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HIGH MOBILITY GROUP-BOX 1 (HMGB1) LEVELS ARE INCREASED IN AMNIOTIC FLUID OF WOMEN WITH INTRA-AMNIOTIC INFLAMMATION-DETERMINED PRETERM BIRTH, AND THE SOURCE MAY BE THE DAMAGED FETAL MEMBRANES

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Abstract

Background—High Mobility Group Box-1 (HMGB1) is considered a prototype alarmin molecule. Upon its extracellular release, HMGB1 engages pattern recognition receptors and the Receptor for Advanced Glycation End (RAGE) followed by an outpouring of inflammatory cytokines, including interleukin (IL)-6.

Methods—We assayed the amniotic fluid (AF) levels of HMGB1 and IL-6 in 255 women that either had a normal pregnancy outcome or delivered preterm. Immunohistochemistry on fetal membranes was used for cellular localization and validation of immunoassay findings. HMGB1 also was analyzed in amniochorion tissue explants subjected to endotoxin.

Results—AF HMGB1 levels are not gestational age regulated but increased in women with intra-amniotic inflammation and preterm birth. The likely source is the damaged amniochorion, as demonstrated by immunohistochemistry and explant experiments.

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Conflict of interests

The authors have no conflicts to declare.

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Conclusions—Our research supports a role for HMGB1 in the inflammatory response leading to preterm birth. As a delayed phase cytokine, in utero exposure to elevated HMGB1 levels may have an impact on the newborn beyond the time of birth.

Graphical abstract

1. Introduction

Originally described as a DNA-binding protein that stabilizes nucleosomes and facilitates transcription, high-mobility group box-1 (HMGB1), is expressed in all cells and preferentially localized in the cell nucleus.¹ When released in response to cell and tissue injury HMGB1 acts as a late-phase cytokine engaging pattern recognition receptors, such as TLR2, TLR4² and the Receptor for Advanced Glycation End-Products (RAGE),³ which in turn activate innate immunity via NF- κ B transactivation.⁴ Although HMGB1 protein by itself can cause an acute inflammatory response with release of cytokines and chemokines, its delayed kinetics of passive release from injured cells makes HMGB1 a distal mediator of acute inflammatory processes initiated by both infectious or non-infectious (i.e. trauma) etiologic agents.^{5,6}

In an animal model of endotoxin-induced fetal damage and preterm birth our group demonstrated that HMGB1 was significantly over expressed outside the nucleus at the site of inflammation-induced damage of vital fetal organs.⁷ Furthermore, our group demonstrated for the first time that components of the Damage Associated Molecular Pattern & Receptor for Advance Glycation End Product (DAMP-RAGE) system, in particular the alarmin S100A12 (EN-RAGE) and the RAGE antagonist soluble RAGE (sRAGE), are present in human amniotic fluid (AF). In women with intra-amniotic infection, levels of S100A12 were found to be determined by the severity of intra-amniotic inflammation (IAI). In contrast, AF sRAGE levels were primarily driven by gestational age (GA).⁸ Here we evaluated the levels and GA regulation of AF HMGB1 in human gestation and pregnancies complicated by intra-amniotic inflammation (IAI) leading to preterm birth. We further provided insight into the possible source of AF HMGB1 by employing immunohistochemistry and a tissue explant system of endotoxin induced inflammation.

2. Materials and methods

2.1. Patients and amniotic fluid collection

Using a prospective study design we investigated AF samples from 255 women pregnant with singletons who had a clinically indicated amniocentesis. Samples were retrieved by trans-abdominal amniocentesis for the purpose of 2nd trimester genetic karyotyping (GA, median [range]: 18 [17–20] weeks, n=25); 3rd trimester fetal lung maturity testing (GA: 36 [35–37] weeks, n=25) or to rule-out AF infection in women who had preterm labor contractions refractory to tocolysis, preterm premature rupture of membranes (PPROM) or advanced cervical dilatation (3 cm) (GA: 29 [25–31] weeks, n=205).

Exclusion criteria were the presence of anhydramnios, human immunodeficiency or hepatitis viral infections, congenital anomalies or abnormal karyotype. Gestational age was determined based on last menstrual period confirmed by an ultrasound examination prior to 20 weeks.¹⁵ Preterm labor was defined as the presence of regular uterine contractions and documented cervical effacement and/or dilatation in patients <37 weeks of gestation. PPRM was confirmed by vaginal AF “pooling”, “nitrazine”, “ferning” or an amniocentesis-dye positive test. Corticosteroids and antibiotics were administered as clinically indicated. The neonatology resuscitation team was present at the time of delivery for all neonates.

All women were recruited at Yale New Haven Hospital (YNHH) and were followed prospectively until delivery. The Human Investigation Committee of Yale University approved the study protocol. All patients provided written informed consent.

2.2. Chemical and microbiological studies of the amniotic fluid

Following retrieval under sterile conditions, AF was analyzed by the YNHH clinical and microbiological laboratories for glucose concentration, lactate dehydrogenase (LDH) activity, white blood cell (WBC) count, Gram stain and standard culturing methods for aerobic and anaerobic bacteria, including *Ureaplasma* and *Mycoplasma* species. These results were available to the clinical team for management of the case. An AF glucose cut-off of 15 mg/dL, an LDH level 419 U/L, a positive Gram stain and/or culture result were considered suggestive of intra-amniotic infection. The results of the microbiological tests were available for case management and were reported as final 5 days after culturing. The remaining AF was transported to the research laboratory, spun at 3000g at 4°C for 20 min., aliquoted in polypropylene cryotubes and stored at –80°C until analysis.

2.3. Mass spectrometry of the amniotic fluid

To confirm or exclude the presence of IAI, an AF proteomic fingerprint (Mass Restricted [MR] score) was generated using Surface Enhanced Laser Desorption Ionization Time-of-Flight (SELDI-TOF) mass spectrometry. The method for generation of the MR score has been previously described.⁹ Briefly, the MR score is comprised of 4 proteomic biomarkers: defensin-2, defensin-1, S100A12 (calgranulin C) and S100A8 (calgranulin A). The MR score ranges from 0 to 4, depending upon the presence or absence of each of the 4 protein biomarkers. A value of 1 was assigned if a biomarker peak was present and 0 if absent.

Based on our previous results, we stratified the study population in two classes based on the severity of inflammation with MR 3 or 4 being considered severe IAI. Scorings of the AF SELDI-TOF tracings were performed without knowledge of the maternal outcome, results of the placental histological examination, or interleukin (IL)-6 levels. The results of the MR score were not used for patient clinical management.

2.4. Histologic evaluation of the fetal membranes

Placental tissues were available for all patients included in this analysis. Hematoxylin and eosin-stained sections of the amniochorion membranes and umbilical cord were read by a perinatal pathologist unaware of the results of the MR score, fetal outcome or umbilical cord blood analyses. Each section was examined systematically for the presence or absence of inflammation and funisitis was diagnosed when neutrophils infiltrated the umbilical vessels walls or Wharton's jelly. Three histological stages of chorioamnionitis were complemented by the histological grading system devised by Salafia et. al., which includes four grades of inflammation of the amnion, chorion-decidua and umbilical cord.^{10,11} Histopathologic evidence of maternal and/or fetal immuno-response was assessed as previously described by Ghidini and collaborators¹² and scored as either absent, mild (grades 1–2) or severe (grades 3–4). Severe inflammation in the amnion was considered a marker of activation of the maternal immune system in response to ascending infection, whereas severe inflammation of umbilical cord and/or chorionic vessels was considered to indicate a response of the fetal immune system.¹²

2.5. Enzyme-linked immunosorbent assays of human amniotic fluid HMGB1 and IL-6

IL-6 (Pierce-Endogen, Rockford, IL) and HMGB1 (IBL International, Hamburg, Germany) immunoassays were performed in duplicate according to manufacturers' instructions by investigators unaware of the clinical presentation. The minimal detectable concentration for IL-6 and HMGB1 was 1 pg/mL, and 1 ng/mL, respectively. The inter- and intra-assay coefficients of variation were <10%.

2.6. Immunohistochemistry for HMGB1 and RAGE

Five µm paraffin sections were deparaffinized in xylene and rehydrated with graded ethanol to potassium-phosphate-buffered saline solution, pH 7.2. Following antigen retrieval with basic solution buffer pH 10, the sections were pretreated with 1% hydrogen peroxide for 15 minutes followed by one-hour incubation in 5% donkey serum. The sections were then incubated overnight at 4°C with rabbit polyclonal anti-HMGB1 (Shino-Test, Kanagawa, Japan 1:1000 dilution) and goat polyclonal anti-RAGE (R&D Systems, 1:100 dilution) antibodies. In pilot experiments, specificity of the anti-HMGB1 antibody was confirmed by Western blot. Detection was performed with biotinylated donkey anti-rabbit or goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, 1:600 dilution) as appropriate followed by avidin-biotin staining (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) and incubated with 3,3'-diaminobenzidine/nickel sulfate as chromogen solution. Specificity of staining was confirmed by omitting the primary antibodies. Specific staining was evaluated semi-quantitatively in a blinded fashion by examining six fields per slide and subjectively scoring on a scale from 0 (no staining) to 5 (intense blue-black staining) the intensity of the chromogen deposited in the amnio-chorion epithelium and umbilical cord.

2.7. Placental explant experiments

Amniochorion and placental villous trophoblast were obtained from healthy women (n=4) undergoing elective cesarean delivery at term in the absence of labor (GA: 38 [38–40] weeks). Tissues were incubated in the presence of 1 µg/mL lipopolysaccharide (LPS) to mimic inflammation. Explant medium was collected at 18 hours of incubation for measurements of HMGB1 by enzyme-linked immunosorbent assays.

2.8. Statistical analysis

Statistical analyses were performed with Sigma Stat, version 2.03 (SPSS Inc., Chicago, IL) and MedCalc (Broekstraat, Belgium) statistical software. Normality testing was performed using the Kolmogorov-Smirnov test. Data were compared with Mann-Whitney Rank Sum test, 1-way ANOVA followed by Student-Newman-Keuls tests (parametric) or Kruskal-Wallis ANOVA on ranks followed by Dunn's tests (non-parametric). Statistical analysis of the immunoassays data was performed after logarithmic transformation of data. Spearman correlations were used to measure co-linearity between the selected independent variables. Comparisons between proportions were done with Chi-square tests. A *P* value of <0.05 was considered significant throughout the analysis.

3. Results

3.1. Clinical and laboratory characteristics of the study population

Demographic and outcome characteristics of the women at amniocentesis are presented in Table 1. Women who had a genetic amniocentesis were older and, similar to those who had lung maturity testing, more often delivered a healthy baby at term. Clinical chorioamnionitis and PPROM were encountered only in the group of women who had an amniocentesis to rule-out infection. This group of women delivered babies of lower birthweight than the other two groups.

The clinical, laboratory and outcome characteristics for the “rule-out infection” group were analysed separately and are presented in Table 2. Women with positive IAI were enrolled and delivered at earlier GAs, had shorter amniocentesis-to-delivery interval, higher frequency of clinical chorioamnionitis and more often delivered babies of lower birthweight. The results of the chemical and microbiological studies of the AF showed the group of women with IAI had lower AF glucose levels, higher AF LDH activity, higher white blood cell (WBC) count, higher frequency of a positive microbial culture or Gram stain and higher AF IL-6 levels. Histological examination of the chorionic plate, fetal membranes and chorio-decidua demonstrated significantly higher stages and grades of histological inflammation in the group of women with IAI.

3.2 HMGB1 levels are increased in amniotic fluid of women with intra-amniotic inflammation and preterm birth

We first asked the question whether the HMGB1 concentration in AF exhibits GA regulation in the absence of infection or inflammation. For this purpose, we analysed only AF from women who had an amniocentesis for either genetic indications (n=25), lung maturity (n=25) or suspicion of intra-amniotic infection that was ruled out and eventually

resulted in term delivery (n=29). We found that AF HMGB1 concentration did not vary significantly across human gestation (Fig. 1A, $r = -0.020$, $P = 0.861$).

To provide evidence that HMGB1 is a component of the IAI process in response to infection leading to PTB, we next analyzed levels in AF retrieved from women with signs or symptoms of preterm birth who had an amniocentesis to rule out infection. We determined that women with severe IAI by proteomic analysis (MR 3–4) had significantly increased amniotic fluid HMGB1 concentration compared to women with either absent (MR 0) or mild inflammation (MR 1–2) (Kruskal Wallis ANOVA $P < 0.001$; Fig. 1B). There was no difference in AF HMGB1 concentration between women with absent and mild IAI. A direct and significant correlation between AF HMGB1 and AF IL-6 levels was noted ($r = 0.738$; $P < 0.001$; Fig. 1C).

As shown in Table 2, positive AF microbial cultures were identified in 9 women who did not have severe IAI by proteomics analysis. The levels of HMGB1 in these cases were similar to those of women with no IAI and negative cultures (no IAI & positive AF cultures: median [interquartile range]: 8.2 [6.4–14.5] vs. no IAI & negative AF cultures: 7.5 [5.6–11.7] ng/mL, Mann-Whitney Rank Sum test $P = 0.397$). The AF IL-6 levels in these 2 groups were also low (no IAI & positive AF cultures: 2.1 [0.6–3.2] vs. no IAI & negative AF cultures: 0.7 [0.2–2.3] ng/mL, $P = 0.190$).

3.3. HMGB1 expression is increased in damaged fetal membranes

By immunohistochemistry we found that cells in the fetal membranes express low levels of HMGB1 in the absence of IAI (Fig. 2A). In contrast, women with severe IAI had intense HMGB1 staining in amniochorion (Fig. 2B). Areas with particularly intense staining were the amnion epithelium and necrotic areas in the choriodecidua. By staining serial sections of fetal membranes from women with histologic chorioamnionitis, we observed that HMGB1 (Fig. 3A) and RAGE receptor (Fig. 3B) display a similar staining pattern, supporting the notion that the HMGB1-RAGE axis may play a role in the damage of the amniochorion in the context of IAI.

3.4. HMGB1 is released by human fetal membranes ex-vivo in response to endotoxin

To further determine whether the amniochorion contributes to the AF pool of HMGB1 or whether the highly hydrophobic HMGB1 formed elsewhere simply infiltrates the damaged amnion and deposits within the chorio-decidua, we challenged explants of either human fetal membranes or placental villous tissue obtained from elective Cesarean sections at term (n=4) with endotoxin *ex-vivo* (LPS, 1 mg/mL). We observed a significant elevation in HMGB1 released in amniochorion medium (Fig. 5 $P = 0.008$), while release by the placental villous explants remained virtually unchanged (not shown, $P > 0.05$).

4. Discussion

Our research supports a role for fetal HMGB1-RAGE system activation in IAI secondary to infection. In this study, we observed that in normal gestation the levels of AF HMGB1 are not GA regulated. However, women with severe IAI had significantly increased AF HMGB1 compared to women with absent and mild inflammation. The presence of HMGB1

in AF from women with severe IAI displayed a positive correlation with the levels of AF IL-6 and the proteomics MR score. We further observed that AF HMGB1 is likely the result of the damaged amniochorion in the context of severe IAI. Therefore, our findings identify elevated levels of HMGB1 as a potential marker for amniochorion cell damage and injury in IAI and infection. HMGB1 is the most highly expressed of all High-Mobility-Group family members, is ubiquitously present in all nucleated cell types and is the most mobile nuclear protein, crossing from nucleus into the cytosol within 1–2 seconds.^{13,14} Traditionally, in cells active in DNA replication, HMGB1 displays a nuclear localization suggesting that this non-histone chromosomal protein has an important role in maintenance of nucleosome structure, regulation of gene replication, transcription and translation.^{15,16} Electrophoretic analysis of nuclear and cytoplasmic fibroblasts fractions indicated that HMGB1 is present in both cell cytoplasm and nucleus with a predominance of the cytoplasmic fraction.¹⁷ Considered a prototype “alarmin” with critical roles in pathogenicity of many conditions, HMGB1 can play key physiologic roles when passively released in the extracellular space by dying cells.¹⁸ Extracellular HMGB1 has cell growth, pro-angiogenic, mitotic activity and antibacterial roles.^{19,20} The mechanisms by which HMGB1 exerts its bactericidal function is not well defined. The current findings that HMGB1 is present in the AF of women with normal pregnancy outcomes suggest that HMGB1 released by amnion cells could be part of the physiologic antimicrobial defense mechanism of the gestational sac. HMGB1 is a RAGE ligand antagonized by the decoy receptor sRAGE.²¹ Our previous studies investigating the presence of the RAGE axis in human pregnancy demonstrated that sRAGE is present in AF and increases with GA.⁸ Because levels of HMGB1 are not GA dependent, it is likely that GA regulation of its antagonist sRAGE, with higher levels at term, may contribute along with other mechanisms to a higher incidence of inflammation-related PTBs at early GAs.⁸

As pathologic mediator, HMGB1 signals tissue and cell loss when released in the extracellular compartment passively from damaged cells or actively secreted by various immune cells, including dendritic, macrophages and natural killer cells.^{22,23} Once outside the cell, HMGB1 acts as a potent and rapid inflammatory signal to set in motion and augment an innate immune response via a positive feedback loop.²⁴ This effect is accomplished through engagement of various receptors including TLR2, TLR4, TLR9 and RAGE.^{25,26} Most recently it was shown that HMGB1 may trigger an inflammatory response via activation of p38MAPK pathway.²⁷ Our original work on the RAGE-HMGB1 system was followed by additional studies confirming that AF HMGB1 is elevated in women with IAI and infection,^{28,29} where it was similarly reported that the amniochorion in pregnancies complicated by IAI and AF infection displays increased immunoreactivity for HMGB1. Interestingly, we showed that several women who delivered preterm had positive AF cultures but no IAI by both MR score and IL-6. The AF levels of HMGB1 were low. Our interpretation is that these women may have had: 1) a recent microbial invasion of the AF sac with insufficient time for full blown inflammatory response; 2) bacterial contamination of the AF sample leading to a false positive culture result; or 3) invasion of the AF cavity with low virulence bacteria (i.e. *Ureaplasma* spp.). Conversely, several women with severe IAI had negative AF cultures. This could be the result of a robust IAI response secondary to uncultivated or difficult to cultivate AF bacteria.³⁰

Our observation of overlapping staining pattern between HMGB1 and RAGE in the amniochorion of women with histologic chorioamnionitis supports the data suggesting that the RAGE-HMGB1 system is an active participant in inflammation-induced PTB and perhaps PPRM. We showed that exposure of fetal membranes to LPS *in vitro* results in significant HMGB1 release, a finding recently confirmed by Bredeson et. al.²⁷ Collectively, the current and previously generated body of evidence suggests that the fetal membranes are a possible source of AF HMGB1 in IAI. The observed increase of HMGB1 in the AF of women with severe IAI and its direct correlation with IL-6 is consistent with our previous work that demonstrated a similar dynamic in the premature fetus exposed to IAI.⁷ Lastly, as HMGB1 is known to bind DNA as well as microbial constituents while facilitating their diffusion through cell membranes³¹, the effects of in utero exposure of the fetus to the combination of bacteria and elevated amniotic fluid HMGB1 may extend over the peripartum period to impact on neonatal outcome.

5. Conclusions

Our findings reveal that the levels of AF HMGB1 are not GA regulated in pregnancies with normal outcome. In pregnancies complicated by IAI secondary to infection, the high levels of HMGB1 correlated with levels of AF IL-6 and to the damage of the fetal membranes as shown by a higher intensity of immunostaining in areas of amnio-chorion necrosis. Our *in vitro* and *in vivo* data suggest inflamed fetal membranes may be a source of elevated HMGB1.

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Highlights

- Amniotic fluid levels of HMGB1 are not gestational age regulated
- Amniotic fluid HMGB1 levels are upregulated in intra-amniotic inflammation
- The damaged amniochorion is likely the source of elevated amniotic fluid HMGB1

Fig 1.

(A) Gestational age (GA) regulation of amniotic fluid HMGB1; (B) HMGB1 levels in amniotic fluid of women with preterm birth and absent (MR 0), mild (MR 1–2) or severe (MR 3–4) intra-amniotic inflammation (IAI); (C) Relationship of amniotic fluid HMGB1 to the acute phase cytokine IL-6 in the same patient population.

Fig 2.

(**A**) Representative immunohistochemical staining for HMGB1 in fetal membranes of woman with spontaneous preterm birth and absent (MR 0) intra-amniotic inflammation (IAI) as compared to (**B**) a patient with severe (IAI) (MR 3–4) and histological chorioamnionitis. Note the intense positive staining of the amnion epithelium (AE, arrows) and of the necrotic zones in choriodecidua (arrowheads). Bar: 50 μ m. Chromogen: Vector NovaRed. Hematoxylin counterstain. am: amnion; cd: choriodecidua

Fig 3.

Representative micrographs illustrating (A) HMGB1 and (B) RAGE staining in fetal membranes of the same patient with preterm birth, severe intra-amniotic inflammation and necrotizing chorioamnionitis. (C) Negative control slide with omitted primary antibody. Note the intense positive staining of both HMGB1 and RAGE in the amnion. Bar: 50 μ m. Chromogen: (A) Vector NovaRed and Hematoxylin counterstain; (B & C) Ni-DAB, no counterstain. am: amnion; cd: choriodecidua

Fig 4.

HMGB1 release was measured by ELISA in the medium of amniochorion explants at 18 hours of incubation. Tissues were obtained under sterile conditions from 4 women undergoing elective C-sections at term. Data was normalized for total tissue protein.

Table 1

Demographic characteristics of women in the study (n=265)

Variables	2nd trimester genetic n = 25	Rule-out infection amniocenteses n = 215	3rd trimester lung maturity n = 25	P value
<i>Clinical and outcome characteristics</i>				
Maternal age, years [†]	33 [28 – 37]	29 [23 – 33]	29 [25 – 33]	0.021
Parity [†]	1 [0 – 1]	1 [1 – 2]	1 [0 – 2]	0.037
Gravidity [†]	2 [1 – 3]	2 [1 – 4]	[2 – 5]	0.187
GA at amniocentesis, weeks [†]	18 [17 – 20]	29 [25 – 31]	36 [35 – 37]	<0.001
Clinical chorioamnionitis [‡]	0 (0)	16 (8)	0 (0)	<0.001
PPROM, [‡]	0 (0)	108 (53)	0 (0)	<0.001
Spontaneous preterm birth <37 weeks [‡]	0 (0)	186 (91)	0 (0)	<0.001
GA at delivery, weeks [†]	39 [38 – 40]	30 [26 – 33]	37 [36 – 38]	<0.001
Cesarean delivery [‡]	6 (30)	77 (38)	20 (83)	<0.001
Birthweight, grams [†]	3,095 [2,807 – 3,742]	1,405 [920 – 2,013]	3,042 [2,708 – 3,321]	<0.001

[†]Data presented as median [interquartile range] and analyzed by Kruskal-Wallis ANOVA on ranks.

[‡]Data presented as n (%) and analyzed by Chi-square tests.

GA: gestational age; PPRM: Preterm Premature Rupture of Membranes

Table 2

Demographic, amniotic fluid and histologic characteristics of cases who had an amniocentesis to rule out intra-amniotic infection (n=215).

Variables	No IAI & TD n = 29	No IAI & PTB n = 86	Yes IAI & PTB n = 100	P value
<i>Clinical and outcome characteristics</i>				
Maternal age, years [†]	26 [22 – 29]	29 [23 – 33]	29 [22 – 34]	0.197
Parity [†]	1 [0 – 2]	1 [0 – 1]	1 [0 – 2]	0.493
Gravidity [†]	2 [1 – 4]	2 [1 – 3]	3 [2 – 4]	0.237
GA enrollment, weeks [†]	29 [25 – 32]	30 [27 – 32]	27 [24 – 30]	<0.001
GA delivery, weeks [†]	39 [38 – 40]	31 [29 – 33]	27 [25 – 30]	<0.001
Amnio - delivery interval, days	63 [46 – 91]	2 [0.7 – 6]	0.3 [0.2 – 0.6]	<0.001
PPROM [‡]	0 (0)	57 (66)	51 (51)	<0.001
Clinical chorioamnionitis [‡]	0 (0)	1 (1)	15 (15)	0.002
Cesarean delivery [‡]	5 (26)	31 (36)	41 (41)	0.446
Birthweight, grams [†]	3,415 [2,909 – 3,570]	1,725 [1,275 – 1,410]	980 [770 – 1,410]	<0.001
<i>Amniotic fluid analysis (microbiological and inflammatory status)</i>				
Glucose, mg/dL [†]	30 [24 – 45]	26 [18 – 40]	3 [2 – 10]	<0.001
LDH, U/L [†]	166 [115 – 232]	171 [113 – 254]	811 [532 – 1,830]	<0.001
WBC, cells/mm ³ [†]	5 [3 – 11]	6 [2 – 18]	890 [216 – 1,880]	<0.001
Positive AF cultures [‡]	0 (0)	9 (10)	74 (74)	<0.001
Positive Gram stain [‡]	0 (0)	6 (7)	62 (62)	<0.001
MR score 3 – 4 [‡]	0 (0)	0 (0)	100 (100)	<0.001
IL-6 ng/mL [†]	0.2 [0.05 – 0.2]	0.7 [0.2 – 2.4]	34.9 [10.3 – 85.9]	<0.001
<i>Histological inflammation</i>				
Chorionic plate, stage [†]	0 [0 – 0]	0 [0 – 2]	3 [2 – 3]	<0.001
Chorionic plate (stage 2–3) [‡]	0 (0)	24 (30)	86 (86)	<0.001
Amnionitis, grade [†]	0 [0 – 0]	0 [0–1]	3 [2 – 3]	<0.001
Amnionitis (grade 2–4) [‡]	0 (0)	16 (16)	80 (93)	<0.001
Chorionodecidualitis, grade [†]	0 [0 – 0]	1 [0 – 3]	3 [3 – 4]	<0.001
Chorionodecidualitis (grade 2–4) [‡]	0 (0)	38 (44)	94 (94)	<0.001

[†]Data presented as median [interquartile range] and analyzed by Kruskal-Wallis one-way analysis of variance.

[‡]Data presented as n (%) and analyzed by Chi-square tests;

IAI=Intra Amniotic Inflammation defined by a Mass Restricted (MR) score; For the purpose of presenting the demographic data, women with MR 0–2 were classified together as “No IAI” while only women with MR 3–4 were categorized as “Yes IAI”. GA=gestational age; PPRM=Preterm Premature Rupture of Membranes.