupture of membranes,[25] (3) 10% of patients with sonographic short cervix,[26] and (4) 43% of patients with cervical insufficiency with prolapsed fetal membranes.[29] In this study, sterile intraamniotic inflammation was observed in 33% of the cases,

FIGURE 2
Concentrations of interleukin 6 in amniotic fluid



Subsets of patients who underwent a follow-up amniocentesis and had idiopathic bleeding in the second trimester with: **A,** intraamniotic inflammation, **B,** intraamniotic infection, and **C,** sterile intraamniotic inflammation. Description: (*full diamond*)=intraamniotic inflammation; (*full circle*)=intraamniotic infection; (*empty circle*)=sterile intraamniotic inflammation; and (*dotted line*)=cutoff value of 3000 pg/mL.

IL-6, interleukin 6.

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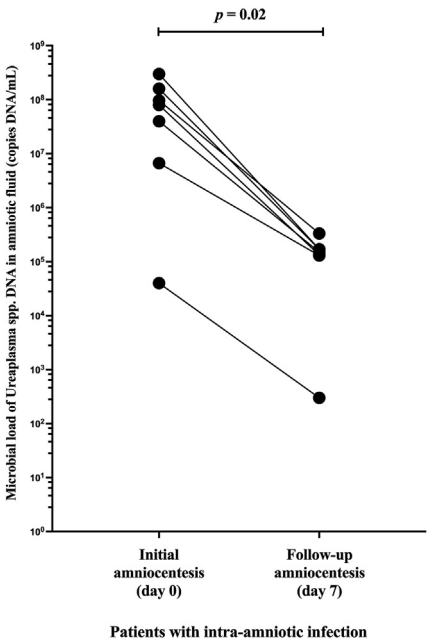
FIGURE 3
Microbial load of *Ureaplasma* spp DNA in amniotic fluid



T subset of patients with idiopathic bleeding in the second trimester of pregnancy with intra-amniotic infection caused by *Ureaplasma* spp who underwent a follow-up amniocentesis.

spp, species.

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almost equal to those with intraamniotic infection.

Antibiotic treatment has been shown to be effective in treating intraamniotic infections and sterile intraamniotic inflammation in the second and third trimesters of pregnancy.[9–13,23,30] In this study, the combination of antibiotics consisting of ceftriaxone, metronidazole, and clarithromycin diminished the intensity of intraamniotic inflammation in 100% of the patients with intraamniotic infection and sterile intraamniotic inflammation in whom follow-up amniocentesis was performed. In the subset of women with intraamniotic infection, in cases of intra-amniotic infection caused by *Ureaplasma* spp, antibiotic therapy diminished both concentrations of IL6 and loads of *Ureaplasma* spp DNA in amniotic fluid. This aligns with our previous observations in women with preterm prelabor rupture of membranes treated with antibiotic monotherapy (intravenous clarithromycin).[23] This observation further supports the rationale for the use of clarithromycin, as a representative macrolide family drug, in combination with antibiotics, given that clarithromycin has: (1) a lower resistance to *Ureaplasma* spp[31] and (2) a higher transplacental rate[32] compared with erythromycin or azithromycin.

Sterile intraamniotic inflammation observed in one-third of the cases in this study was resolved after antibiotic therapy in 86% of the patients who underwent follow-up amniocentesis. We hypothesized that the presence of infection/inflammation localized in the choriodecidual niche leading to the alteration of local hemostasis could: (1) trigger the production of inflammatory mediators from the fetal membranes and release them into the amniotic fluid,[8] (2) damage the fetal membranes by releasing endogenous molecules called alarmins (eg, HMGB1 [high mobility group box 1] protein) into the amniotic fluid, which results in the subsequent development of an intraamniotic inflammatory response through pattern recognition receptors,[25,33–37] or (3) trigger a combination of both mechanisms. The high efficacy of antibiotic treatments for sterile intraamniotic inflammation supports these theories. Their effect on sterile intraamniotic inflammation diminishing/resolution might be explained by: (1) a direct antimicrobial effect on local infection in the choriodecidual niche, (2) the effect of clarithromycin on sterile inflammation by dampening HMGB1-induced inflammation,[38] and (3) the effect of clarithromycin on sterile inflammation through the reduction in the production of cytokines,[39] chemokines,[40] and transcription factors.[41]

Clinical implications

This study found that intraamniotic inflammation complicates more than two-thirds of pregnancies with idiopathic bleeding in the second trimester. We described that a combination of ceftriaxone, metronidazole, and clarithromycin attenuates the intensity of intraamniotic inflammation in patients with pregnancy-related pathology. This is clinically vital and relevant information, particularly in light of the absence of clear recommendations for treating idiopathic bleeding in the second trimester of pregnancy from national or international obstetrics and gynecology societies.[14] The high prevalence of intraamniotic inflammation among cases raises the question of whether the management of this pregnancy complication should be personalized on the basis of the absence or presence of intraamniotic inflammation given that it can be positively affected by antibiotic treatment.

Research implications

This study found placental lesions consistent with maternal vascular malperfusion in 75% and 80% of cases with idiopathic bleeding in the second trimester, with sterile intraamniotic and without intraamniotic inflammation, respectively. This observation is noteworthy because these lesions have thus far been expected in cases of preeclampsia,[42,43] fetal growth restriction,[44] preterm labor with intact membranes,[44] preterm prelabor rupture of membranes,[44] and stillbirth.[45] Recently, the presence of these serious complications (belonging under the umbrella of "great obstetrical syndromes"[46,47]) associated with maternal vascular malperfusion has been shown to involve an alteration of the maternal serum concentrations of angiogenic and antiangiogenic markers such as placental growth factor (PlGF) and soluble vascular endothelial growth factor receptor-1, known as soluble fms-like tyrosine kinase (sFlt-1) or their ratio, known as angiogenic index-1 (PlGF/sFlt-1).[44] Therefore, assessing whether this association is relevant for a subset of patients with idiopathic bleeding in the second trimester and placental lesions consistent with maternal vascular malperfusion would be interesting.

Strengths and limitations

The main strength of this study is the use of paired amniotic fluid samples obtained before and after antibiotic treatment in pregnancies complicated by idiopathic bleeding in the second trimester of pregnancy. Similarly, a

TABLE 2

Details of presentations of the initial and follow-up amniocenteses, and outcomes of women with idiopathic bleeding in the second trimester of pregnancy with intraamniotic inflammation who were treated with ceftriaxone, clarithromycin, and metronidazole and underwent a follow-up amniocentesis

	Gestational age at admission (wk+d)	Initial amniocentesis at the time of admission			Follow-up amniocentesis after 7 d			Gestational age at delivery (wk+d)	PPROM (Yes/No)	PTL (Yes/No)	HCA (Yes/No)	Funisitis (Yes/No)	MVM (Yes/No)	Neonatal morbidity
		Culture	PCR	IL6 (pg/mL)	Culture	PCR	IL6 (pg/mL)							
1.	18+0	Negative	*Ureaplasma* spp	26,405	Negative	*Ureaplasma* spp	12,017	31+0	Yes	No	Yes	Yes	Yes	RDS
2.	18+1	Negative	*Ureaplasma* spp	50,000	Negative	*Ureaplasma* spp	33,242	19+4	No	No	Yes	No	Yes	Spontaneous abortion
3.	20+1	*Streptococcus parasanguinis*	Negative	18,034	Negative	Negative	12,831	39+6	No	No	N/A	N/A	N/A	None
4.	21+1	Negative	*Ureaplasma* spp	50,000	Negative	*Ureaplasma* spp	3998	27+0	No	Yes	Yes	Yes	No	RDS, LOS, BPD, NEC
5.	22+1	Negative	*Ureaplasma* spp	50,000	Negative	*Ureaplasma* spp	36,677	25+1	No	Yes	Yes	No	No	RDS, BPD, ROP
6.	25+2	Negative	*Ureaplasma* spp	29,537	Negative	*Ureaplasma* spp	6354	30+5	Yes	No	Yes	No	No	RDS
7.	27+6	Negative	*Ureaplasma* spp	8176	Negative	*Ureaplasma* spp	2445	30+4	Yes	No	Yes	Yes	Yes	RDS
8.	27+6	Negative	*Ureaplasma* spp	13,884	Negative	*Ureaplasma* spp	2850	33+6	Yes	No	Yes	Yes	Yes	RDS
9.	18+3	Negative	Negative	24,867	Negative	Negative	14,318	39+4	No	No	N/A	N/A	N/A	None
10.	22+3	Negative	Negative	20,445	Negative	Negative	2952	37+0	No	No	N/A	N/A	N/A	None
11.	22+5	Negative	Negative	4185	Negative	Negative	1695	39+2	No	No	N/A	N/A	N/A	None
12.	24+1	Negative	Negative	13,239	Negative	Negative	2497	39+6	Yes	No	N/A	N/A	N/A	None
13.	24+2	Negative	Negative	19,179	Negative	Negative	2900	36+0	Yes	No	Yes	No	No	None
14.	26+2	Negative	Negative	3576	Negative	Negative	1926	35+5	Yes	No	Yes	No	Yes	None

BPD, bronchopulmonary dysplasia; *HCA*, histologic chorioamnionitis; *IL6*, interleukin 6; *LOS*, late-onset sepsis; *MVM*, maternal vascular malperfusion; *N/A*, not available; *NEC*, necrotizing enterocolitis; *PCR*, polymerase chain reaction; *PPROM*, preterm prelabor rupture of membranes; *PTL*, preterm labor with intact membranes; *RDS*, respiratory distress syndrome; *ROP*, retinopathy of prematurity; *Spp*, species.

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TABLE 3
Details of presentations of the initial amniocentesis and outcomes of women with idiopathic bleeding in the second trimester of pregnancy without intraamniotic inflammation

	Gestational age at admission (wk+d)	Initial amniocentesis at the time of admission			Gestational age at delivery or abortion (wk+d)	PPROM (Yes/No)	PTL (Yes/No)	HCA (Yes/No)	Funisitis (Yes/No)	MVM (Yes/No)	Neonatal morbidity
		Culture	PCR	IL6 (pg/mL)							
1.	19+3	Negative	Negative	123	39+5	No	No	N/A	N/A	N/A	None
2.	19+4	Negative	Negative	836	40+3	No	No	N/A	N/A	N/A	None
3.	19+4	Negative	Negative	1288	31+0	No	Yes	No	No	Yes	RDS
4.	19+4	Negative	Negative	348	40+4	No	No	N/A	N/A	N/A	None
5.	20+4	Negative	Negative	1542	41+3	No	No	N/A	N/A	N/A	None
6.	21+0	Negative	Negative	1713	30+6	Yes	No	Yes	No	No	RDS
7.	25+4	Negative	Negative	1594	37+0	No	No	N/A	N/A	N/A	None
8.	26+6	Negative	Negative	635	35+5	No	Yes	No	No	Yes	RDS
9.	26+6	Negative	Negative	1117	29+0	No	Yes	No	No	Yes	RDS
10.	27+6	Negative	Negative	2376	30+4	Yes	No	Yes	No	Yes	RDS
11.	27+6	Negative	Negative	254	40+3	No	No	N/A	N/A	N/A	None

HCA, histologic chorioamnionitis; *IL6*, interleukin 6; *MVM*, maternal vascular malperfusion; *N/A*, not available; *PCR*, polymerase chain reaction; *PPROM*, preterm prelabor rupture of membranes; *PTL*, preterm labor with intact membranes; *RDS*, respiratory distress syndrome.

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combination of noncultivation (specific PCR for *Ureaplasma* spp, *M hominis*, and *C trachomatis* and nonspecific PCR for detection of 16S rRNA gene) and cultivation approaches used to evaluate the presence of MIAC provided us a unique opportunity to dissect the subsets of patients with intraamniotic infection and sterile intraamniotic inflammation.

This study has some limitations. First, follow-up amniocentesis was not performed in patients without intraamniotic inflammation or those who delivered within 7 days of amniocentesis. Second, the number of women with intraamniotic inflammation who underwent follow-up amniocentesis was relatively small. Therefore, the results should be interpreted cautiously and validated in a large cohort. Third, changes in the intensity of intraamniotic inflammation associated with antibiotic treatment in women with idiopathic bleeding complicated by intraamniotic inflammation were characterized by the concentration of only 1 amniotic fluid protein (IL6). Modern high-throughput "−omics" technologies are needed to provide a more complex, precise, and comprehensive determination of the pathophysiology of the intensity of intraamniotic inflammatory responses associated with antibiotic treatment. Fourth, the retrospective design of the study prevented us from assessing the effect of the antibiotic treatment on the perinatal outcomes in the subset of women with idiopathic bleeding in the second trimester with intraamniotic inflammation. Fifth, some patients (29% [4/14]) who underwent follow-up amniocenteses received a course of corticosteroids after the initial amniocentesis. Glucocorticoids, particularly dexamethasone, have been shown to inhibit the production of IL6 by amnion cells in the cell culture model[48] but not in the explant tissue model from term fetal membranes.[49] Nevertheless, betamethasone administration did not alter the concentrations of IL6 in the amniotic fluid of the sheep model.[50] Regardless of this conflicting evidence, it is not possible to fully exclude any potential impact of corticosteroids on IL6 concentrations in amniotic fluid samples collected from the subset of women who received a course of corticosteroids during follow-up amniocenteses. However, it should also be noted that a reduction of the microbial load of *Ureaplasma* spp DNA in the amniotic fluid could not be achieved by the administration of betamethasone. Finally, the absence of a cultivation method to assess the presence of *Ureaplasma* spp and/or selective quantitative real-time PCR in amniotic fluid[51,52] prevented us from determining whether the antibiotic treatments used could eradicate *Ureaplasma* spp from amniotic fluid because their DNA in amniotic fluid can originate from live or dead bacteria.

Conclusion

Idiopathic bleeding in the second trimester of pregnancy is complicated by intraamniotic inflammation in two-thirds of cases. Antibiotic treatment with ceftriaxone, metronidazole, and clarithromycin reduces the intensity or resolves intraamniotic inflammation in patients with idiopathic bleeding during the second trimester of pregnancy. This treatment also eradicates microorganisms or diminishes their amniotic fluid load in patients with idiopathic bleeding in the second trimester of pregnancy. ■

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SUPPLEMENTAL TABLE

Maternal and clinical characteristics of women with idiopathic bleeding in the second trimester of pregnancy with intraamniotic infection and sterile intraamniotic inflammation with respect to delivery within or after 7 days

Characteristic	Patients with intraamniotic infection			Patients with sterile intraamniotic inflammation		
	Delivery/abortion within 7 d (n=5)	Delivery after 7 d (n=8)	*P* value[a]	Delivery/abortion within 7 d (n=6)	Delivery after 7 d (n=6)	*P* value[b]
Maternal age (y), median (IQR)	37 (31–39)	33 (27–34)	.16	32 (31–33)	35 (30–37)	.21
Primiparous, n (%)	0 (0%)	3 (38%)	.23	2 (33%)	2 (33%)	1.00
Bleeding in the first trimester of pregnancy	5 (100%)	5 (63%)	.23	6 (100%)	6 (100%)	1.00
Prepregnancy body mass index (kg/m^2), median (IQR)	24.3 (20.9–28.8)	24.8 (21.5–27.7)	.60	22.5 (19.3–30.9)	26.1 (22.7–28.8)	.49
Smoking, n (%)	0 (0%)	1 (13%)	1.00	0 (0%)	0 (0%)	—
Gestational age at admission (wk), median (IQR)	21+4 (20+1–26+2)	21+1 (18+5–27+2)	.86	24+4 (22+6–26+2)	23+0 (20+5–24+6)	.26
Length of the cervix at admission (mm), median (IQR)	37 (18–49)	32 (25–32)	.62	27 (20–40)	40 (29–44)	.23
Oligohydramnios at admission, n (%)	3 (60%)	3 (38%)	1.00	0 (0%)	2 (33%)	.46
Placenta previa minor at admission, n (%)	3 (60%)	3 (38%)	1.00	1 (17%)	2 (33%)	1.00
Subchorionic hematoma at admission, n (%)	3 (60%)	3 (38%)	1.00	3 (50%)	4 (67%)	1.00
Amniotic fluid IL6 levels at admission (pg/mL), median (IQR)	33,824 (12,875–50,000)	27,881 (14,992–50,000)	.95	28,452 (9731–39,334)	16,209 (4033–21,551)	.18
CRP levels at admission (mg/L), median (IQR)	15.3 (3.4–46.5)	15.6 (5.3–28.5)	.83	9.4 (4.1–12.9)	12.5 (8.6–29.8)	.24
WBC count at admission ($\times 10^9$ L), median (IQR)	12.6 (10.5–17.9)	11.3 (9.5–17.6)	.62	15.3 (11.5–19.8)	11.6 (11.5–12.5)	.11
Gestational age at delivery (wk), median (IQR)	22+1 (20+3–26+4)	30+5 (25+6–33+1)	.06	24+5 (23+5–26+5)	38+1 (36+0–39+5)	**.002**[c]
Abortion before gestational age of 22+0 wk, n (%)	2 (40%)	1 (13%)	.51	0 (0%)	0 (0%)	—
PPROM, n (%)	1 (20%)	3 (38%)	1.00	2 (33%)	2 (33%)	1.00
PTL, n (%)	2 (40%)	2 (25%)	1.00	4 (67%)	0 (0%)	.06
Term delivery, n (%)	0 (0%)	1 (13%)	1.00	0 (0%)	4 (67%)	.06
Cesarean delivery, n (%)	2 (40%)	2 (25%)	1.00	3 (50%)	3 (50%)	1.00
Birthweight (g), median (IQR)	530 (273–825)	1290 (813–3750)	**.03**[c]	700 (577–933)	3295 (2448–3715)	**.002**[c]
Apgar score <7; 5 min, n (%)	2 (40%)	3 (38%)	1.00	3 (50%)	0 (0%)	.18
Apgar score <7; 10 min, n (%)	2 (40%)	2 (25%)	1.00	3 (50%)	0 (0%)	.18

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(continued)

SUPPLEMENTAL TABLE

Maternal and clinical characteristics of women with idiopathic bleeding in the second trimester of pregnancy with intraamniotic infection and sterile intraamniotic inflammation with respect to delivery within or after 7 days *(continued)*

Characteristic	Patients with intraamniotic infection			Patients with sterile intraamniotic inflammation		
	Delivery/abortion within 7 d (n=5)	Delivery after 7 d (n=8)	*P* value[a]	Delivery/abortion within 7 d (n=6)	Delivery after 7 d (n=6)	*P* value[b]
Acute histologic chorioamnionitis, n (%)	5 (100%)	7 (100%)[d]	1.00	6 (100%)	2 (100%)[e]	1.00
Funisitis, n (%)	4 (60%)	4 (50%)	.64	4 (67%)	0 (0%)[e]	.43
Maternal vascular malperfusion, n (%)	2 (40%)	1 (13%)	.37	4 (67%)	2 (100%)[e]	1.00

Continuous variables were compared using a nonparametric Mann—Whitney *U* test. Categorical variables were compared using the Fisher exact test. Continuous variables are presented as median (IQR) and categorical variables as number (percentage).

CRP, C-reactive protein; *IL6*, interleukin 6; *IQR*, interquartile range; *PPROM*, preterm prelabor rupture of membranes; *PTL*, preterm labor with intact membranes; *WBC*, white blood cells.

[a] Comparison between the subgroups of patients with intraamniotic infection; [b] Comparison between the subgroups of patients with sterile intraamniotic inflammation; [c] Statistically significant results; [d] Data available for 88% (7/8) of patients; [e] Data available for 67% (2/6) of patients.

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