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**Human  $\beta$ -defensin-1: a natural antimicrobial peptide present in amniotic fluid that is increased in spontaneous preterm labor with intra-amniotic infection**

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**Short title:** Human  $\beta$ -defensin-1 in amniotic fluid

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**ABSTRACT**

**Problem:** Human  $\beta$ -defensins (HBD) are antimicrobial peptides that participate in the soluble innate immune mechanisms against infection. Herein, we determined whether HBD-1 was present in amniotic fluid during normal pregnancy and whether its concentrations change with intra-amniotic inflammation and/or infection.

**Method of Study:** Amniotic fluid was collected from 219 women in the following groups: 1) midtrimester who delivered at term (n=35); 2) term with (n=33) or without (n=17) labor; 3) preterm labor with intact membranes who delivered at term (n=29) or who delivered preterm with (n=19) and without (n=29) intra-amniotic inflammation and infection or with intra-amniotic inflammation but without infection (n=21); and 4) preterm prelabor rupture of membranes (pPROM) with (n=19) and without (n=17) intra-amniotic inflammation/infection. Amniotic fluid HBD-1 concentrations were determined using a sensitive and specific ELISA kit.

**Results:** 1) HBD-1 was detectable in all amniotic fluid samples; 2) amniotic fluid concentrations of HBD-1 were changed with gestational age (midtrimester vs. term no labor), being higher in midtrimester; 3) amniotic fluid concentrations of HBD-1 were similar between women with and without spontaneous labor at term; 4) among patients with spontaneous preterm labor, amniotic fluid concentrations of HBD-1 were greater in women with intra-amniotic inflammation/infection and in women with intra-amniotic inflammation without infection compared to those without intra-amniotic inflammation or infection who delivered preterm and those who delivered at term; and 5) the presence of intra-amniotic inflammation and infection in patients with pPROM did not change amniotic fluid concentrations of HBD-1.

**Conclusions:** HBD-1 is a physiological constituent of amniotic fluid that is increased in midtrimester during normal pregnancy and in the presence of culturable microorganisms in the amniotic cavity. These findings provide insight into the soluble host defense mechanisms against intra-amniotic infection.

**Keywords:** acute chorioamnionitis, cytokines, danger signals, fetal immunity, funisitis, innate immunity, microbial invasion of the amniotic cavity, neutrophils, preterm PROM, sterile intra-amniotic inflammation

## **INTRODUCTION**

The amniotic fluid has several antimicrobial properties and functions as an immunological barrier to the growing fetus<sup>1-9</sup>. It contains soluble components such as electrolytes, carbohydrates, lipids, and peptides which can act as a defense against pathogens invading the amniotic cavity<sup>10-30</sup>. The amniotic fluid also contains cellular

components<sup>31-45</sup> that have been recently characterized using immunophenotyping<sup>46</sup>. It is now clear that amniotic fluid includes both innate and adaptive immune cells such as monocytes/macrophages, neutrophils, innate lymphoid cells, B cells, natural killer cells, and T cells<sup>46</sup>. Most of these immune cells are more abundant in women with intra-amniotic infection and/or inflammation<sup>46</sup>. Among innate immune cells, neutrophils are considered a marker of intra-amniotic inflammation since their number is increased in women with intra-amniotic infection<sup>47, 48</sup>. While amniotic fluid neutrophils are mostly of fetal origin in preterm gestations, they can also be of maternal origin at term<sup>49</sup>. Regardless of their origin, amniotic fluid neutrophils participate in the host defense mechanisms against intra-amniotic infection by performing phagocytosis<sup>50</sup>, forming neutrophil extracellular traps or NETs<sup>51</sup>, and releasing inflammatory mediators<sup>48, 52-78</sup> including an array of antimicrobial peptides<sup>18, 79-84</sup>.

Antimicrobial peptides represent soluble mediators of the innate immune system, which provide protection against pathogens<sup>85-91</sup>. Several families of antimicrobial peptides have been described in mammals, such as cathelicidins and defensins<sup>92, 93</sup>. The latter are small cationic peptides synthesized by neutrophils, Paneth cells, and epithelial cells<sup>85, 94-99</sup>, and are regulated primarily by microbial signals, cytokines, and in some cases by neuroendocrine signals in a tissue-specific manner<sup>90, 95, 100</sup>. Defensins can be of different types based on their function and location of expression in the human body<sup>90</sup>. Two main types of defensins have been identified in humans:  $\alpha$  and  $\beta$  defensins<sup>101, 102</sup>. Alpha defensins are seen in azurophilic granules in neutrophils<sup>85, 94, 103, 104</sup> and have also been identified in the Paneth cells of intestinal crypts<sup>95, 98, 105, 106</sup>. These mediators have six subtypes referred to as human neutrophil peptide (HNP)-1 to 4 and human defensin-5 and -6<sup>90</sup>. Increased concentrations of HNP-1 in amniotic fluid have been associated with intra-uterine infection and acute histologic chorioamnionitis<sup>107, 108</sup>.

Human  $\beta$ -defensins (HBD) are expressed in the epithelial cells of mucosal surfaces<sup>86, 99, 109</sup>. HBD-1, -2 and -3 proteins have all been localized in the reproductive tissues including the amnion epithelium, chorion trophoblast, decidua, and placental syncytiotrophoblast<sup>110</sup>. HBD-2 has also been detected in amniotic fluid and its concentration is increased in women with intra-amniotic inflammation/infection who

underwent spontaneous preterm labor with intact membranes and in those with preterm prelabor rupture of membranes (pPROM)<sup>84</sup>. However, whether HBD-1 can be detected in amniotic fluid during normal pregnancy and its complications is unknown.

The aims of this study were to: 1) detect HBD-1 in amniotic fluid of women in mid-gestation and at term with or without labor; 2) investigate whether HBD-1 was increased in amniotic fluid of women who underwent spontaneous preterm labor with intact membranes and intra-amniotic inflammation and/or infection; and 3) determine whether HBD-1 concentrations were different between women who underwent pPROM with or without intra-amniotic inflammation/infection.

## **MATERIALS AND METHODS**

### **Study design and population**

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

We utilized 219 amniotic fluid samples which were divided into 4 groups. Group 1 comprised amniotic fluid from women who underwent amniocentesis during mid-trimester (14-18 weeks) for genetic indications and delivered a normal full term neonate (n=35) (Table 1). Group 2 included amniotic fluid samples from women with normal pregnancies who underwent amniocentesis at term (>37 weeks) with (n=33) and without (n=17) spontaneous labor (Table 1). Group 3 included women who underwent spontaneous preterm labor with intact membranes and were further classified into women who underwent 1) spontaneous preterm labor who proceeded to have a term delivery with a negative amniotic fluid culture and interleukin (IL)-6 <2.6ng/mL (n=29), 2) spontaneous preterm labor without intra-amniotic inflammation or infection (n=29) (see diagnostic criteria below), 3) spontaneous preterm labor with intra-amniotic inflammation and a negative amniotic fluid culture (n=21), and 4) spontaneous preterm labor with

intra-amniotic inflammation and infection proven by a positive amniotic fluid culture (n=19) (Table 2). Lastly, Group 4 consisted of amniotic fluid samples from women who underwent pPROM with (n=19) and without (n=17) intra-amniotic inflammation and infection (Table 3).

The inclusion criteria for uncomplicated pregnancies were: 1) no medical, obstetrical, or surgical complications, 2) absence of microbial invasion of the amniotic cavity (MIAC) (see diagnostic criteria below), 3) amniotic fluid IL-6 concentrations <2.6ng/mL, 4) intact membranes, and 5) delivery of a term neonate with birth weight appropriate for gestational age.

### **Clinical definitions**

Spontaneous preterm labor was defined as regular uterine contractions (at least two contractions every 10 minutes) associated with cervical changes. MIAC was defined by a positive amniotic fluid culture for microorganisms<sup>111-123</sup>. Intra-amniotic inflammation was defined as an amniotic fluid IL-6 concentration  $\geq 2.6\text{ng/mL}$ <sup>118</sup>. Intra-amniotic infection was defined as the presence of MIAC with intra-amniotic inflammation<sup>77, 118, 120, 121, 124-138</sup>. Preterm prelabor rupture of membranes (pPROM) had to occur prior to the onset of labor before 37 weeks and was diagnosed by vaginal pooling of amniotic fluid, amniotic fluid ferning patterns, and a positive nitrazine test<sup>139-142</sup>.

### **Sample collection**

Amniotic fluid was retrieved by transabdominal amniocentesis, under antiseptic conditions and monitored by ultrasound. Transabdominal amniocentesis in patients from Groups 2, 3 and 4 was performed for the detection of intra-amniotic inflammation and/or infection and fetal lung maturity tests. The remaining amniotic fluid that was not required for clinical purposes was centrifuged at 4°C and 1300 x g for 10 minutes for the removal of cellular and particulate matter, and the supernatants were stored at -80°C. A portion of this amniotic fluid was also transported to the laboratory for culture of aerobic/anaerobic bacteria and genital mycoplasmas. The clinical tests also included the determination of amniotic fluid white blood cell (WBC) count<sup>47</sup>, glucose concentration<sup>143</sup>, Gram stain<sup>144</sup>, and IL-6 concentration<sup>118</sup>.

### **Determination of IL-6 in amniotic fluid**

Amniotic fluid concentrations of IL-6 were determined by using a sensitive and specific enzyme immunoassay obtained from R&D systems (Minneapolis, MN, USA). The IL-6 concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The detection limit of the IL-6 assay was 0.09 pg/mL. The IL-6 concentrations in amniotic fluid were determined for clinical purposes.

### **Detection of HBD-1 by ELISA**

HBD-1 was detected in amniotic fluid using the Total Beta Defensin 1 ELISA kit from Aviscera Bioscience (Cat#SK00858-06, Santa Clara, CA). This ELISA kit was validated in our laboratory prior to execution of the study. Amniotic fluid concentrations of HBD-1 were obtained by interpolation from the standard curve. The inter- and intra-assay coefficients of variation were 14.56% and 11.47%, respectively. The sensitivity of the assay was 8.96 pg/mL.

### **Statistical Analysis**

Statistical analysis was performed using the SPSS v19 software (SPSS Inc., IBM Corporation, Armonk, NY, USA). Normality of the data was tested using the Shapiro-Wilk test. Non-parametric testing was applied for comparisons, and adjustments for multiple comparisons were performed when indicated. Comparisons of the proportions were made using Fisher's exact tests. A P-value of <0.05 was used to determine statistical significance.

## **RESULTS**

The demographic and clinical characteristics of the study populations are displayed in Tables 1, 2, and 3. Nulliparity rate was similar among the study groups. The maternal age and race were significantly different between women in the mid-

trimester group and those at term with or without labor (Table 1), but did not differ among the preterm labor or pPROM groups (Tables 2 and 3). The neonatal birthweights were significantly different among the preterm labor groups (Table 2) and pPROM groups (Table 3). Amniotic fluid IL-6 concentrations were elevated in women with spontaneous labor at term compared to those who delivered at term without labor (Table 1) and, as expected, were increased in women with intra-amniotic inflammation and/or infection compared to those without this clinical condition (Tables 2 and 3). HBD-1 was detected in all 219 amniotic fluid samples.

### **Amniotic fluid HBD-1 concentration in normal pregnancy**

The concentration of HBD-1 in amniotic fluid was higher in the midtrimester group than in the term no labor group [midtrimester: median 13.73 ng/mL (IQR 6.87-20.44 ng/mL) vs. term no labor: median 9.07 ng/mL (IQR 6.93-12.15 ng/mL),  $p=0.03$ ] (Figure 1).

The concentration of HBD-1 in amniotic fluid was similar between the term no labor and term labor groups [term no labor: median 9.07 ng/mL (IQR 6.93-12.15 ng/mL) vs. term labor: median 6.44 ng/mL (IQR 3.59-11.45 ng/mL),  $p=0.1$ ] (Figure 2).

### **Amniotic fluid HBD-1 concentration in women with spontaneous preterm labor**

Among women who presented with spontaneous preterm labor, the median amniotic fluid concentration of HBD-1 was higher in women with intra-amniotic inflammation and infection than in those who delivered at term [preterm labor with intra-amniotic inflammation and infection who delivered preterm: median 24.36 ng/mL (IQR 17.34-38.91 ng/mL) vs. preterm labor who delivered at term: median 10.87 ng/mL (IQR 8.82-13.94 ng/mL),  $p<0.001$ ] (Figure 3). The median amniotic fluid concentration of HBD-1 was also greater in women with intra-amniotic inflammation and infection than in those without intra-amniotic inflammation or infection [preterm labor with intra-amniotic inflammation and infection who delivered preterm: median 24.36 ng/mL (IQR 17.34-38.91 ng/mL) vs. preterm labor without intra-amniotic inflammation or infection who delivered preterm: median 12.2 ng/mL (IQR 8.02-18.58 ng/mL),  $p=0.006$ ] (Figure 3).



Patients who underwent spontaneous preterm labor with intra-amniotic inflammation but without detected microorganisms had a higher median amniotic fluid concentration of HBD-1 than those with preterm labor who delivered at term [preterm labor with intra-amniotic inflammation but without infection who delivered preterm: median 18.31 ng/mL (IQR 12.17-32.15 ng/mL) vs. preterm labor who delivered at term: median 10.87 ng/mL (IQR 8.82-13.94 ng/mL),  $p=0.003$ ] (Figure 3). Patients who underwent spontaneous preterm labor with intra-amniotic inflammation but without infection also had a higher median amniotic fluid concentration of HBD-1 than those with preterm labor without intra-amniotic inflammation or infection [preterm labor with intra-amniotic inflammation but without infection who delivered preterm: median 18.31 ng/mL (IQR 12.17-32.15 ng/mL) vs. preterm labor without intra-amniotic inflammation or infection who delivered preterm: median 12.2 ng/mL (IQR 8.02-18.58 ng/mL),  $p=0.02$ ] (Figure 3). Yet, there were no differences between women with preterm labor and intra-amniotic inflammation and infection and those with intra-amniotic inflammation but without infection [preterm labor with intra-amniotic inflammation and infection who delivered preterm: median 24.36 ng/mL (IQR 17.34-38.91 ng/mL) vs. preterm labor with intra-amniotic inflammation but without infection who delivered preterm: median 18.31 ng/mL (IQR 12.17-32.15 ng/mL),  $p=0.9$ ] (Figure 3). There were no differences between women who underwent spontaneous preterm labor without intra-amniotic inflammation or infection and those with preterm labor who delivered at term [preterm labor without intra-amniotic inflammation or infection who delivered preterm: median 12.2 ng/mL (IQR 8.02-18.58 ng/mL) vs. preterm labor who delivered at term: median 10.87 ng/mL (IQR 8.82-13.94 ng/mL),  $p=0.9$ ] (Figure 3).

### **Amniotic fluid HBD-1 concentration in women with preterm prelabor rupture of membranes (pPROM)**

Patients with pPROM had similar median amniotic fluid concentrations of HBD-1, irrespective of their intra-amniotic inflammation/infection status [pPROM without intra-amniotic inflammation or infection: median 12.4 (IQR 9.46-14.77) vs. pPROM with intra-amniotic inflammation and infection: median 15.47 (IQR 8.68-19.51),  $p=0.2$ ] (Figure 4).

## **DISCUSSION**

**The principal findings of this study were:** 1) HBD-1 is a physiological constituent of amniotic fluid; 2) amniotic fluid concentrations of HBD-1 changed with gestational age (midtrimester vs. term no labor), being higher in midtrimester; 3) amniotic fluid concentrations of HBD-1 were similar between women with and without spontaneous labor at term; 4) among patients with spontaneous preterm labor, amniotic fluid concentrations of HBD-1 were greater in women with intra-amniotic inflammation and infection than in those without intra-amniotic inflammation or infection who delivered preterm and those who delivered at term; 5) amniotic fluid concentrations of HBD-1 were also higher in women with intra-amniotic inflammation but without infection than in those without intra-amniotic inflammation or infection who delivered preterm or those who delivered at term; and 6) the presence of intra-amniotic inflammation and infection in patients with pPROM did not change amniotic fluid concentrations of HBD-1.

### **HBD-1 is present in amniotic fluid and its concentration changes with gestational age**

HBD-1 was present in amniotic fluid of women with normal pregnancy. Consistently, previous studies have shown that other human defensins (HBD-2 and -3) are present in the second trimester amniotic fluid<sup>84, 145</sup>. Given that fetal tissues including the lungs, placenta and chorioamniotic membranes, as well as the decidua express human defensins<sup>110, 146-150</sup>, it is likely that both fetal and maternal tissues serve as a source for these natural antimicrobial peptides.

The amniotic fluid concentration of HBD-1 was elevated in midtrimester compared to women who delivered at term without labor, and in those who delivered at term with labor (comparison not shown), suggesting that this antimicrobial peptide is more required in the second trimester than at term pregnancy. This is in line with the evidence showing that in midtrimester; most of the innate immune cells that participate in host defense mechanisms against infection (e.g. neutrophils) are low in number<sup>46</sup>. Therefore, amniotic fluid HBD-1 may serve as an innate soluble component that protects the fetus against invading pathogens during midtrimester when neutrophils are rare. As gestation progresses towards term, the number of neutrophils in amniotic fluid increases until these immune cells become the dominant leukocyte subset<sup>46</sup> and may

take over the host defense mechanisms in the amniotic cavity through performing phagocytosis<sup>50</sup> and NET formation<sup>51</sup>, as well as releasing other antimicrobial peptides<sup>18, 79-84</sup>.

These data indicate that HBD-1 is a physiological component of amniotic fluid during normal pregnancy and that it may be implicated in the soluble innate immune mechanisms which occur in the amniotic cavity early in gestation.

### **Amniotic fluid HBD-1 concentrations do not differ between women with and without spontaneous labor at term**

The amniotic fluid concentration of HBD-1 was similar between women with and without spontaneous labor at term. Labor is considered a state of physiologic inflammation<sup>151-167</sup> since intra-amniotic infection is absent in most women who deliver at term<sup>117</sup>. Therefore, spontaneous labor at term is a sterile inflammatory process and may not need to be associated with an increase in the concentration of HBD-1. This is supported by a previous report showing that the expression of HBD-1 is unchanged in the placenta and chorioamniotic membranes by the process of labor at term<sup>110</sup>. These data show that amniotic fluid HBD-1 is unchanged by the physiological process of labor at term.

### **Amniotic fluid HBD-1 concentration is increased in women with spontaneous preterm labor and intra-amniotic inflammation and/or infection**

Women with spontaneous preterm labor and intra-amniotic inflammation and infection (amniotic fluid IL-6 $\geq$ 2.6 ng/mL and a positive microbial culture) had higher amniotic fluid concentrations of HBD-1 than those without intra-amniotic inflammation or infection (amniotic fluid IL-6 $<$ 2.6 ng/mL and a negative microbial culture) and those who delivered at term. These findings are consistent with a previous report demonstrating that amniotic fluid concentration of HBD-2 is increased in women with MIAC and inflammation<sup>84</sup>. The data presented herein are also consistent with *in vitro* studies indicating that *Candida albicans* can increase the release of HBD-1 by the chorioamniotic membranes<sup>168</sup>. The intra-amniotic inflammatory process associated with elevated amniotic fluid concentrations of HBD-1 may be mediated by toll-like receptors

(TLRs) such as TLR-4 and TLR-2, which had been implicated in the mechanisms that lead to secretion of defensins by epithelial cells<sup>169</sup>. Our data, along with previous studies demonstrating that MIAC results in an increased amniotic fluid concentration of lactoferrin<sup>81, 82</sup>, lysozyme<sup>19, 21</sup>, and bacterial/permeability-increasing protein<sup>20</sup> among others<sup>107, 170-172</sup>, suggest that antimicrobial peptides participate in the soluble host defense mechanisms against intra-amniotic infection<sup>173, 174</sup> in women who underwent spontaneous preterm labor and delivered preterm.

Women with spontaneous preterm labor with intra-amniotic inflammation but without infection (amniotic fluid IL-6 $\geq$ 2.6 ng/mL but a negative microbial culture) had higher amniotic fluid concentrations of HBD-1 than those without intra-amniotic inflammation or infection (amniotic fluid IL-6 $<$ 2.6 ng/mL and a negative microbial culture) and those who delivered at term. Given that molecular microbiological detection of nonculturable microbes was not attainable in this study, we cannot discard the possibility that the intra-amniotic inflammatory process in the absence of culturable microbes is sterile in nature. In the case that nonculturable microbes are present in the amniotic cavity, the resulting intra-amniotic inflammatory response seems to be milder than in cases with culturable microorganisms, suggesting that MIAC potentiates the host response in the amniotic cavity.

In patients with preterm labor, intra-amniotic inflammation can also occur in the absence of microbes detected by cultivation and molecular microbiological techniques (i.e. sterile intra-amniotic inflammation)<sup>126, 128</sup>. We and others have proposed that sterile intra-amniotic inflammation can be induced by danger signals derived by the fetal tissues<sup>175-179</sup>. Women with sterile intra-amniotic inflammation have a similar rate of preterm birth and adverse neonatal outcomes as those with proven intra-amniotic infection<sup>128</sup>. In addition, both sterile intra-amniotic inflammation and intra-amniotic infection are associated with acute histologic chorioamnionitis and funisitis (i.e. acute histologic lesions of the placenta)<sup>128</sup> and high concentrations of inflammatory cytokines in the amniotic cavity<sup>76</sup>. In line with these findings, our data showed that both women with intra-amniotic inflammation/infection and those with intra-amniotic inflammation but without infection have high amniotic fluid concentrations of HBD-1. These data suggest

that HBD-1 participates in the host response against nonculturable microbes or danger signals in the amniotic cavity.

### **Amniotic fluid HBD-1 concentration in women with preterm prelabor rupture of membranes (pPROM) is unchanged by the presence of intra-amniotic inflammation and infection**

Women with pPROM and intra-amniotic inflammation and infection (amniotic fluid IL-6 $\geq$ 2.6 ng/mL and a positive microbial culture) had similar amniotic fluid concentrations of HBD-1 compared to those without intra-amniotic inflammation or infection (amniotic fluid IL-6 $<$ 2.6 ng/mL but a negative microbial culture). Our previous report showed that amniotic fluid concentrations of HBD-2 are higher in patients with pPROM and MIAC than in those without MIAC<sup>84</sup>. These findings are explained by evidence indicating that HBD-1 and HBD-2 show differential expression in inflammatory diseases (e.g. periodontitis<sup>180</sup>) and in human peripheral blood cells stimulated with microbial products<sup>181</sup>. In addition, patients with pPROM usually received antibiotic treatment<sup>182, 183</sup>, which could directly reduce the release of antimicrobial peptides and neutrophil-mediated antimicrobial activity, in addition to decreasing the microbial burden<sup>184, 185</sup>. Further research is required to investigate whether antibiotics given to women with pPROM can directly inhibit the secretion of HBD-1 in the presence of bacteria associated with this clinical condition.

### **CONCLUSIONS**

The findings described herein indicate that HBD-1 is a physiological constituent of amniotic fluid that is increased in midtrimester during normal pregnancy as part of the soluble innate immune response early in gestation. Amniotic fluid HBD-1 concentrations are increased in the presence of culturable microorganisms in the amniotic cavity, which may participate in the soluble host defense mechanisms against intra-amniotic infection or other inflammatory stimuli (e.g. danger signals or nonculturable microorganisms). Further studies may investigate the mechanisms whereby HBD-1 is released into the amniotic cavity, its antimicrobial properties against microbes associated with intra-

amniotic infection, and whether danger signals could induce its release in the setting of sterile intra-amniotic inflammation.

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### Figure Legends

**Figure 1.** Amniotic fluid concentrations of human  $\beta$ -defensin-1 (HBD-1) in normal pregnancy. The median concentration of amniotic fluid HBD-1 in midtrimester (n=35) was significantly higher than that of women at term without labor (n=17) [midtrimester: median 13.73 (IQR 6.87-20.44 ng/mL) vs. term no labor: median 9.07 ng/mL (IQR 6.93-12.15 ng/mL); p=0.03]. IQR = interquartile range.

**Figure 2.** Amniotic fluid concentrations of human  $\beta$ -defensin-1 (HBD-1) at term pregnancy. There was no difference between the median amniotic fluid concentration of HBD-1 between women with (n=33) and without labor (n=17) at term [term no labor: median 9.07 ng/mL (IQR 6.93-12.15 ng/mL) vs. term labor: median 6.44 ng/mL (IQR 3.59-11.45 ng/mL); p=0.1]. IQR = interquartile range.

**Figure 3.** Amniotic fluid concentrations of human  $\beta$ -defensin-1 (HBD-1) in spontaneous preterm labor with intact membranes. The median amniotic fluid concentration of HBD-1 in women with preterm labor and intra-amniotic inflammation and infection who delivered preterm (n=19) was significantly higher than that of women with preterm labor who delivered at term (n=29) [preterm labor with intra-amniotic inflammation and infection who delivered preterm: median 24.36 ng/mL (IQR 17.34-38.91) vs. preterm labor who delivered at term: median 10.87 ng/mL (IQR 8.82-13.94 ng/mL); p<0.001] and women with preterm labor without intra-amniotic inflammation or infection who delivered preterm (n=29) [preterm labor with intra-amniotic inflammation and infection who delivered preterm: median 24.36 ng/mL (IQR 17.34-38.91) vs. preterm labor without intra-amniotic inflammation or infection who delivered preterm: median 12.2 ng/mL (IQR 8.02-18.58 ng/mL); p=0.006]. The median amniotic fluid concentration of HBD-1 in women with preterm labor and intra-amniotic inflammation but without infection who delivered preterm (n=21) was elevated compared to that of women with preterm labor

who delivered at term [preterm labor with intra-amniotic inflammation but without infection who delivered preterm: median 18.31 ng/mL (IQR 12.17-32.15 ng/mL) vs. preterm labor who delivered at term: median 10.87 ng/mL (IQR 8.82-13.94 ng/mL);  $p=0.003$ ] and women with preterm labor without intra-amniotic inflammation or infection who delivered preterm [preterm labor with intra-amniotic inflammation but without infection who delivered preterm: median 18.31 ng/mL (IQR 12.17-32.15 ng/mL) vs. preterm labor without intra-amniotic inflammation or infection who delivered preterm: median 12.2 ng/mL (IQR 8.02-18.58 ng/mL);  $p=0.02$ ]. IQR = interquartile range. P-values were adjusted for multiple comparisons.

**Figure 4.** Amniotic fluid concentrations of human  $\beta$ -defensin-1 (HBD-1) in preterm prelabor rupture of membranes (pPROM). The median amniotic fluid concentrations of HBD-1 were similar between women with pPROM and intra-amniotic inflammation and infection ( $n=19$ ) and those without intra-amniotic inflammation or infection ( $n=17$ ) [pPROM with intra-amniotic inflammation and infection: median 15.47 (IQR 8.68-19.51) vs. pPROM without intra-amniotic inflammation or infection: median 12.4 (IQR 9.46-14.77);  $p=0.2$ ]. IQR = interquartile range.

**Table 1.** Clinical and demographic characteristics of women who underwent an amniocentesis during midtrimester or at term pregnancy

	Normal Pregnancy Midtrimester (n=35)	Term no labor (n=17)	Term in labor (n=33)	P-value
Maternal Age (years) <sup>a</sup>	37 (35-39)	24 (21-32)	23 (20-29)	<0.001
Nulliparous <sup>b</sup>	17.1% (6/35)	23.5% (4/17)	42.4% (14/33)	0.058
Race <sup>b</sup>				<0.001
African-American	85.7% (30/35)	0	0	
Caucasian	(2/35)	0	0	
Hispanic	0	100% (17/17)	100% (33/33)	
Other	8.6 (3/35)	0	0	
Gestational age at amniocentesis (weeks) <sup>a</sup>	16 (16-17)	39 (38.5-40)	39 (38-40)	<0.001
Gestational age at delivery (weeks) <sup>a</sup>	39 (38-40)	39 (38.5-40)	39 (38-40)	0.874
Birthweight (grams) <sup>a</sup>	3344 (3176-3621)	3260 (3130-3790)	3280 (3085-3715)	0.734
Amniotic fluid IL-6 (ng/mL) <sup>a</sup>	0.68 (0.34-1.06)	0.41 (0.22-0.85)	1.04 (0.5-1.37)	0.046

Data are given as median (interquartile range) and percentage (n/N)

<sup>a</sup>Kruskal-Wallis / Mann-Whitney U test.

<sup>b</sup>Fisher's exact test.

**Table 2.** Clinical and demographic characteristics of women who underwent spontaneous preterm labor

	Preterm labor delivered at term (n=29)	Preterm labor without intra-amniotic inflammation or infection (n=29)	Preterm labor with intra-amniotic inflammation but without infection (n=21)	Preterm labor with intra-amniotic inflammation and infection (n=19)	P-value
Maternal Age (years) <sup>a</sup>	23 (20-30)	25 (22-30)	22 (20-26)	25 (20-29)	0.65
Nulliparous <sup>b</sup>	24.1% (7/29)	34.5% (10/29)	42.9% (9/21)	63.2% (12/19)	0.061
Race <sup>b</sup>					0.48
African-American	86.2 (25/29)	85.1% (23/27) <sup>d</sup>	85% (17/20) <sup>c</sup>	100% (19/19)	
Caucasian	10.3% (3/29)	14.8% (4/27) <sup>d</sup>	10% (2/20) <sup>c</sup>	0	
Hispanic	3.4% (1/29)	0	5% (1/20) <sup>c</sup>	0	
Gestational age at amniocentesis (weeks) <sup>a</sup>	29.3 (27.6-31.2)	28.7 (26.6-32)	25 (22-28.1)	24.6 (20.35-27)	<0.001
Gestational age at delivery (weeks) <sup>a</sup>	38 (37-40)	33 (31-36)	28 (22.3-31)	25 (20.4-29.25)	<0.001
Birthweight (grams) <sup>a</sup>	2930 (2600-3150)	1960 (1390-2320)	830 (480-1300)	610 (430-1177.5)	<0.001
Amniotic fluid IL-6 (ng/mL) <sup>a</sup>	0.55 (0.23-0.76)	0.92 (0.36 - 1.32)	28.43 (9.54-60.7)	172.4 (87.74-350.91)	<0.001
Amniotic fluid glucose (mg/dL) <sup>a</sup>	37 (28.5-51.5) <sup>e</sup>	32 (27-36.5) <sup>f</sup>	27 (21.5-29) <sup>e</sup>	16 (10-28)	<0.001

Amniotic fluid white blood cells (/mm <sup>3</sup> ) <sup>a</sup>	1 (0-4.5)	1 (0-4)	9 (2-119.3) <sup>c</sup>	486 (7-1152)	<0.001
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Data are given as median (interquartile range) and percentage (n/N)

<sup>a</sup>Kruskal-Wallis test.

<sup>b</sup>Fisher's exact test.

<sup>c</sup>One missing data.

<sup>d</sup>Two missing data.

<sup>e</sup>Four missing data.

<sup>f</sup>Eight missing data.

**Table 3.** Clinical and demographic characteristics of women with preterm prelabor rupture of membranes (pPROM)

	pPROM without intra-amniotic inflammation or infection (n=17)	pPROM with intra-amniotic inflammation and infection (n=19)	P-value
Maternal Age (years) <sup>a</sup>	23 (20.5-31.5)	29 (21-32)	0.60
Nulliparous <sup>b</sup>	35.3% (6/17)	31.6% (6/19)	1
Race <sup>b</sup>			0.603
African-American	94.1% (16/17)	83.3% (15/18) <sup>c</sup>	
Caucasian	5.9% (1/17)	16.7% (3/18) <sup>c</sup>	
Gestational age at amniocentesis (weeks) <sup>a</sup>	30.6 (26.8-32.75)	28.3 (24.4-30.5)	0.076
Gestational age at delivery (weeks) <sup>a</sup>	34 (31.7-36)	29 (24-31)	<0.001
Birthweight (grams) <sup>a</sup>	2200 (1807-2444)	1330 (660-1710)	<0.001
Amniotic fluid IL-6	0.64 (0.25-1)	64.3 (20.08-172.8)	<0.001

(ng/mL) <sup>a</sup>			
Amniotic fluid glucose (mg/dL) <sup>a</sup>	28 (24.25-34.5) <sup>d</sup>	18 (16-22)	0.001
Amniotic fluid white blood cells (/mm <sup>3</sup> ) <sup>a</sup>	4 (0-19)	411 (20-912)	<0.001

Data are given as median (interquartile range) and percentage (n/N)

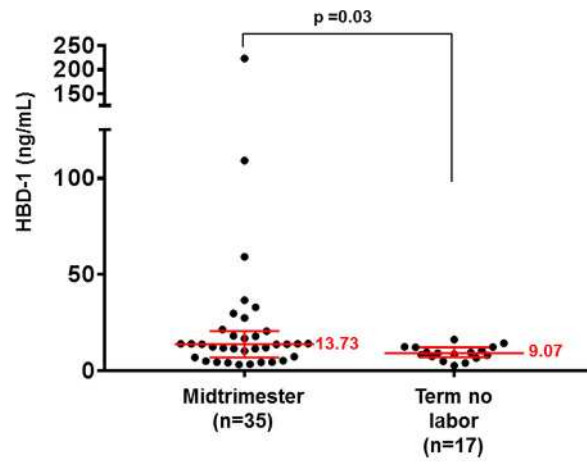
<sup>a</sup>Mann-Whitney U test.

<sup>b</sup>Fisher's exact test.

<sup>c</sup>One missing data.

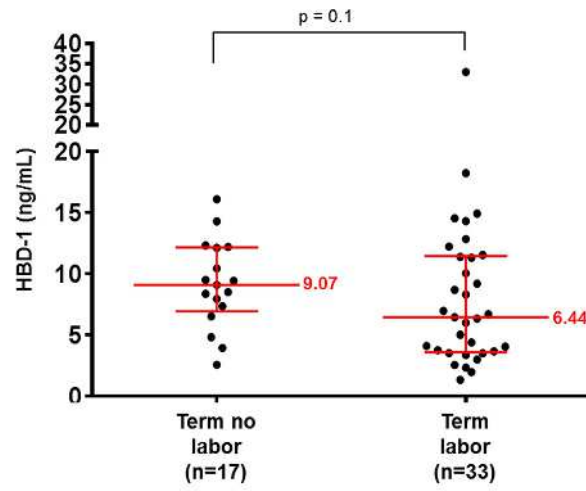
<sup>d</sup>Three missing data.

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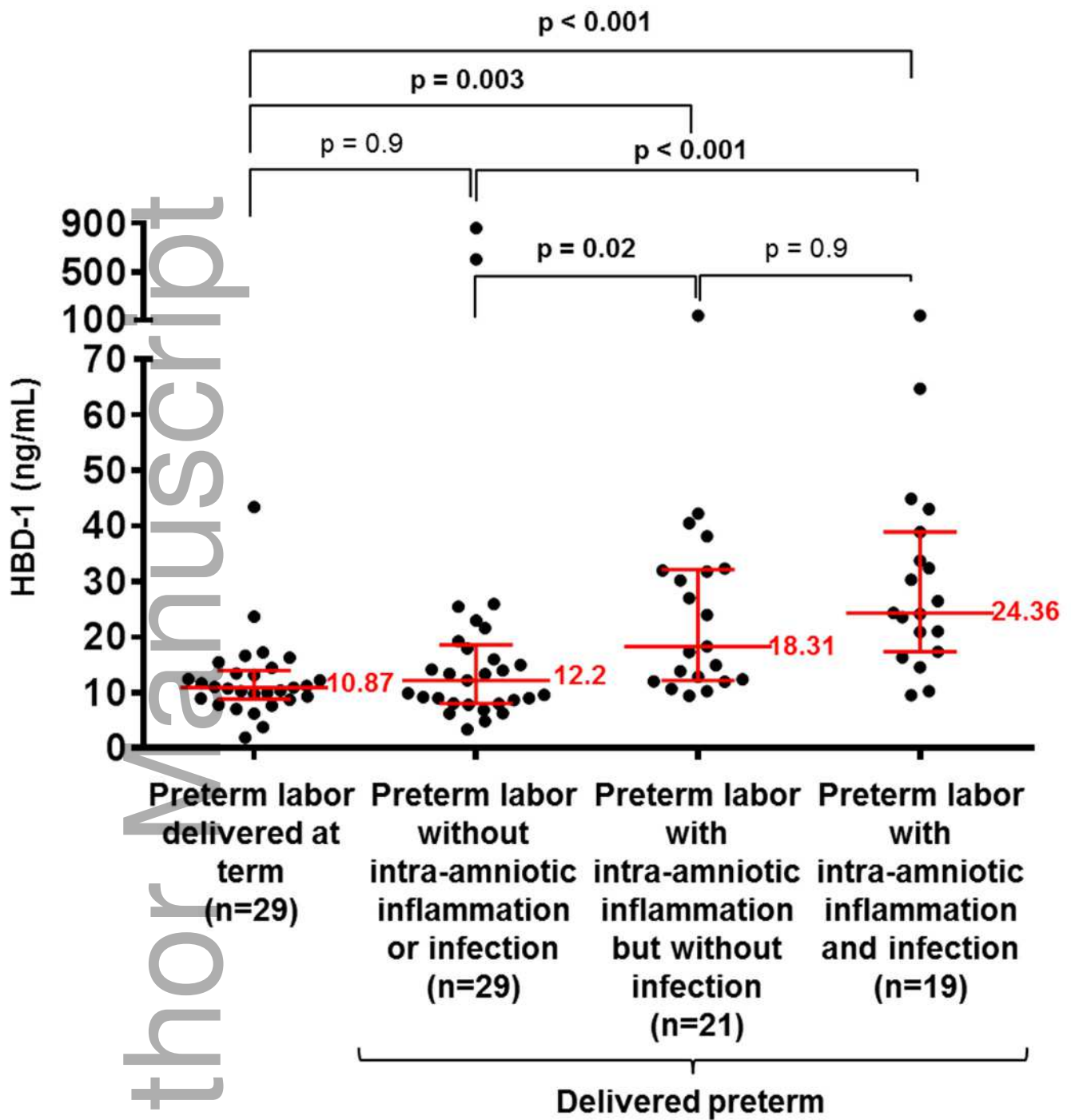


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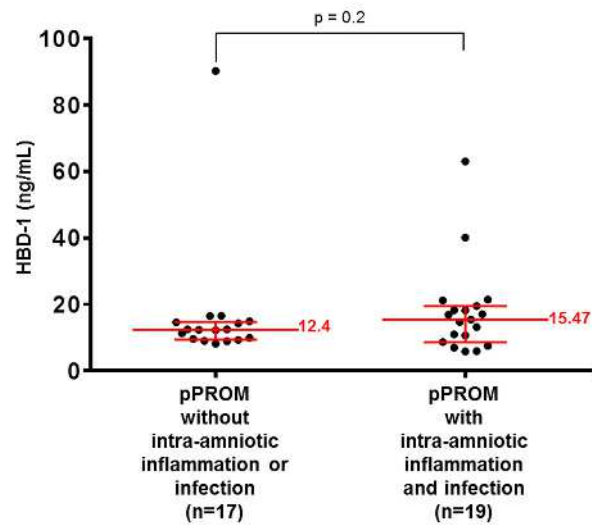




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