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Prevalence and Clinical Significance of Sterile Intra-amniotic Inflammation in Patients with Preterm Labor and Intact Membranes

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Abstract

Problem—Inflammation and infection play a major role in preterm birth. The purpose of this study was to: 1) determine the prevalence and clinical significance of sterile intra-amniotic inflammation; and 2) examine the relationship between amniotic fluid (AF) concentrations of high mobility group box-1 (HMGB1) and the interval from amniocentesis-to-delivery in patients with sterile intra-amniotic inflammation.

Method of Study—AF samples obtained from 135 women with preterm labor and intact membranes were analyzed using cultivation techniques as well as broad-range PCR and mass spectrometry (PCR/ESI-MS). Sterile intra-amniotic inflammation was defined when patients with negative AF cultures and without evidence of microbial footprints had intra-amniotic inflammation (AF interleukin-6 ≥ 2.6 ng/mL).

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Results—1) The frequency of sterile intra-amniotic inflammation was significantly greater than that of microbial-associated intra-amniotic inflammation [26% (35/135) vs. 11% (15/135); (p=0.005)]; 2) patients with sterile intra-amniotic inflammation delivered at comparable gestational ages, had similar rates of acute placental inflammation and adverse neonatal outcomes as patients with microbial-associated intra-amniotic inflammation; and 3) patients with sterile intra-amniotic inflammation and high AF concentrations of HMGB1 (≥ 8.55 ng/mL) delivered earlier than those with low AF concentrations of HMGB1 (p=0.02).

Conclusions—1) sterile intra-amniotic inflammation is more frequent than microbial-associated intra-amniotic inflammation; and 2) we propose that danger signals participate in sterile intra-amniotic inflammation in the setting of preterm labor.

Keywords

alarmins; danger signal; HMGB1; polymerase chain reaction with electrospray ionization mass spectrometry; pregnancy; preterm delivery

Introduction

Preterm labor is the leading cause of perinatal morbidity and mortality worldwide.[1-6] Two-thirds of preterm deliveries occur after the spontaneous onset of preterm labor, with either intact or ruptured membranes.[7-11] Preterm labor is one of the great obstetrical syndromes,[11-15] in which the presenting symptoms and signs largely represent activation of the common pathway of parturition [i.e. increased uterine contractility,[16-26] cervical remodeling,[27-39] and membrane/decidual activation[40-55]]. Activation of the common pathway of parturition can be the result of multiple pathological processes.[11,14] Intra-amniotic inflammation due to microbial invasion of the amniotic cavity (MIAC) is an important cause of spontaneous preterm delivery,[56-80] and the molecular mechanisms responsible for parturition in this scenario have been extensively studied.[81-119]

Intra-amniotic inflammation can be due to microorganisms (bacteria, parasites or viruses) or to other mechanisms of disease in which necrosis or cellular stress induces a release of mediators which activate the innate immune system.[120-123] We have used the term “sterile intra-amniotic inflammation” to refer to an inflammatory process in which microorganisms cannot be detected.[124-130] The precise stimuli for sterile intra-amniotic inflammation in patients with preterm labor and intact membranes have not been identified. One possibility is that Damage-associated molecular patterns (DAMPs) are responsible for this inflammatory process, as well as in a subset of patients with clinical chorioamnionitis at term.[127, 129, 131] Several alarmins, including interleukin (IL)-1α,[89, 132] S100 calcium binding protein B (S100B),[125] high mobility group box-1 (HMGB1),[129, 131] and heat shock proteins,[127] are elevated in the amniotic fluid (AF) of women with intra-amniotic inflammation.[126] HMGB1 is a prototypic alarmin,[133-142] and elevated concentrations of this alarmin may reflect engagement of DAMPs-induced inflammation.[141-151] HMGB1 plays a key role in mediating inflammation in response to microorganisms, as well as sterile inflammation due to cell injury. HMGB1 is secreted actively during microbial invasion or cellular stress and passively by damage of cellular integrity.[152-154] In this study, we sought to: 1) determine the frequency and clinical significance of sterile intra-amniotic

inflammation; and 2) examine the relationship between the AF concentrations of HMGB1 and the interval from amniocentesis-to-delivery in patients with sterile intra-amniotic inflammation.

Materials and Methods

Study population

A prospective cohort of women with singleton pregnancies who presented with spontaneous preterm labor and intact membranes was selected from the clinical database and Bank of Biological Samples maintained by Wayne State University, the Detroit Medical Center, and the Perinatology Research Branch of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) if they met the following criteria: 1) had an amniocentesis (transabdominal) performed between 20 and 35 weeks of gestation prior to the rupture of the chorioamniotic membranes; 2) absence of chromosomal or structural fetal anomalies; and 3) the pregnancy outcome was known. This population represents a subset of that included in a study previously reported in this journal[130] focusing on the use of molecular microbiologic techniques. Women with an insufficient volume of AF required for the determination of HMGB1 were excluded from this analysis [5%, (7/142)]. Each patient provided written informed consent and the use of biological specimens and clinical data for research purposes was approved by the Institutional Review Boards of NICHD and Wayne State University.

Clinical definitions

Preterm labor was diagnosed by the presence of regular uterine contractions (at least 3 in 30 minutes) and documented cervical changes in patients with a gestational age between 20 and 36 6/7 weeks. Preterm delivery was defined as birth prior to the 37th week of gestation. MIAC was defined as either a positive culture for bacteria in AF or the detection of microbial footprints for either viruses or bacteria, using polymerase chain reaction (PCR) coupled with electrospray ionization mass spectrometry (ESI-MS) (Ibis® technology - Athogen, Carlsbad, CA). Intra-amniotic inflammation was diagnosed when AF IL-6 concentration was ≥ 2.6 ng/mL.[72,155] Microbial-associated intra-amniotic inflammation was defined as the presence of MIAC with intra-amniotic inflammation. Sterile intra-amniotic inflammation was diagnosed when the AF IL-6 concentration was ≥ 2.6 ng/mL and there was no evidence of microbial footprints for viruses or bacteria (negative AF culture and no detection of microbial footprints using PCR/ESI-MS).

Composite neonatal morbidity was defined as the presence of: respiratory distress syndrome, bronchopulmonary dysplasia, grade III or IV intraventricular hemorrhage, periventricular leukomalacia, proven neonatal sepsis, necrotizing enterocolitis or perinatal mortality. The diagnostic criteria of these complications have been previously reported.[156] Acute placental inflammation was diagnosed based on the presence of inflammatory cells in the chorionic plate, chorioamniotic membranes (histologic chorioamnionitis),[157-159] and/or umbilical cord (funisitis).[157,158]

Amniocentesis and amniotic fluid processing

Patients with preterm labor and intact membranes who had transabdominal ultrasound-guided amniocentesis to evaluate possible intra-amniotic infection (within the standard of care at the Detroit Medical Center) were eligible for the study. AF was immediately transported in a capped sterile syringe to the clinical laboratory. Evaluation of white blood cell (WBC) count, glucose concentration and Gram stain of AF were also performed shortly after collection. AF not required for clinical assessment was centrifuged for 10 minutes at 4°C shortly after the amniocentesis, and the supernatant was aliquoted and stored at -70°C until analysis. The current management for preterm labor in our hospital is to administer corticosteroids (betamethasone or dexamethasone) between 24 and 34 weeks of gestation. Betamethasone is given intramuscularly in two doses (12 mg), 24-hours apart and dexamethasone is administered intramuscularly in 4 doses (6 mg), 12-hours apart.

Detection of microorganisms with cultivation and molecular methods

AF was analyzed using cultivation techniques (aerobic, anaerobic and genital mycoplasmas) as well as with PCR/ESI-MS (Ibis®). Briefly, DNA was extracted from 300 uL of AF using a method that combines bead-beating cell lysis with a magnetic-bead based extraction method.[160,161] Extracted DNA was amplified by the previously described broad bacteria and candida (BAC) detection assay according to the manufacturer's instructions.[162] PCR/ESI-MS can identify 3400 bacteria and 40 Candida spp, which are represented in the platform's signature database.[163-165] For viral detection, the nucleic acids were extracted from 300 uL of AF using a method that combines chemical lysis with a magnetic-bead based extraction method. The extracted RNA/DNA was amplified on the broad viral assay according to the manufacturer's instructions. In the eight wells, there were 14 primer pairs used to detect the following viruses: *Herpes simplex virus* 1 (HHV-1), *Herpes simplex virus* 2 (HHV-2), *Varicella-zoster virus* (HHV-3), *Epstein-Barr virus* (HHV-4), *Cytomegalovirus* (HHV-5), *Kaposi's sarcoma-associated herpes virus* (HHV-8), human adenoviruses, human enteroviruses, *BK polyomavirus*, *JC polyomavirus* and *Parvovirus B19*.[165]

After PCR amplification, 30-μL aliquots of each PCR product were desalted and analyzed via ESI-MS as previously described.[163,166] The presence of microorganisms was determined by signal processing and triangulation analysis of all base composition signatures obtained from each sample and compared to a database. The sensitivity [level of detection (LOD)] of the assay for the detection of bacteria in blood is on average 100 colony-forming units (CFU) per mL [95% confidence interval (CI), 6 – 600 CFU/mL].[164] A comparison of detection limits between blood and AF show comparable detection limits (100 CFU/mL). The sensitivity for the broad viral in plasma ranges from 400 to 6600 copies/mL.[167] Detection limits in AF ranged from ~800 to 1600 copies/mL (depending upon the specific microorganism).

IL-6 and HMGB1 concentrations in amniotic fluid

AF concentrations of IL-6 and HMGB1 were determined by sensitive and specific enzyme immunoassays obtained from R&D Systems (Minneapolis, MN) and IBL International (Toronto, Canada), respectively. An initial assay validation was performed in our laboratory prior to conducting this study. Briefly, the immunoassays utilized a quantitative sandwich

enzyme immunoassay technique and the concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, and for HMGB1 3.1% and 4.4%, respectively. The detection limits of the assay for IL-6 and HMGB1 were 0.09 pg/mL and 0.2 ng/mL, respectively.

Statistical analysis

The Kolmogorov-Smirnov test and visual plot inspection were used to assess the normality of continuous data distributions. A Kruskal-Wallis test and a two-tailed Mann–Whitney U test were used to compare differences of arithmetic variables among and between the groups. Comparisons of proportions were performed using Chi-square or Fisher's exact tests. Differences in the frequency of sterile intra-amniotic inflammation and microbial-associated inflammation were examined using McNemar's test. A receiver operating characteristic (ROC) curve for the identification of patients who delivered within seven days was used to select a cutoff for AF concentrations of HMGB1. Kaplan-Meier survival curves were plotted and the log rank test was used to determine whether there were differences in time-to-delivery (censoring observations for patients delivered for maternal or fetal indications). Logistic and Cox proportional hazard regression models were fit to examine magnitudes of association. Spearman's correlation was used to assess the relationship between two continuous variables. A two tailed p-value of <0.05 was considered statistically significant. The statistical package used was SPSS v.15.0 (SPSS, Chicago, IL).

Results

Prevalence of sterile and microbial-associated intra-amniotic inflammation

Clinical characteristics are displayed in Table I. The frequency of sterile intra-amniotic inflammation was significantly greater than that of microbial-associated intra-amniotic inflammation [26% (35/135) vs. 11% (15/135) (p=0.005)] (Figure 1). When analysis was restricted to patients who delivered before 37, 34, 32 and 30 weeks of gestation, the frequency of sterile intra-amniotic inflammation was higher than that of microbial-associated intra-amniotic inflammation in all gestational age subgroups (p=0.01, 0.03, 0.02 and 0.02, respectively) (see Figure 1). The earlier the gestational age at delivery, the higher the prevalence of both microbial-associated and sterile intra-amniotic inflammation.

Clinical characteristics of sterile intra-amniotic inflammation

The median gestational age [interquartile range, (IQR)] at amniocentesis was significantly lower in patients with sterile and microbial associated intra-amniotic inflammation than in women without intra-amniotic inflammation [25 (IQR: 23 – 32) and 26 (IQR: 23 – 32) weeks vs. 32 (29 – 33) weeks, respectively (each $p<0.001$); Table I]. There were no significant differences in other clinical characteristics, such as maternal age, race, tobacco use or cervical dilatation at admission among these three clinical groups (Table I).

The median (IQR) AF concentrations of IL-6 and WBC count in patients with sterile intra-amniotic inflammation were lower than those of patients with microbial-associated intra-amniotic inflammation [AF IL-6: 12 (5 - 21) vs. 96 (17 - 266) ng/mL; $p<0.001$; and WBC count: 3 (1 - 17) vs. 295 (2 - 960) cells/mm^3; p=0.02]. There was no significant difference in

the median (IQR) gestational age at delivery between patients with microbial-associated intra-amniotic inflammation and those with sterile intra-amniotic inflammation [26 (24 – 33) vs. 27 (24 – 32) weeks; p=0.6]. The prevalence of acute placental inflammation (acute histologic chorioamnionitis and/or funisitis) was similar in patients with intra-amniotic inflammation with or without detectable microorganisms [79% (11/14) vs. 61% (19/31); p=0.3].

Neonatal morbid events (assessed by composite neonatal morbidity) were significantly more common in patients with microbial-associated intra-amniotic inflammation and sterile intra-amniotic inflammation than in those without intra-amniotic inflammation [67% (10/15) and 68% (24/35) vs. 11% (9/85); each p<0.001; see Table I]. Importantly, there was no significant difference in the prevalence of neonatal morbid events between neonates born to mothers with sterile intra-amniotic inflammation and those born to mothers with microbial-associated intra-amniotic inflammation (p=1.0).

Amniotic fluid concentrations of HMGB1 in women with sterile intra-amniotic inflammation and the interval to delivery

The median (IQR) AF concentration of HMGB1 was significantly higher in patients with sterile intra-amniotic inflammation (n=35) than in those without intra-amniotic inflammation (n=85) [7.6 (5.8 – 10.4) vs. 6 (4 – 7.7) ng/mL; p=0.007]. Among patients with sterile intra-amniotic inflammation, those who delivered within seven days after amniocentesis (n=23) had a significantly higher median (IQR) AF concentration of HMGB1 than patients who delivered after seven days (n=12) [8.8 (5.9 - 17.3) vs. 6.7 (3.7 - 8.1) ng/mL; p=0.03) (Figure 2)]. A cutoff of 8.55 ng/mL was selected to define an elevated AF HMGB1 concentration upon examining a ROC curve for the identification of patients who delivered within seven days of amniocentesis, excluding women with microbial-associated intra-amniotic inflammation (Figure 3). Patients with AF concentrations of HMGB1 at or above this cutoff were 5-fold more likely (than those with concentrations below 8.55 ng/mL) to deliver within seven days of amniocentesis [adjusted odds ratio (OR): 5.1; 95% CI 1.6 – 15.5], adjusting for gestational age at amniocentesis and cervical dilatation with logistic regression.

The amniocentesis-to-delivery interval of women with sterile intra-amniotic inflammation who had AF HMGB1 concentrations > 8.55 ng/mL was significantly shorter than that of women with sterile intra-amniotic inflammation who had AF HMGB1 concentrations below 8.55 ng/mL and women without intra-amniotic inflammation [median 3, IQR: 1 – 7 days vs. median 8, IQR: 4 – 18 days, and median 31, IQR: 20 – 59 days (p=0.02 and p<0.0001, respectively, Figure 4)]. Importantly, there was no significant difference in the amniocentesis-to-delivery interval between patients with sterile intra-amniotic inflammation and AF HMGB1 > 8.55 ng/mL compared to patients with microbial-associated intra-amniotic inflammation [median 3, IQR: 1 – 7 days vs. median 1, IQR: 0- 8 days; p=0.6, Figure 4)].

Multivariable survival analysis (Cox proportional hazard modeling) was used to explore the amniocentesis-to-delivery interval among different clinical groups taking into account microbial invasion, intra-amniotic inflammation, and AF concentration of HMGB1 while

adjusting for cervical dilatation at admission and gestational age at amniocentesis. Patients with either microbial-associated or sterile intra-amniotic inflammation with AF HMGB1 ≥ 8.55 ng/mL had a shorter amniocentesis-to-delivery interval than those without intra-amniotic inflammation [hazard ratio of 14.6 (95% CI, 6.2 - 34), and 17.7 (95% CI, 7.3 - 42), respectively]. Moreover, patients with sterile intra-amniotic inflammation and AF HMGB1 < 8.55 ng/mL also had a shorter amniocentesis-to-delivery interval than those without intra-amniotic inflammation [hazard ratio of 6.5 (95% CI%, 3.2 – 13.3)].

There was an association between AF HMGB1 concentrations and acute inflammatory lesions in the placenta (acute histologic chorioamnionitis and/or funisitis). The median (IQR) AF concentration of HMGB1 was significantly higher in patients with acute placental inflammation (n=48) than that of patients without these lesions (n=77) [8.5 (5.1 – 17.8) vs. 6.6 (4.7 – 8.3) ng/mL; p=0.009]. The prevalence of acute placental inflammation was significantly higher in patients with elevated AF HMGB1 (> 8.55 ng/mL) than in those with a low AF concentration of HMGB1 [58% (24/41) vs. 29% (24/84); p=0.001].

Amniotic fluid concentrations of IL-6 in patients with sterile intra-amniotic inflammation and elevated HMGB1 concentrations

Figure 5 displays AF IL-6 concentrations in patients with sterile intra-amniotic inflammation according to the AF concentration of HMGB1 [below 8.55 ng/mL; (n=20) and above 8.55 ng/mL; (n=15)]. The median (IQR) AF concentration of IL-6 was significantly higher in patients with AF HMGB1 ≥ 8.55 ng/mL compared to those with AF HMGB1 < 8.55 ng/mL [21 (10 – 27) vs. 7 (3.5 – 15.3) ng/mL; p=0.006; Figure 5]. AF concentrations of HMGB1 were moderately correlated with AF IL-6 concentrations (Spearman's Rho 0.6; p<0.001) and inversely correlated with the interval from amniocentesis to delivery (Spearman's Rho −0.4; p=0.03) in patients with sterile intra-amniotic inflammation.

Antibiotics were administered in 29% (39/135) of patients before amniocentesis. Most patients [60% (81/135)] did not receive corticosteroids before amniocentesis. We repeated the analysis adjusting for the administration of antibiotics (within 6 hours) and corticosteroids (within 7 days) before amniocentesis using analysis of covariance, and the results of amniotic fluid IL-6 and HMGB1 concentrations remained significant among groups.

Discussion

Principal findings of the study

1) Sterile intra-amniotic inflammation is more common than microbial-associated intra-amniotic inflammation in patients with preterm labor and intact membranes; 2) patients with sterile intra-amniotic inflammation had early preterm deliveries at a comparable gestational age to women with microbial-associated intra-amniotic inflammation; 3) the AF glucose concentration and WBC count in cases of sterile intra-amniotic inflammation were within normal ranges; 4) acute histologic chorioamnionitis and/or funisitis were identified in patients with sterile intra-amniotic inflammation, as well as in those with microbial-associated intra-amniotic inflammation; 5) the intensity of the inflammatory response

(assessed by AF IL-6 concentrations) was stronger in patients with microbial-associated intra-amniotic inflammation than in patients with sterile intra-amniotic inflammation; and 6) patients with sterile intra-amniotic inflammation and high AF concentrations of HMGB1, delivered earlier than those with low AF concentrations of HMGB1. This is compelling evidence that sterile intra-amniotic inflammation is of major importance in preterm parturition.

Sterile vs. microbial-associated intra-amniotic inflammation

The key finding of this study is that in patients with preterm labor and intact membranes sterile intra-amniotic inflammation is more common than microbial-associated intra-amniotic inflammation. We have previously proposed that intra-amniotic inflammation without detectable microorganisms is a mechanism of disease in preterm labor[71,72,74,76,125-129,131,168] based on observations of elevated AF concentrations of inflammatory mediators [IL-6,[71,72,74,91,96,168-175] IL-8,[106] matrix metalloproteinase 8 (MMP-8)[76,128], monocyte chemotactic protein-1 (MCP-1),[113, 176] and other inflammatory markers[79,80,107,172,177]] in the absence of detectable microorganisms. The designation of "sterile intra-amniotic inflammation" depends on the techniques employed to exclude the presence of microbes. In this study, we used a new sensitive method which uses electrospray ionization mass spectrometry (ESI-MS) for base composition analysis of polymerase chain reaction (PCR) products to rapidly create a signature that allows for the identification of a large number of microorganisms (bacteria and viruses) at the genus and species level within 8 hours. Therefore, the classification of intra-amniotic inflammation as "sterile" was determined with a high degree of confidence. It is possible that future technological advances will allow discovery of microorganisms which have escaped detection. The use of next generation sequencing may result in the detection of low quantities of microbial nucleic acids in biological fluids. Yet, determining whether this reflects the true presence of bacteria and viruses (rather than contamination) is a challenge.

Clinical and laboratory characteristics of sterile intra-amniotic inflammation

Although the clinical characteristics of patients with sterile intra-amniotic inflammation were similar to those with microbial-associated intra-amniotic inflammation (e.g. gestational age at presentation, short interval-to-delivery, and a high rate of adverse neonatal events), the laboratory findings were different. Specifically, the AF WBC count and AF glucose concentrations of patients with sterile intra-amniotic inflammation were within normal range (see Table I). Therefore, a work-up based on AF WBC count and AF glucose concentration would fail to identify the most common type of intra-amniotic inflammation in patients with preterm labor and intact membranes. Interestingly, the AF concentrations of IL-6 are significantly lower in patients with sterile intra-amniotic inflammation than in those with microbial-associated intra-amniotic inflammation. Further studies are required to characterize the behavior of the cytokinechemokine network in amniotic fluid and maternal blood in patients with sterile intra-amniotic inflammation.

Acute histologic chorioamnionitis can occur in the absence of microbial-associated intra-amniotic inflammation

Acute histologic chorioamnionitis and funisitis are generally considered to be due to amniotic fluid infection.[91,95,170,178-187] Early studies using amniocentesis in patients with spontaneous preterm labor who delivered within 48 hours showed that 71% (27/38) of patients with acute histologic chorioamnionitis and 81% (29/36) with funisitis had positive AF cultures for bacteria.[188] Hillier et al. reported that in 73% (16/22) of placentas with acute histologic chorioamnionitis, bacteria could be isolated from the chorioamniotic space.[92] The findings reported herein indicate that 58% (n=18/31) of patients with sterile intra-amniotic inflammation have acute histologic chorioamnionitis, and that of all patients with acute histologic chorioamnionitis, 38% (n=18/48) had sterile intra-amniotic inflammation at the time of amniocentesis. We propose that DAMPs-induced inflammation occurs after a danger signal engages a pattern recognition receptor (PRR), such as toll like receptors (TLRs)-2, -4, and -9, leading to the release of HMGB1 and other alarmins that can induce inflammation by stimulating the production of inflammatory cytokines such as IL-6, IL-1β, IL-8 and TNF-α.[144,145,189,190]

Although our observations suggest that MIAC was not present at the time of amniocentesis in patients with sterile intra-amniotic inflammation who subsequently delivered and had acute histologic chorioamnionitis, it is possible that microorganisms may have invaded the amniotic cavity at some point before the procedure and induced intra-amniotic inflammation, which eradicated the microbes. This possibility is extremely difficult to exclude in humans. It is also possible that danger-associated intra-amniotic inflammation led to preterm labor, and that a secondary microbial invasion of the amniotic cavity occurred which was not detected at the time of amniocentesis. Studies in which placentas and membranes are examined for microorganisms using culture, sequence-based techniques and morphologic approaches are required to address this possibility. The biology of infection during pregnancy is extremely complex. Recent observations in animal models suggest that systemic and localized infections in the lower genital tract during pregnancy may increase the susceptibility to microbial products (i.e. endotoxin) or bacteria (two-hit hypothesis model).[191-195] Whether this is the case in humans remains to be determined.

Danger signals in patients with sterile intra-amniotic inflammation and preterm labor

Inflammation is a mechanism of host defense in response to infection and non-infection related insults.[196-200] The innate immune system initiates an inflammatory response when PRRs sense exogenous or endogenous signals considered potentially harmful to the host.[146,196,199-213] During infection, these signals include molecular structures that are conserved among microbial species, known as pathogen-associated molecular patterns (PAMPs).[203,207,214] Tissue damaged from non-infectious origins (e.g. trauma, ischemia-reperfusion injury, urate crystals, chemical injuries etc.) induce the release alarmins or “danger signals”,[154,203,207,214] known as DAMPs. When recognized by PRRs, these multifunctional proteins are also capable of inducing an inflammatory response.[139,144,145,189,207,212,215]

In this study, AF concentrations of HMGB1, a prototypical “alarmin”, were significantly higher in patients with sterile intra-amniotic inflammation than in patients without intra-amniotic inflammation. In addition, we report that patients with sterile intra-amniotic inflammation and high AF concentrations of HMGB1 (≥ 8.55 ng/mL) had a significantly shorter amniocentesis-to-delivery interval than patients with AF HMGB1 < 8.55 ng/mL. These findings suggest that alarmins can play a role in preterm labor in the setting of sterile intra-amniotic inflammation.

HMGB1 is a chromatin component located in the nucleus of eukaryotic cells, whose functional role is to inform other cells that damage has occurred.[133,135,149,189,216-218] This protein has chemotactic activity for monocytes,[152,153,217, 219, 220] macrophages,[221, 222] neutrophils,[221-224] and dendritic cells.[137,138,217,225,226] HMGB1 is released into the extracellular space either actively or passively. Active secretion occurs from stressed cells of the immune system (e.g. macrophages, monocytes and cells at the frontline of host defense).[133,135,137,145,149] Passive release occurs during sterile cell injury and is almost immediate.[147] During infection, HMGB1 is transferred from the nucleus of monocytes, macrophages and other immune cells to the cytosol, where it accumulates in intracellular vesicles prior to secretion which takes approximately 8 hours.[147,153227] In mice, serum concentrations of HMGB1 increase 8 to 32 hours after endotoxin exposure; thus, HMGB1 has been considered a “late” mediator of inflammation.[227-230] Alarmins are potential therapeutic targets. Blocking HMGB1 with neutralizing antibodies reduces mortality in animal models of endotoxemia[227,230] and sepsis,[228,231] this has generated considerable interest in HMGB1 as a potential pharmacologic target in sepsis and sterile inflammation.

Evidence that “alarmins” can induce preterm labor

Systemic administration of IL-1α has been shown to induce preterm delivery in mice,[232,233] an effect that was abrogated by pretreatment with the IL-1 receptor antagonist.[233] Subsequently, Bry et al. reported that intra-amniotic injection of IL-1α in rabbits similarly induces preterm labor.[234] Elevated AF concentrations of other alarmins have been reported in women with preterm labor with intact membranes or preterm prelabor rupture of membranes, including S100B,[125] HMGB1,[129,131] and heat shock proteins.[126,127] Whether these multifunctional proteins can induce preterm labor and delivery remains to be determined. Further studies of the effect of HMGB1 on the chorioamniotic membranes and pregnancy are required.

Although we have reported that HMGB1 concentrations are elevated in a subset of patients with preterm labor,[129] the mechanisms whereby HMGB1 may lead to the onset of labor are unknown. Additional research is required to answer these questions. Necrotic or stress cells can release alarmins including HMGB1. These alarmins would be responsible, at least in part, for inducing inflammation and the onset of labor. The effect of IL-1α in inducing parturition[232-234] may be mediated through the activation of the components of the inflammasome.[235-237]

Conclusion

1) Sterile intra-amniotic inflammation is more frequent than microbial-associated inflammation in patients with preterm labor and intact membranes; 2) we propose that danger signals participate in sterile intra-amniotic inflammation in the setting of preterm labor with intact membranes; and 3) future research on the mechanisms responsible for sterile intra-amniotic inflammation, biomarkers, and therapeutic interventions are required to address the challenges offered by this clinical condition.

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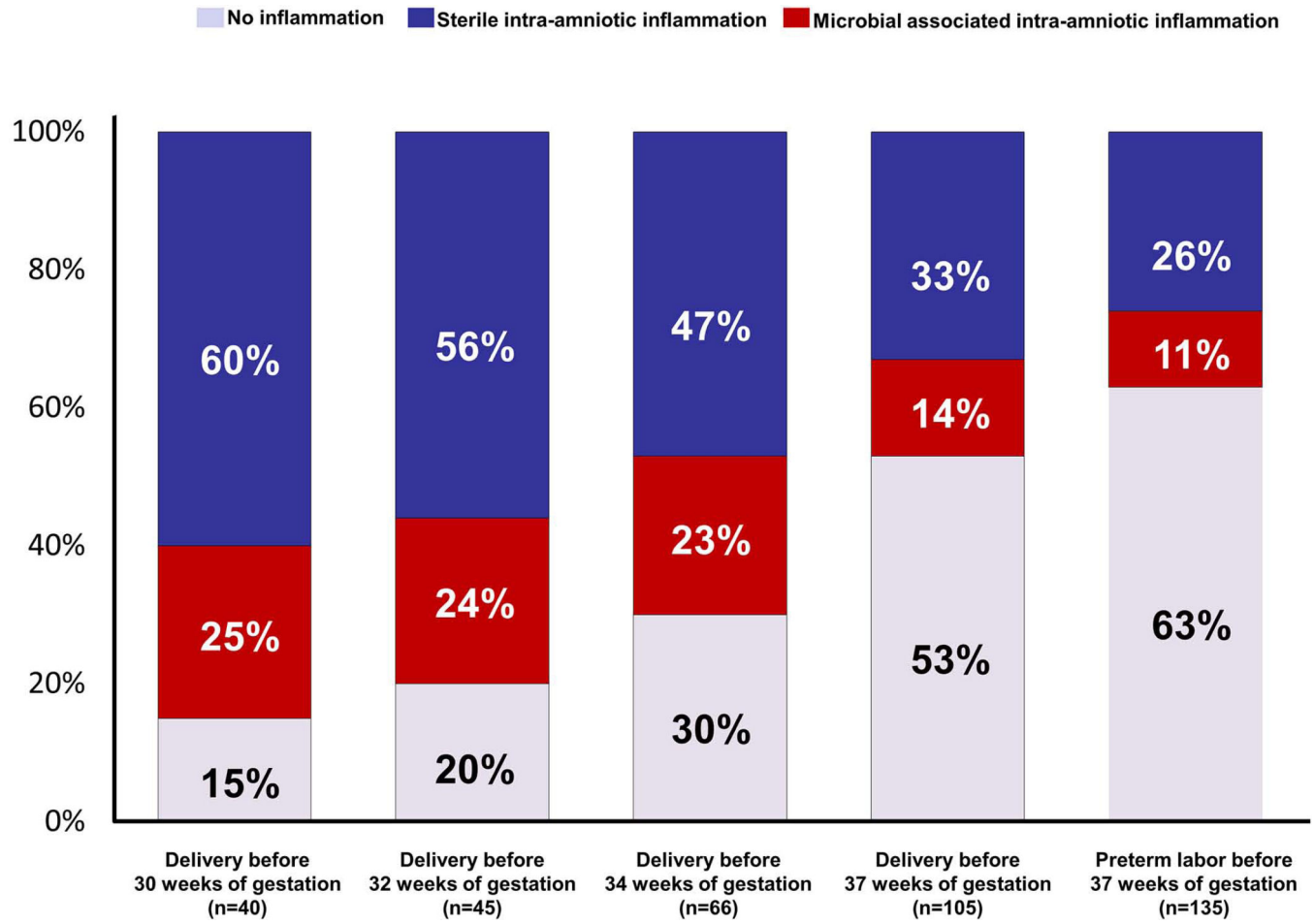


Figure 1.

Frequency of sterile and microbial-associated intra-amniotic inflammation in patients with preterm labor and intact membranes as a function of the gestational age at delivery. The frequency of sterile intra-amniotic inflammation was significantly greater than that of microbial-associated intra-amniotic inflammation [26% (35/135) vs. 11% (15/135) (p= 0.005)].

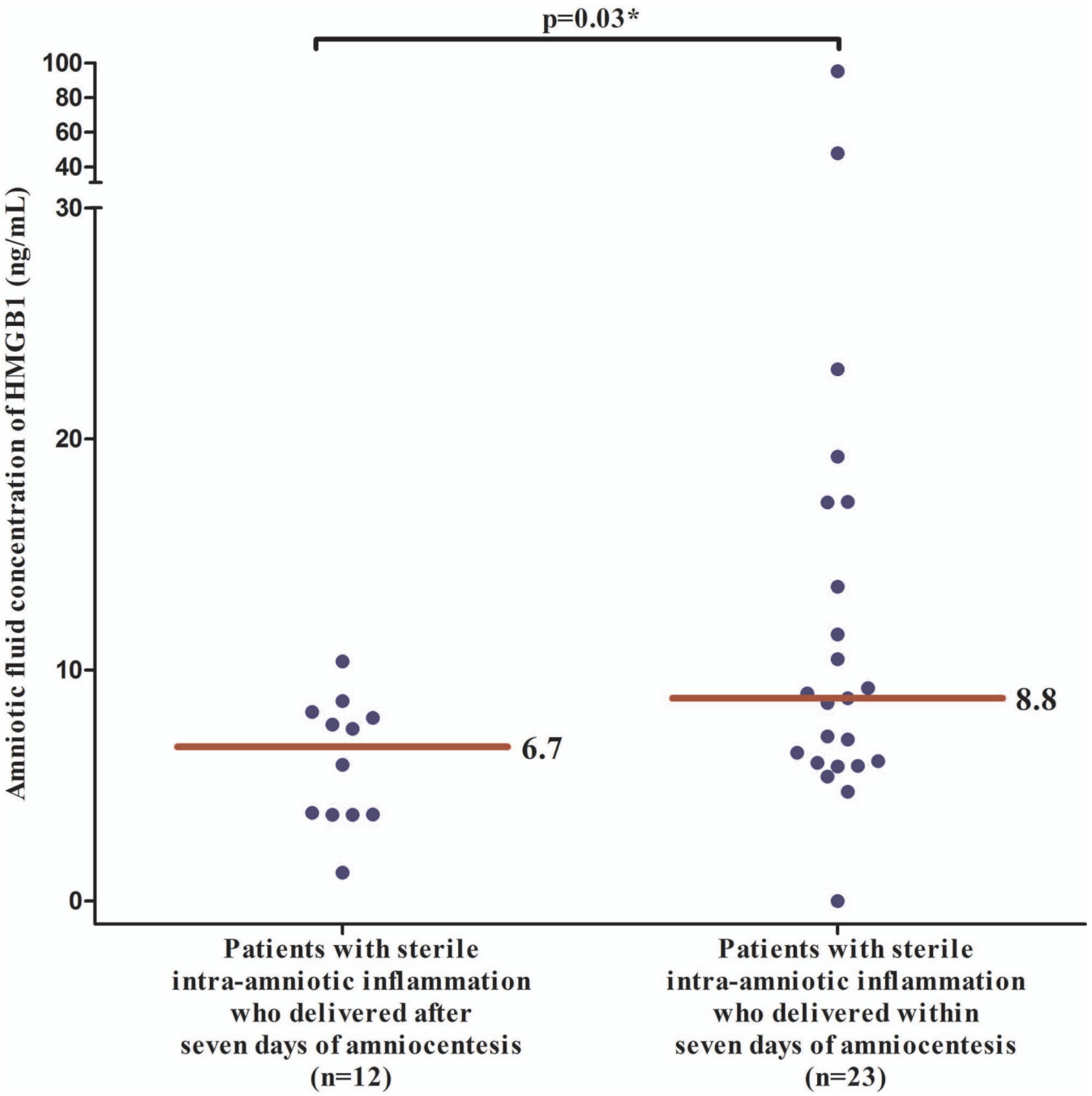


Figure 2.

AF concentrations of HMGB1 in patients with sterile intra-amniotic inflammation according to whether delivery occurred within or after seven days of amniocentesis. Patients who delivered within seven days after amniocentesis had a significantly higher median (IQR) AF concentration of HMGB1 than patients who delivered after seven days [8.8 (5.9 - 17.3) vs. 6.7 (3.7 - 8.1); p=0.03].

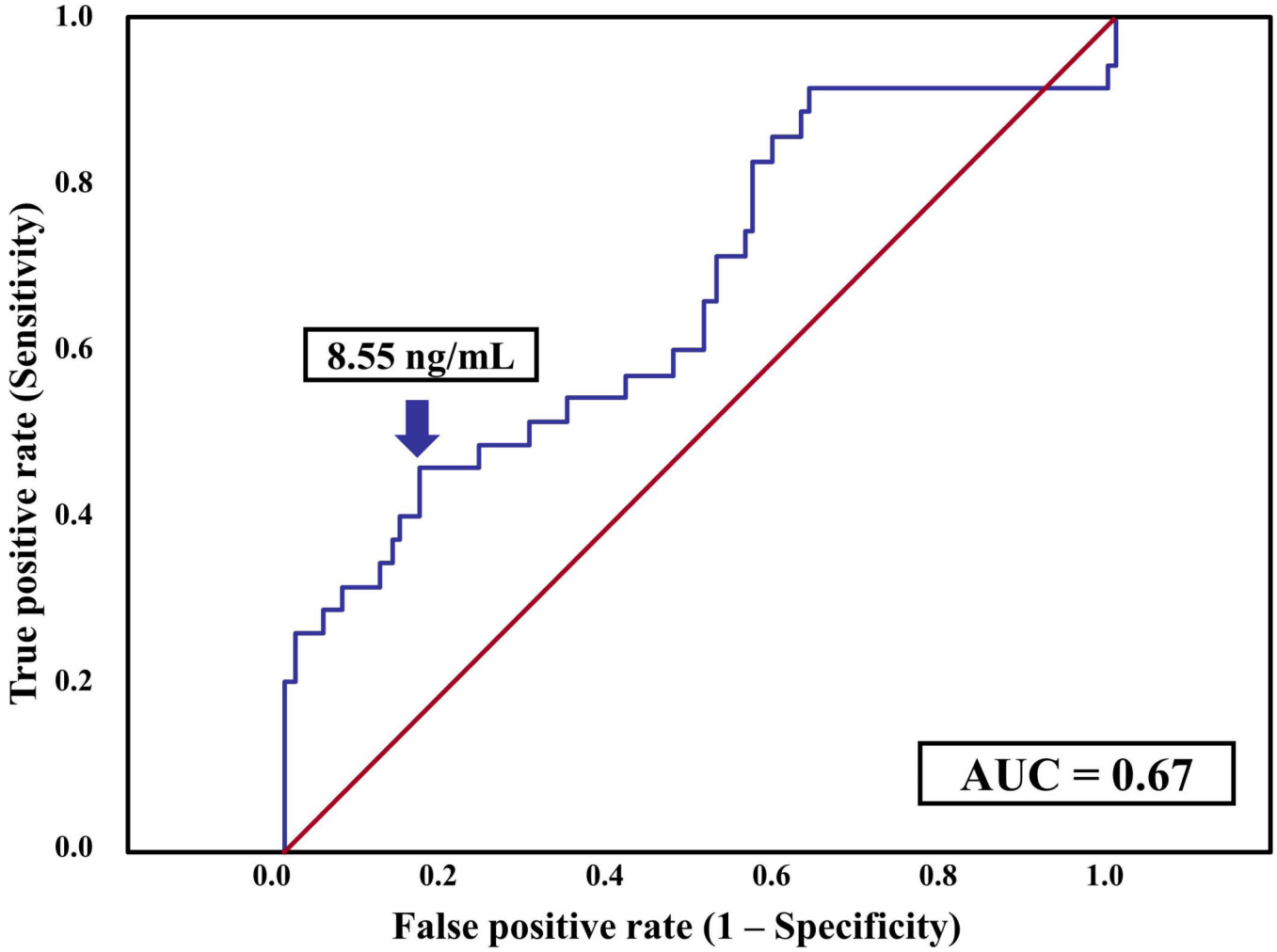
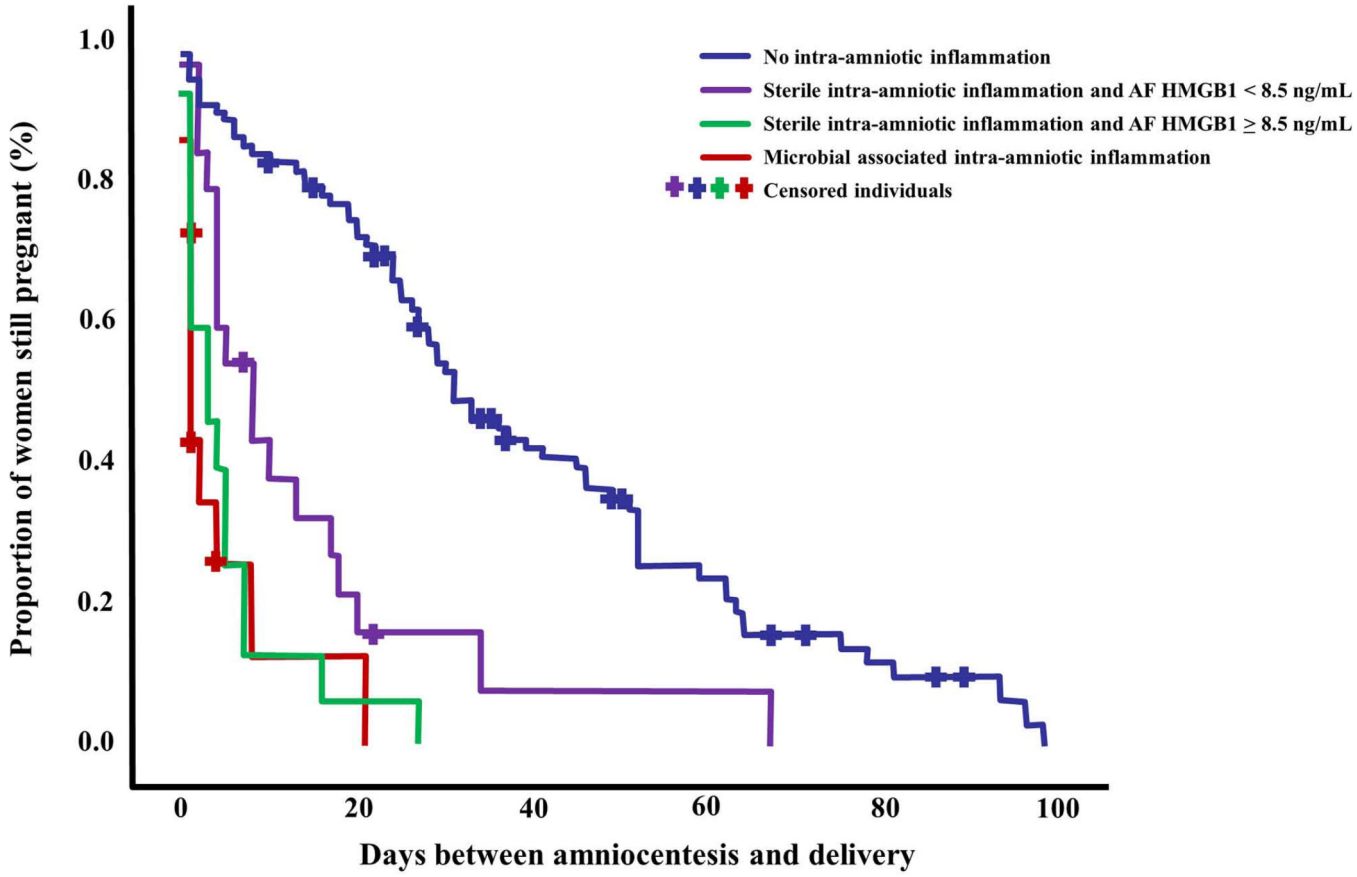


Figure 3.

Receiver operator characteristic curve analysis of AF concentrations of HMGB1 for the identification of patients without microbial-associated intra-amniotic inflammation who subsequently delivered within seven days after amniocentesis. AF HMGB1 concentration of 8.55 ng/mL had an area under the ROC curve of 0.67 (95% CI 0.55 – 0.78; p=0.005), a sensitivity of 46% (16/35), a specificity of 84% (71/85), a positive predictive value of 53% (16/30), and a negative predictive value of 80% (71/90) for the identification of patients who subsequently delivered within 7 days.



Groups	Median interval days	Interquartile range
Microbial associated intra-amniotic inflammation	1	0 - 8
Sterile intra-amniotic inflammation and AF HMGB1 ≥ 8.55 ng/mL	3	1 – 7
Sterile intra-amniotic inflammation and AF HMGB1 < 8.55 ng/mL	8	4 – 18
No intra-amniotic inflammation	31	20 – 59

Figure 4.

Survival analysis of amniocentesis-to-delivery interval (days) according to the presence of microbial-associated intra-amniotic inflammation or sterile intra-amniotic inflammation with the presence of higher concentration of *alarmins*. Patients in whom labor was induced were censored and are represented by crosses. The amniocentesis-to-delivery interval among women with sterile intra-amniotic inflammation who had elevated AF HMGB1 concentrations (≥8.55 ng/mL) was significantly shorter than that of: 1) women with sterile intra-amniotic inflammation who had AF HMGB1 concentrations below 8.55 ng/mL; and 2) women without intra-amniotic inflammation [median 3, IQR: 1 – 7 days vs. median 8, IQR: 4 – 18 days and median 31, IQR: 20 – 59 days ($p=0.02$ and $p<0.0001$), respectively]. There was no significant difference in the amniocentesis-to-delivery interval between patients with sterile intra-amniotic inflammation and AF HMGB1 concentrations ≥ 8.55 ng/mL and those with microbial-associated intra-amniotic inflammation.

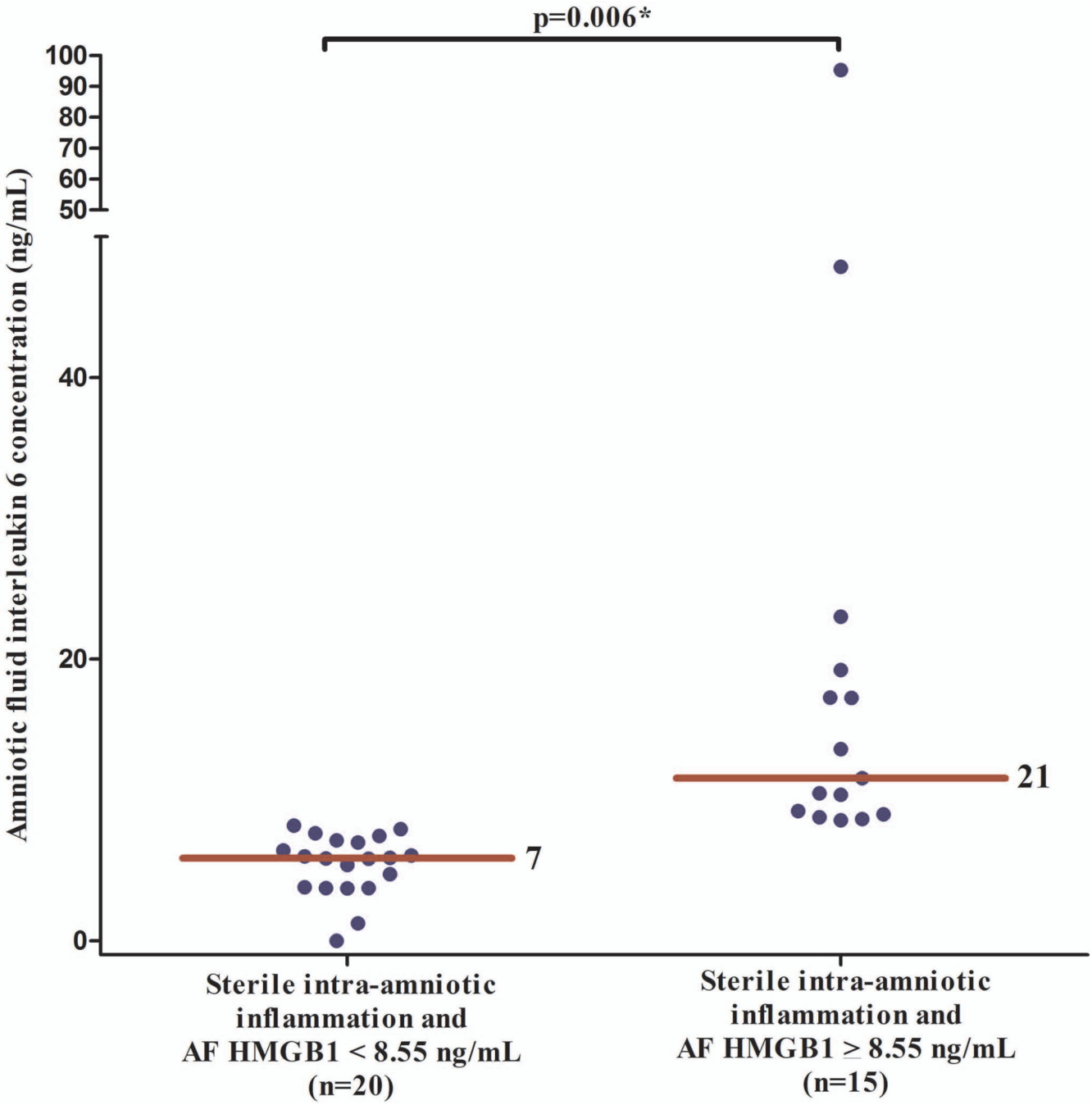


Figure 5.

AF concentrations of IL-6 in patients with sterile intra-amniotic inflammation according to the AF concentration of HMGB1 (above or below 8.55 ng/mL). The median (IQR) AF concentration of IL-6 was significantly higher in patients with AF HMGB1 ≥ 8.55 ng/mL than in those with AF HMGB1 < 8.55 ng/mL [21 (10 – 27) vs. 7 (3.5 – 15.3) ng/mL; p=0.006].

Table I

Clinical and demographic characteristics of the study population.

	No intra-amniotic inflammation (n=85)	Microbial-associated intra-amniotic inflammation (n=15)	Sterile intra-amniotic inflammation (n=35)	p value
Maternal age (years)	23 (20 – 26)	24 (20 – 30)	23 (20 – 26.2)	0.8
BMI (kg/m^2)	23 (20 – 29)	27 (23 – 29)	23 (20 – 32)	0.1
Nulliparity	32% (27)	73% (11)	40% (14)	0.009
Race				0.1
African-American	89% (76)	73% (11)	91% (30)	
Caucasian	6% (5)	20% (3)	6% (2)	
Hispanic	0	7% (1)	3% (1)	
Others	5% (4)	0	0	
Tobacco use during pregnancy	20% (17)	13% (2)	21% (7)	0.8
Cervical dilatation at admission (cms)	3 (2 – 4)	4 (2 – 4)	3 (2 – 4)	0.1
Gestational age at amniocentesis (weeks)	32 (29 – 33)	26 (23 – 32)	25 (23 – 32)	<0.001*
AF white blood cells count (cells/mm^3)	1 (0 – 5)	295 (2 – 960)	3 (1 – 17)	<0.001*
AF glucose (mg/dL)	29 (24 – 34)	11 (10 – 20)	22 (18 – 28)	<0.001*
AF interleukin-6 (ng/mL)	0.8 (0.5 – 1.1)	96 (17 – 266)	12 (5 – 21)	<0.001*
Gestational age at delivery (weeks)	36 (34 – 38)	26 (24 – 33)	27 (24 – 32)	<0.001*
Composite neonatal morbidity	11% (9)	67% (10)	68% (24)	<0.001
Acute placental inflammation¥	22.5% (18/80)	79% (11/14)	61% (19/31)	<0.001*
Acute histologic chorioamnionitis	21% (17/80)	79% (11/14)	58% (18/31)	
Funisitis	13% (10/80)	57% (8/14)	29% (9/31)	

Data presented as median (interquartile) and percentage and (n); AF: amniotic fluid; BMI: body mass index.

¥Acute placental inflammation was calculated over a total of 125 specimens.

*Kruskal Wallis test.