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Human β-defensin-1: A natural antimicrobial peptide present in amniotic fluid that is increased in spontaneous preterm labor with intra-amniotic infection

Aneesha Varrey[1,2] | Roberto Romero[1,3,4,5] (iD) | Bogdan Panaitescu[1,2] | Derek Miller[1,2] |
Tinnakorn Chaiworapongsa[1,2] | Manasi Patwardhan[2] | Jonathan Faro[1,2] |
Percy Pacora[1,2] | Sonia S. Hassan[1,2,6] | Chaur-Dong Hsu[2] | Nardhy Gomez-Lopez[1,2,7] (iD)

[1]Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U. S. Department of Health and Human Services, Bethesda, Maryland, and Detroit, Michigan

[2]Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, Michigan

[3]Department of Obstetrics and Gynecology, University of Michigan, Ann Arbor, Michigan

[4]Department of Epidemiology and Biostatistics, Michigan State University, East Lansing, Michigan

[5]Center for Molecular Medicine and Genetics, Wayne State University, Detroit, Michigan

[6]Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan

[7]Department of Immunology, Microbiology and Biochemistry, Wayne State University School of Medicine, Detroit, Michigan

Correspondence: Nardhy Gomez-Lopez, PhD, Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Perinatology Research Branch, NICHD/NIH/DHHS, 275 E. Hancock, Detroit, MI 48201 (nardhy.gomez-lopez@wayne.edu; ngomezlo@med.wayne.edu).
and
Roberto Romero, MD, D. Med. Sci., Perinatology Research Branch, NICHD/NIH/DHHS, Wayne State University/Hutzel Women's Hospital 3990 John R, Box 4, Detroit, MI 48201 (prbchiefstaff@med.wayne.edu).

Abstract

Problem: Human β-defensins (HBDs) 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: (a) midtrimester who delivered at term (n = 35); (b) term with (n = 33) or without (n = 17) labor; (c) 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 (d) 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: (a) HBD-1 was detectable in all amniotic fluid samples; (b) amniotic fluid concentrations of HBD-1 were changed with gestational age (midtrimester vs term no labor), being higher in midtrimester; (c) amniotic fluid concentrations of HBD-1 were similar between women with and without spontaneous labor at term; (d) among patients with spontaneous preterm labor, amniotic fluid concentrations of HBD-1 in women with intra-amniotic inflammation/infection and in those with intra-amniotic inflammation without infection were greater than in women without intra-amniotic inflammation or infection who delivered preterm or at term; and (e) the presence of

intra-amniotic inflammation and infection in patients with pPROM did not change amniotic fluid concentrations of HBD-1.

Conclusion: 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.

1 | INTRODUCTION

The amniotic fluid and placenta have antimicrobial properties and function as an immunological barrier to the growing fetus.[1-9] Specifically, the amniotic fluid contains soluble components such as electrolytes, carbohydrates, lipids, and peptides which can act as a defense against pathogens invading the amniotic cavity.[10-30] The amniotic fluid also includes cellular components[31-45] that have been recently characterized using immunophenotyping.[46] It is now clear that amniotic fluid contains both innate and adaptive immune cells such as monocytes/macrophages, neutrophils, innate lymphoid cells, B cells, natural killer cells, and T cells.[46] Most of these immune cells are more abundant in women with intra-amniotic infection and/or inflammation.[46] Among innate immune cells, neutrophils are considered a marker of intra-amniotic inflammation as their number is increased in women with intra-amniotic infection.[47,48] While amniotic fluid neutrophils are mostly of fetal origin in preterm gestations, they can also be of maternal origin at term.[49] Regardless of their origin, amniotic fluid neutrophils participate in the host defense mechanisms against intra-amniotic infection by performing phagocytosis,[50] forming neutrophil extracellular traps or NETs,[51] and releasing inflammatory mediators[48,52-78] including an array of antimicrobial peptides.[18,79-84]

Antimicrobial peptides represent soluble mediators of the innate immune system, which provide protection against pathogens.[85-91] Several families of antimicrobial peptides have been described in mammals, such as cathelicidins and defensins.[92,93] The latter are small cationic peptides synthesized by neutrophils, Paneth cells, and epithelial cells[85,94-99] and are regulated primarily by microbial signals and cytokines, and in some cases by neuroendocrine signals in a tissue-specific manner.[90,95,100] Defensins can be of different types based on their function and location of expression in the human body.[90] Two main types of defensins have been identified in humans: α- and β-defensins.[101,102] Alpha defensins are seen in azurophilic granules in neutrophils[85,94,103,104] and have also been identified in the Paneth cells of intestinal crypts.[95,98,105,106] These mediators have six subtypes referred to as human neutrophil peptide (HNP)-1 to HNP-4 and

human defensin-5 and human defensin-6.[90] Increased concentrations of HNP-1 in amniotic fluid have been associated with intrauterine infection and acute histologic chorioamnionitis.[107,108]

Human β-defensins (HBD) are expressed in the epithelial cells of mucosal surfaces.[86,99,109] HBD-1, HBD-2, and HBD-3 proteins have all been localized in the reproductive tissues including the amnion epithelium, chorion trophoblast, decidua, and placental syncytiotrophoblast.[110] 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).[84] 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 (a) detect HBD-1 in amniotic fluid of women in midgestation and at term with or without labor; (b) 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 (c) determine whether HBD-1 concentrations were different between women who underwent pPROM with or without intra-amniotic inflammation/infection.

2 | MATERIALS AND METHODS

2.1 | 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

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 (y)[a]	37 (35-39)	24 (21-32)	23 (20-29)	<0.001
Nulliparous[b]	17.1% (6/35)	23.5% (4/17)	42.4% (14/33)	0.058
Race[b]				
African American	85.7% (30/35)	0	0	<0.001
Caucasian	5.7% (2/35)	0	0	
Hispanic	0	100% (17/17)	100% (33/33)	
Other	8.6 (3/35)	0	0	
Gestational age at amniocentesis (wk)[a]	16 (16-17)	39 (38.5-40)	39 (38-40)	<0.001
Gestational age at delivery (wk)[a]	39 (38-40)	39 (38.5-40)	39 (38-40)	0.8
Birthweight (g)[a]	3344 (3176-3621)	3260 (3130-3790)	3280 (3085-3715)	0.7
Amniotic fluid IL-6 (ng/mL)[a]	0.68 (0.34-1.06)	0.41 (0.22-0.85)	1.04 (0.5-1.37)	0.04

Data are given as median (interquartile range) and percentage (n/N).
[a]Kruskal-Wallis/Mann-Whitney U test.
[b]Fisher's exact test.

amniocentesis during midtrimester (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 (a) spontaneous preterm labor who proceeded to have a term delivery with a negative amniotic fluid culture and interleukin (IL)-6 <2.6 ng/mL (n = 29), (b) spontaneous preterm labor without intra-amniotic inflammation or infection (n = 29; see diagnostic criteria below), (c) spontaneous preterm labor with intra-amniotic inflammation and a negative amniotic fluid culture (n = 21), and (d) 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 as follows: (a) no medical, obstetrical, or surgical complications, (b) absence of microbial invasion of the amniotic cavity (MIAC; see diagnostic criteria below), (c) amniotic fluid IL-6 concentrations <2.6 ng/mL, (d) intact membranes, and (e) delivery of a term neonate with birthweight appropriate for gestational age.

2.2 | 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.[111-123] Intra-amniotic inflammation was defined as an amniotic fluid IL-6 concentration ≥2.6 ng/mL.[118] Intra-amniotic infection was defined as the presence of MIAC with intra-amniotic inflammation.[77,118,120,121,124-138] 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.[139-142]

2.3 | 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× 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,[47] glucose concentration,[143] Gram stain,[144] and IL-6 concentration.[118]

2.4 | Determination of IL-6 in amniotic fluid

Amniotic fluid concentrations of IL-6 were determined 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.

2.5 | 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, USA). This ELISA kit was validated in our laboratory prior to execution of the study. Amniotic fluid concentrations of HBD-1

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 (y)[a]	23 (20-30)	25 (22-30)	22 (20-26)	25 (20-29)	0.6
Nulliparous[b]	24.1% (7/29)	34.5% (10/29)	42.9% (9/21)	63.2% (12/19)	0.06
Race[b]					
African American	86.2 (25/29)	85.1% (23/27)[d]	85% (17/20)[c]	100% (19/19)	0.4
Caucasian	10.3% (3/29)	14.8% (4/27)[d]	10% (2/20)[c]	0	
Hispanic	3.4% (1/29)	0	5% (1/20)[c]	0	
Gestational age at amniocentesis (wk)[a]	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 (wk)[a]	38 (37-40)	33 (31-36)	28 (22.3-31)	25 (20.4-29.25)	<0.001
Birthweight (grams)[a]	2930 (2600-3150)	1960 (1390-2320)	830 (480-1300)	610 (430-1177.5)	<0.001
Amniotic fluid IL-6 (ng/mL)[a]	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)[a]	37 (28.5-51.5)[e]	32 (27-36.5)[f]	27 (21.5-29)[e]	16 (10-28)	<0.001
Amniotic fluid white blood cells (/mm^3)[a]	1 (0-4.5)	1 (0-4)	9 (2-119.3)[c]	486 (7-1152)	<0.001

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

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.

2.6 | 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. Nonparametric 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.

3 | RESULTS

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

3.1 | 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).

3.2 | 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

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 (y)[a]	23 (20.5-31.5)	29 (21-32)	0.6
Nulliparous[b]	35.3% (6/17)	31.6% (6/19)	1
Race[b]			
African American	94.1% (16/17)	83.3% (15/18)[c]	0.6
Caucasian	5.9% (1/17)	16.7% (3/18)[c]	
Gestational age at amniocentesis (wk)[a]	30.6 (26.8-32.75)	28.3 (24.4-30.5)	0.07
Gestational age at delivery (wk)[a]	34 (31.7-36)	29 (24-31)	<0.001
Birthweight (grams)[a]	2200 (1807-2444)	1330 (660-1710)	<0.001
Amniotic fluid IL-6 (ng/mL)[a]	0.64 (0.25-1)	64.3 (20.08-172.8)	<0.001
Amniotic fluid glucose (mg/dL)[a]	28 (24.25-34.5)[d]	18 (16-22)	0.001
Amniotic fluid white blood cells (/mm^3)[a]	4 (0-19)	411 (20-912)	<0.001

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

[a]Mann-Whitney U test.

[b]Fisher's exact test.

[c]One missing data.

[d]Three missing data.

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 who delivered preterm (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 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; 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 who delivered preterm and those 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). A linear regression model was fit with HBD-1 amniotic fluid concentration as the dependent variable and gestational age (continuous) and spontaneous preterm labor groups as independent variables. This analysis showed that HBD-1 concentration was not significantly associated with gestational age.

3.3 | 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).

4 | DISCUSSION

4.1 | The principal findings of this study were as follows

(a) HBD-1 is a physiological constituent of amniotic fluid; (b) amniotic fluid concentrations of HBD-1 changed with gestational age (midtrimester vs term no labor), being higher in midtrimester; (c) amniotic fluid concentrations of HBD-1 were similar between women with and

without spontaneous labor at term; (d) 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 or at term; (e) 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 at term; and (f) the presence of intra-amniotic inflammation and infection in patients with pPROM did not change amniotic fluid concentrations of HBD-1.

4.2 | 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 HBD-3) are present in the second trimester
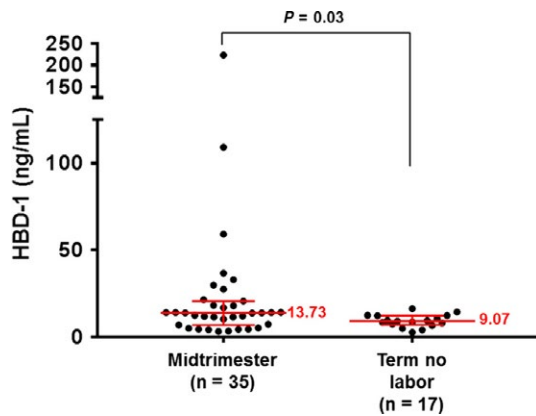
FIGURE 1 Amniotic fluid concentrations of human β-defensin-1 (HBD-1) in normal pregnancy. Amniotic fluid concentrations of HBD-1 in midtrimester and at term without labor. Medians and interquartile ranges are shown in red
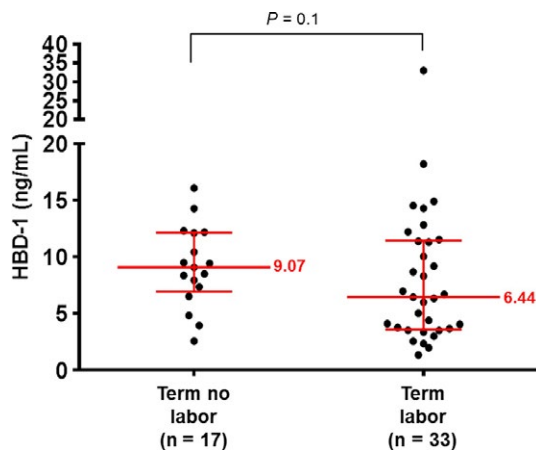
FIGURE 2 Amniotic fluid concentrations of human β-defensin-1 (HBD-1) at term pregnancy. Amniotic fluid concentrations of HBD-1 in women with and without spontaneous labor at term. Medians and interquartile ranges are shown in red
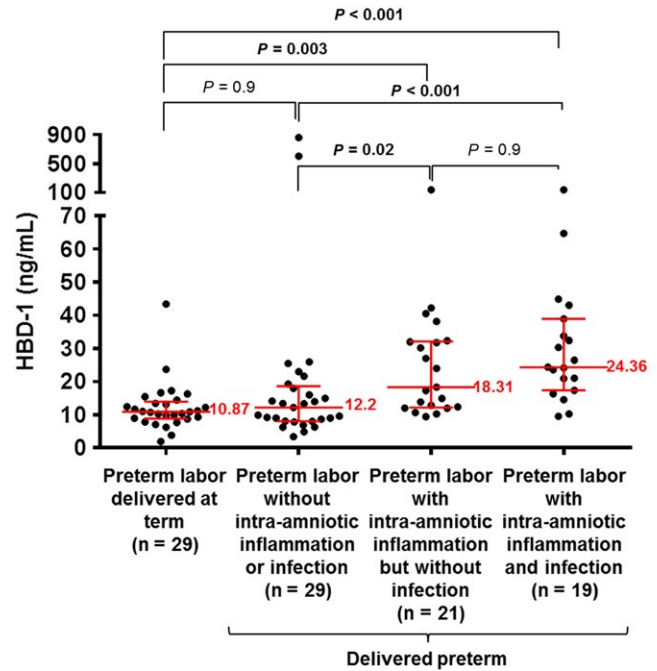
FIGURE 3 Amniotic fluid concentrations of human β-defensin-1 (HBD-1) in women with spontaneous preterm labor and intact membranes. Amniotic fluid concentrations of HBD-1 in women who underwent spontaneous preterm labor and delivered at term or preterm with and without intra-amniotic inflammation and/or infection. Medians and interquartile ranges are shown in red. *P*-values were adjusted for multiple comparisons

amniotic fluid.[84,145] Given that fetal tissues including the lungs, placenta, and chorioamniotic membranes, as well as the decidua express human defensins,[9, 110,146-150] 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.[46] 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 toward term, the number of neutrophils in amniotic fluid increases until these immune cells become the dominant leukocyte subset[46] and may take over the host defense mechanisms in the amniotic cavity through performing phagocytosis[50] and NET formation,[51] as well as releasing other antimicrobial peptides.[18,79-84]

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.

4.3 | 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[151-167] as intra-amniotic infection is absent in most women who deliver at term.[117] 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.[110] These data show that amniotic fluid HBD-1 is unchanged by the physiological process of labor at term.

4.4 | 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 ≥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.[84] 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.[168] 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.[169] Our data, along with previous studies demonstrating that MIAC results in an increased concentration of lactoferrin,[81,82] lysozyme,[19,21] and bacterial/permeability-increasing protein[20] in amniotic fluid and other compartments,[107,170-172] suggest that antimicrobial peptides participate in the soluble host defense mechanisms against intra-amniotic infection[173,174] in women with spontaneous preterm labor and birth.

Women with spontaneous preterm labor with intra-amniotic inflammation but without infection (amniotic fluid IL-6 ≥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 this intra-amniotic inflammatory process 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
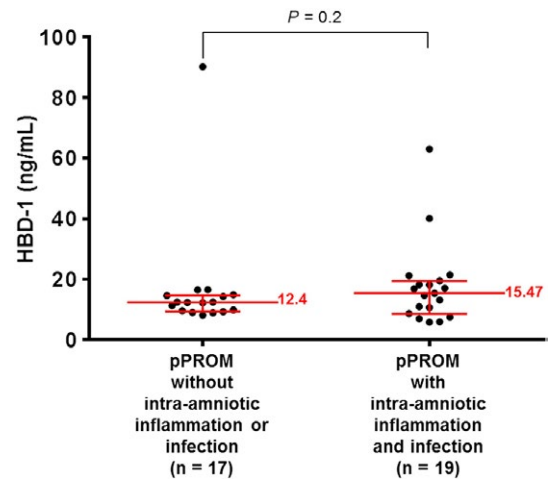

FIGURE 4 Amniotic fluid concentrations of human β-defensin-1 (HBD-1) in preterm prelabor rupture of membranes (pPROM). Amniotic fluid concentrations of HBD-1 in women who underwent pPROM with and without intra-amniotic inflammation/ infection. Medians and interquartile ranges are shown in red

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).[126,128] We and others have proposed that this sterile inflammatory process can be induced by danger signals derived by the fetal tissues.[175-179] The importance of sterile intra-amniotic inflammation is underscored by the observation that women with such a clinical condition have a similar rate of preterm birth and adverse neonatal outcomes as those with proven intra-amniotic infection.[128] 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)[128] and high concentrations of inflammatory cytokines in the amniotic cavity.[76] 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. Collectively, these data suggest that HBD-1 participates in the host response against nonculturable microbes or danger signals in the amniotic cavity.

4.5 | 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 ≥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.[84] These findings are explained by evidence indicating that HBD-1 and HBD-2 show differential expression in inflammatory diseases (e.g, periodontitis[180]) and in human peripheral blood cells stimulated with microbial products.[181] Alternatively, patients with pPROM usually received antibiotic treatment,[182,183] which could directly reduce the release of antimicrobial peptides and neutrophil-mediated antimicrobial activity, in addition to decreasing the microbial burden.[184,185] 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.

5 | 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.

CONFLICT OF INTEREST

The authors declared no potential conflict of interests.

ORCID

Roberto Romero iD http://orcid.org/0000-0002-4448-5121

Nardhy Gomez-Lopez iD http://orcid.org/0000-0002-3406-5262

REFERENCES

1. Galask RP, Snyder IS. Antimicrobial factors in amniotic fluid. *Am J Obstet Gynecol*. 1970;106:59-65.
2. Miller J, Michel J, Bercovici B, Argaman M, Sacks T. Studies on the antimicrobial activity of amniotic fluid. *Am J Obstet Gynecol*. 1976;125:212-214.
3. Tafari N, Ross SM, Naeye RL, Galask RP, Zaar B. Failure of bacterial growth inhibition by amniotic fluid. *Am J Obstet Gynecol*. 1977;128:187-189.
4. Thadepalli H, Appleman MD, Maidman JE, Arce JJ, Davidson EC Jr. Antimicrobial effect of amniotic fluid against anaerobic bacteria. *Am J Obstet Gynecol*. 1977;127:250-254.
5. Thadepalli H, Bach VT, Davidson EC Jr. Antimicrobial effect of amniotic fluid. *Obstet Gynecol*. 1978;52:198-204.
6. Thadepalli H, Gangopadhyay PK, Maidman JE. Amniotic fluid analysis for antimicrobial factors. *Int J Gynaecol Obstet*. 1982;20:65-72.
7. Davis LE, McLaren LC, Stewart JA, James CG, Levine MD, Skipper BJ. Immunological and microbiological studies of midtrimester amniotic fluid. *Gynecol Obstet Invest*. 1983;16:261-268.
8. Schmidt W. The amniotic fluid compartment: the fetal habitat. *Adv Anat Embryol Cell Biol*. 1992;127:1-100.
9. Svinarich DM, Gomez R, Romero R. Detection of human defensins in the placenta. *Am J Reprod Immunol*. 1997;38:252-255.
10. Sozanskii AM. The biochemical composition of amniotic fluid and of maternal and fetal blood at various periods of pregnancy. *Biull Eksp Biol Med*. 1961;51:323-326.
11. Niemela A, Kulomaa M, Vija P, Tuohimaa P, Saarikoski S. Lactoferrin in human amniotic fluid. *Hum Reprod*. 1989;4:99-101.
12. Rueda R, Vargas ML, Garcia-Pacheco M, Garcia-Olivares E. Detection of immunoregulatory lipid-like factors in human amniotic fluid. *Am J Reprod Immunol*. 1990;24:40-44.
13. Campbell J, Wathen N, Macintosh M, Cass P, Chard T, Mainwaring Burton R. Biochemical composition of amniotic fluid and extra-embryonic coelomic fluid in the first trimester of pregnancy. *Br J Obstet Gynaecol*. 1992;99:563-565.
14. Romero R, Baumann P, Gonzalez R, et al. Amniotic fluid prostanoid concentrations increase early during the course of spontaneous labor at term. *Am J Obstet Gynecol*. 1994;171:1613-1620.
15. Romero R, Munoz H, Gomez R, et al. Increase in prostaglandin bioavailability precedes the onset of human parturition. *Prostaglandins Leukot Essent Fatty Acids*. 1996;54:187-191.
16. Edwin SS, Romero RJ, Munoz H, Branch DW, Mitchell MD. 5-Hydroxyeicosatetraenoic acid and human parturition. *Prostaglandins*. 1996;51:403-412.
17. Drohse H, Christensen H, Myrhoj V, Sorensen S. Characterisation of non-maternal serum proteins in amniotic fluid at weeks 16 to 18 of gestation. *Clin Chim Acta*. 1998;276:109-120.
18. Petraglia F, Gomez R, Luisi S, et al. Increased midtrimester amniotic fluid activin A: a risk factor for subsequent fetal death. *Am J Obstet Gynecol*. 1999;180:194-197.
19. Yoshio H, Tollin M, Gudmundsson GH, et al. Antimicrobial polypeptides of human vernix caseosa and amniotic fluid: implications for newborn innate defense. *Pediatr Res*. 2003;53:211-216.
20. Espinoza J, Chaiworapongsa T, Romero R, et al. Antimicrobial peptides in amniotic fluid: defensins, calprotectin and bacterial/permeability-increasing protein in patients with microbial invasion of the amniotic cavity, intra-amniotic inflammation, preterm labor and premature rupture of membranes. *J Matern Fetal Neonatal Med*. 2003;13:2-21.
21. Akinbi HT, Narendran V, Pass AK, Markart P, Hoath SB. Host defense proteins in vernix caseosa and amniotic fluid. *Am J Obstet Gynecol*. 2004;191:2090-2096.
22. Cho CK, Shan SJ, Winsor EJ, Diamandis EP. Proteomics analysis of human amniotic fluid. *Mol Cell Proteomics*. 2007;6:1406-1415.

23. Bujold E, Romero R, Kusanovic JP, et al. Proteomic profiling of amniotic fluid in preterm labor using two-dimensional liquid separation and mass spectrometry. *J Matern Fetal Neonatal Med.* 2008;21:697-713.

24. Lee SE, Romero R, Park IS, Seong HS, Park CW, Yoon BH. Amniotic fluid prostaglandin concentrations increase before the onset of spontaneous labor at term. *J Matern Fetal Neonatal Med.* 2008;21:89-94.

25. Perluigi M, Di Domenico F, Cini C, et al. Proteomic analysis for the study of amniotic fluid protein composition. *J Prenat Med.* 2009;3:39-41.

26. Romero R, Mazaki-Tovi S, Vaisbuch E, et al. Metabolomics in premature labor: a novel approach to identify patients at risk for preterm delivery. *J Matern Fetal Neonatal Med.* 2010;23:1344-1359.

27. Witkin SS, Chervenak J, Bongiovanni AM, Herway C, Linhares IM, Skupski D. Influence of mid-trimester amniotic fluid on endogenous and lipopolysaccharide-mediated responses of mononuclear lymphoid cells. *Am J Reprod Immunol.* 2012;67:28-33.

28. Maddipati KR, Romero R, Chaiworapongsa T, et al. Eicosanomic profiling reveals dominance of the epoxygenase pathway in human amniotic fluid at term in spontaneous labor. *FASEB J.* 2014;28:4835-4846.

29. Pierce J, Jacobson P, Benedetti E, et al. Collection and characterization of amniotic fluid from scheduled C-section deliveries. *Cell Tissue Bank.* 2016;17:413-425.

30. Maddipati KR, Romero R, Chaiworapongsa T, et al. Clinical chorioamnionitis at term: the amniotic fluid fatty acyl lipidome. *J Lipid Res.* 2016;57:1906-1916.

31. Votta RA, de Gagneten CB, Parada O, Giulietti M. Cytologic study of amniotic fluid in pregnancy. *Am J Obstet Gynecol.* 1968;102:571-577.

32. Hoyes AD. Ultrastructure of the cells of the amniotic fluid. *J Obstet Gynaecol Br Commonw.* 1968;75:164-171.

33. Wachtel E, Gordon H, Olsen E. Cytology of amniotic fluid. *J Obstet Gynaecol Br Commonw.* 1969;76:596-602.

34. Pasquinucci C, Dambrosio F, Meroni P, Della Torre L. The amniotic fluid. 3. A morphologic study of cytology. *Ann Ostet Ginecol Med Perinat.* 1969;91:90-106.

35. Casadei R, D'Ablaing G 3rd, Kaplan BJ, Schwinn CP. A cytologic study of amniotic fluid. *Acta Cytol.* 1973;17:289-298.

36. Sutherland GR, Bauld R, Bain AD. Observations on human amniotic fluid cell strains in serial culture. *J Med Genet.* 1974;11:190-195.

37. Cutz E, Conen PE. Macrophages and epithelial cells in human amniotic fluid: transmission and scanning electron microscopic study. *Am J Anat.* 1978;151:87-101.

38. Schrage R, Bogelspacher HR, Wurster KG. Amniotic fluid cells in the second trimester of pregnancy. *Acta Cytol.* 1982;26:407-416.

39. Gosden CM. Amniotic fluid cell types and culture. *Br Med Bull.* 1983;39:348-354.

40. Medina-Gomez P, McBride WH. Amniotic fluid macrophages from normal and malformed fetuses. *Prenat Diagn.* 1986;6:195-205.

41. Fauza D. Amniotic fluid and placental stem cells. *Best Pract Res Clin Obstet Gynaecol.* 2004;18:877-891.

42. Lynch W, Rezai S, Henderson CE. Human amniotic fluid: a source of stem cells for possible therapeutic use. *Am J Obstet Gynecol.* 2016;215:401.

43. Marquardt N, Ivarsson MA, Sundstrom E, et al. Fetal CD103+ IL-17-producing group 3 innate lymphoid cells represent the dominant lymphocyte subset in human amniotic fluid. *J Immunol.* 2016;197:3069-3075.

44. Young BK, Chan MK, Liu L, Basch RS. Amniotic fluid as a source of multipotent cells for clinical use. *J Perinat Med.* 2016;44:333-337.

45. Dolin CD, Chan MK, Basch RS, Young BK. Human term amniotic fluid: a novel source of stem cells for regenerative medicine. *Am J Obstet Gynecol.* 2018; pii: S0002-9378(18)30445-9.

46. Gomez-Lopez N, Romero R, Xu Y, et al. The immunophenotype of amniotic fluid leukocytes in normal and complicated pregnancies. *Am J Reprod Immunol.* 2018;79:e12827.

47. Romero R, Quintero R, Nores J, et al. Amniotic fluid white blood cell count: a rapid and simple test to diagnose microbial invasion of the amniotic cavity and predict preterm delivery. *Am J Obstet Gynecol.* 1991;165:821-830.

48. Martinez-Varea A, Romero R, Xu Y, et al. Clinical chorioamnionitis at term VII: the amniotic fluid cellular immune response. *J Perinat Med.* 2017;45:523-538.

49. Gomez-Lopez N, Romero R, Xu Y, et al. Are amniotic fluid neutrophils in women with intraamniotic infection and/or inflammation of fetal or maternal origin? *Am J Obstet Gynecol.* 2017;217:693 e691-693 e616.

50. Gomez-Lopez N, Romero R, Garcia-Flores V, et al. Amniotic fluid neutrophils can phagocytize bacteria: a mechanism for microbial killing in the amniotic cavity. *Am J Reprod Immunol.* 2017;78:e12723.

51. Gomez-Lopez N, Romero R, Xu Y, et al. Neutrophil extracellular traps in the amniotic cavity of women with intra-amniotic infection: a new mechanism of host defense. *Reprod Sci.* 2017;24:1139-1153.

52. Romero R, Brody DT, Oyarzun E, et al. Infection and labor. III. Interleukin-1: a signal for the onset of parturition. *Am J Obstet Gynecol.* 1989;160:1117-1123.

53. Romero R, Parvizi ST, Oyarzun E, et al. Amniotic fluid interleukin-1 in spontaneous labor at term. *J Reprod Med.* 1990;35:235-238.

54. Romero R, Mazor M, Brandt F, et al. Interleukin-1 alpha and interleukin-1 beta in preterm and term human parturition. *Am J Reprod Immunol.* 1992;27:117-123.

55. Hillier SL, Witkin SS, Krohn MA, Watts DH, Kiviat NB, Eschenbach DA. The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection. *Obstet Gynecol.* 1993;81:941-948.

56. Gomez R, Ghezzi F, Romero R, Munoz H, Tolosa JE, Rojas I. Premature labor and intra-amniotic infection. Clinical aspects and role of the cytokines in diagnosis and pathophysiology. *Clin Perinatol.* 1995;22:281-342.

57. Hsu CD, Aversa K, Meaddough E, Lee IS, Copel JA. Elevated amniotic fluid nitric oxide metabolites and cyclic guanosine 3',5'-monophosphate in pregnant women with intraamniotic infection. *Am J Obstet Gynecol.* 1997;177:793-796.

58. Yoon BH, Romero R, Jun JK, et al. Amniotic fluid cytokines (interleukin-6, tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-8) and the risk for the development of bronchopulmonary dysplasia. *Am J Obstet Gynecol.* 1997;177:825-830.

59. Hsu CD, Meaddough E, Hong SF, Aversa K, Lu LC, Copel JA. Elevated amniotic fluid nitric oxide metabolites and interleukin-6 in intra-amniotic infection. *J Soc Gynecol Investig.* 1998;5:21-24.

60. Hsu CD, Meaddough E, Aversa K, et al. Dual roles of amniotic fluid nitric oxide and prostaglandin E2 in preterm labor with intra-amniotic infection. *Am J Perinatol.* 1998;15:683-687.

61. Athayde N, Edwin SS, Romero R, et al. A role for matrix metalloproteinase-9 in spontaneous rupture of the fetal membranes. *Am J Obstet Gynecol.* 1998;179:1248-1253.

62. Hsu CD, Meaddough E, Aversa K, et al. Elevated amniotic fluid levels of leukemia inhibitory factor, interleukin 6, and interleukin 8 in intra-amniotic infection. *Am J Obstet Gynecol.* 1998;179:1267-1270.

63. Hsu CD, Meaddough E, Aversa K, Copel JA. The role of amniotic fluid L-selectin, GRO-alpha, and interleukin-8 in the pathogenesis of intraamniotic infection. *Am J Obstet Gynecol.* 1998;178:428-432.

64. Gonzalez-Bosquet E, Cerqueira MJ, Dominguez C, Gasser I, Bermejo B, Cabero L. Amniotic fluid glucose and cytokines values in the early diagnosis of amniotic infection in patients with preterm labor and intact membranes. *J Matern Fetal Med.* 1999;8:155-158.

65. Hsu CD, Aversa KR, Lu LC, et al. Nitric oxide: a clinically important amniotic fluid marker to distinguish between intra-amniotic mycoplasma and non-mycoplasma infections. *Am J Perinatol*. 1999;16:161-166.

66. Athayde N, Romero R, Gomez R, et al. Matrix metalloproteinases-9 in preterm and term human parturition. *J Matern Fetal Med*. 1999;8:213-219.

67. Lu LC, Hsu CD. Elevated amniotic fluid nucleosome levels in women with intra-amniotic infection. *Obstet Gynecol*. 1999;94:7-10.

68. Maymon E, Romero R, Pacora P, et al. Human neutrophil collagenase (matrix metalloproteinase 8) in parturition, premature rupture of the membranes, and intrauterine infection. *Am J Obstet Gynecol*. 2000;183:94-99.

69. Hsu CD, Aversa K, Meaddough E. The role of amniotic fluid interleukin-6, and cell adhesion molecules, intercellular adhesion molecule-1 and leukocyte adhesion molecule-1, in intra-amniotic infection. *Am J Reprod Immunol*. 2000;43:251-254.

70. Hsu CD, Hong SF, Harirah H, Bahado-Singh R, Lu L. Amniotic fluid soluble fas levels in intra-amniotic infection. *Obstet Gynecol*. 2000;95:667-670.

71. Maymon E, Romero R, Chaiworapongsa T, et al. Value of amniotic fluid neutrophil collagenase concentrations in preterm premature rupture of membranes. *Am J Obstet Gynecol*. 2001;185:1143-1148.

72. Angus SR, Segel SY, Hsu CD, et al. Amniotic fluid matrix metalloproteinase-8 indicates intra-amniotic infection. *Am J Obstet Gynecol*. 2001;185:1232-1238.

73. Maymon E, Romero R, Chaiworapongsa T, et al. Amniotic fluid matrix metalloproteinase-8 in preterm labor with intact membranes. *Am J Obstet Gynecol*. 2001;185:1149-1155.

74. Helmig BR, Romero R, Espinoza J, et al. Neutrophil elastase and secretory leukocyte protease inhibitor in prelabor rupture of membranes, parturition and intra-amniotic infection. *J Matern Fetal Neonatal Med*. 2002;12:237-246.

75. Harirah H, Donia SE, Hsu CD. Amniotic fluid matrix metalloproteinase-9 and interleukin-6 in predicting intra-amniotic infection. *Obstet Gynecol*. 2002;99:80-84.

76. Romero R, Grivel JC, Tarca AL, et al. Evidence of perturbations of the cytokine network in preterm labor. *Am J Obstet Gynecol*. 2015;213:836 e831-836 e818.

77. Romero R, Chaemsaithong P, Korzeniewski SJ, et al. Clinical chorioamnionitis at term II: the intra-amniotic inflammatory response. *J Perinat Med*. 2016;44:5-22.

78. Son GH, You YA, Kwon EJ, Lee KY, Kim YJ. Comparative analysis of midtrimester amniotic fluid cytokine levels to predict spontaneous very pre-term birth in patients with cervical insufficiency. *Am J Reprod Immunol*. 2016;75:155-161.

79. Romero R, Ceska M, Avila C, Mazor M, Behnke E, Lindley I. Neutrophil attractant/activating peptide-1/interleukin-8 in term and preterm parturition. *Am J Obstet Gynecol*. 1991;165:813-820.

80. Heller KA, Greig PC, Heine RP. Amniotic-fluid lactoferrin: a marker for subclinical intraamniotic infection prior to 32 weeks gestation. *Infect Dis Obstet Gynecol*. 1995;3:179-183.

81. Otsuki K, Yoda A, Saito H, et al. Amniotic fluid lactoferrin in intrauterine infection. *Placenta*. 1999;20:175-179.

82. Pacora P, Maymon E, Gervasi MT, et al. Lactoferrin in intrauterine infection, human parturition, and rupture of fetal membranes. *Am J Obstet Gynecol*. 2000;183:904-910.

83. Gravett MG, Novy MJ, Rosenfeld RG, et al. Diagnosis of intra-amniotic infection by proteomic profiling and identification of novel biomarkers. *JAMA*. 2004;292:462-469.

84. Soto E, Espinoza J, Nien JK, et al. Human beta-defensin-2: a natural antimicrobial peptide present in amniotic fluid participates in the host response to microbial invasion of the amniotic cavity. *J Matern Fetal Neonatal Med*. 2007;20:15-22.

85. Rice WG, Ganz T, Kinkade JM Jr, Selsted ME, Lehrer RI, Parmley RT. Defensin-rich dense granules of human neutrophils. *Blood*. 1987;70:757-765.

86. Huttner KM, Bevins CL. Antimicrobial peptides as mediators of epithelial host defense. *Pediatr Res*. 1999;45:785-794.

87. Bals R. Epithelial antimicrobial peptides in host defense against infection. *Respir Res*. 2000;1:141-150.

88. Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol*. 2000;8:402-410.

89. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature*. 2002;415:389-395.

90. Ganz T. Defensins: antimicrobial peptides of innate immunity. *Nat Rev Immunol*. 2003;3:710-720.

91. Tosi MF. Innate immune responses to infection. *J Allergy Clin Immunol*. 2005;116:241-249; quiz 250.

92. Lehrer RI, Ganz T. Defensins of vertebrate animals. *Curr Opin Immunol*. 2002;14:96-102.

93. Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol*. 2004;75:39-48.

94. Ganz T, Selsted ME, Szklarek D, et al. Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest*. 1985;76:1427-1435.

95. Jones DE, Bevins CL. Paneth cells of the human small intestine express an antimicrobial peptide gene. *J Biol Chem*. 1992;267:23216-23225.

96. Harwig SS, Park AS, Lehrer RI. Characterization of defensin precursors in mature human neutrophils. *Blood*. 1992;79:1532-1537.

97. Ganz T, Lehrer RI. Defensins. *Curr Opin Immunol*. 1994;6:584-589.

98. Mallow EB, Harris A, Salzman N, et al. Human enteric defensins. Gene structure and developmental expression. *J Biol Chem*. 1996;271:4038-4045.

99. Zhao C, Wang I, Lehrer RI. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. *FEBS Lett*. 1996;396:319-322.

100. Jarczak J, Kosciuczuk EM, Lisowski P, et al. Defensins: natural component of human innate immunity. *Hum Immunol*. 2013;74:1069-1079.

101. Schneider JJ, Unholzer A, Schaller M, Schafer-Korting M, Korting HC. Human defensins. *J Mol Med (Berl)*. 2005;83:587-595.

102. Semple F, Dorin JR. beta-Defensins: multifunctional modulators of infection, inflammation and more? *J Innate Immun*. 2012;4:337-348.

103. Ganz T, Selsted ME, Lehrer RI. Antimicrobial activity of phagocyte granule proteins. *Semin Respir Infect*. 1986;1:107-117.

104. Gabay JE, Scott RW, Campanelli D, et al. Antibiotic proteins of human polymorphonuclear leukocytes. *Proc Natl Acad Sci U S A*. 1989;86:5610-5614.

105. Porter EM, Liu L, Oren A, Anton PA, Ganz T. Localization of human intestinal defensin 5 in Paneth cell granules. *Infect Immun*. 1997;65:2389-2395.

106. Cunliffe RN. Alpha-defensins in the gastrointestinal tract. *Mol Immunol*. 2003;40:463-467.

107. Heine RP, Wiesenfeld H, Mortimer L, Greig PC. Amniotic fluid defensins: potential markers of subclinical intrauterine infection. *Clin Infect Dis*. 1998;27:513-518.

108. Erez O, Romero R, Tarca AL, et al. Differential expression pattern of genes encoding for anti-microbial peptides in the fetal membranes of patients with spontaneous preterm labor and intact membranes and those with preterm prelabor rupture of the membranes. *J Matern Fetal Neonatal Med*. 2009;22:1103-1115.

109. Selsted ME, Tang YQ, Morris WL, et al. Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. *J Biol Chem*. 1993;268:6641-6648.

110. King AE, Paltoo A, Kelly RW, Sallenave JM, Bocking AD, Challis JR. Expression of natural antimicrobials by human placenta and fetal membranes. *Placenta*. 2007;28:161-169.

111. Romero R, Mazor M, Wu YK, et al. Infection in the pathogenesis of preterm labor. *Semin Perinatol*. 1988;12:262-279.

112. Romero R, Mazor M. Infection and preterm labor. *Clin Obstet Gynecol*. 1988;31:553-584.

113. Romero R, Sirtori M, Oyarzun E, et al. Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes. *Am J Obstet Gynecol*. 1989;161:817-824.

114. Romero R, Shamma F, Avila C, et al. Infection and labor. VI. Prevalence, microbiology, and clinical significance of intraamniotic infection in twin gestations with preterm labor. *Am J Obstet Gynecol*. 1990;163:757-761.

115. Romero R, Ghidini A, Mazor M, Behnke E. Microbial invasion of the amniotic cavity in premature rupture of membranes. *Clin Obstet Gynecol*. 1991;34:769-778.

116. Romero R, Mazor M, Morrotti R, et al. Infection and labor. VII. Microbial invasion of the amniotic cavity in spontaneous rupture of membranes at term. *Am J Obstet Gynecol*. 1992;166:129-133.

117. Romero R, Nores J, Mazor M, et al. Microbial invasion of the amniotic cavity during term labor. Prevalence and clinical significance. *J Reprod Med*. 1993;38:543-548.

118. Yoon BH, Romero R, Moon JB, et al. Clinical significance of intraamniotic inflammation in patients with preterm labor and intact membranes. *Am J Obstet Gynecol*. 2001;185:1130-1136.

119. Seong HS, Lee SE, Kang JH, Romero R, Yoon BH. The frequency of microbial invasion of the amniotic cavity and histologic chorioamnionitis in women at term with intact membranes in the presence or absence of labor. *Am J Obstet Gynecol*. 2008;199(375):e371-e375.

120. Romero R, Miranda J, Chaiworapongsa T, et al. A novel molecular microbiologic technique for the rapid diagnosis of microbial invasion of the amniotic cavity and intra-amniotic infection in preterm labor with intact membranes. *Am J Reprod Immunol*. 2014;71:330-358.

121. Romero R, Miranda J, Kusanovic JP, et al. Clinical chorioamnionitis at term I: microbiology of the amniotic cavity using cultivation and molecular techniques. *J Perinat Med*. 2015;43:19-36.

122. Musilova I, Andrys C, Drahosova M, et al. Amniotic fluid clusterin in pregnancies complicated by the preterm prelabor rupture of membranes. *J Matern Fetal Neonatal Med*. 2017;30:2529-2537.

123. Rowlands S, Danielewski JA, Tabrizi SN, Walker SP, Garland SM. Microbial invasion of the amniotic cavity in midtrimester pregnancies using molecular microbiology. *Am J Obstet Gynecol*. 2017;217:71 e71-71 e75.

124. Romero R, Chaiworapongsa T, Alpay Savasan Z, et al. Damage-associated molecular patterns (DAMPs) in preterm labor with intact membranes and preterm PROM: a study of the alarmin HMGB1. *J Matern Fetal Neonatal Med*. 2011;24:1444-1455.

125. Gervasi MT, Romero R, Bracalente G, et al. Midtrimester amniotic fluid concentrations of interleukin-6 and interferon-gamma-inducible protein-10: evidence for heterogeneity of intra-amniotic inflammation and associations with spontaneous early (<32 weeks) and late (>32 weeks) preterm delivery. *J Perinat Med*. 2012;40:329-343.

126. Combs CA, Gravett M, Garite TJ, et al., ProteoGenix/Obstetrix Collaborative Research N. Amniotic fluid infection, inflammation, and colonization in preterm labor with intact membranes. *Am J Obstet Gynecol*. 2014;210:125 e121-125 e115.

127. Romero R, Miranda J, Chaiworapongsa T, et al. Sterile intra-amniotic inflammation in asymptomatic patients with a sonographic short cervix: prevalence and clinical significance. *J Matern Fetal Neonatal Med*. 2014;28:1343-1359.

128. Romero R, Miranda J, Chaiworapongsa T, et al. Prevalence and clinical significance of sterile intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Reprod Immunol*. 2014;72:458-474.

129. Romero R, Miranda J, Chaemsaithong P, et al. Sterile and microbial-associated intra-amniotic inflammation in preterm prelabor rupture of membranes. *J Matern Fetal Neonatal Med*. 2015;28:1394-1409.

130. Chaemsaithong P, Romero R, Korzeniewski SJ, et al. A point of care test for interleukin-6 in amniotic fluid in preterm prelabor rupture of membranes: a step toward the early treatment of acute intra-amniotic inflammation/infection. *J Matern Fetal Neonatal Med*. 2016;29:360-367.

131. Yoneda N, Yoneda S, Niimi H, et al. Polymicrobial amniotic fluid infection with mycoplasma/ureaplasma and other bacteria induces severe intra-amniotic inflammation associated with poor perinatal prognosis in preterm labor. *Am J Reprod Immunol*. 2016;75:112-125.

132. Chaemsaithong P, Romero R, Korzeniewski SJ, et al. A rapid interleukin-6 bedside test for the identification of intra-amniotic inflammation in preterm labor with intact membranes. *J Matern Fetal Neonatal Med*. 2016;29:349-359.

133. Musilova I, Bestvina T, Hudeckova M, et al. Vaginal fluid interleukin-6 concentrations as a point-of-care test is of value in women with preterm prelabor rupture of membranes. *Am J Obstet Gynecol*. 2016;215:619 e611-619 e612.

134. Park JY, Romero R, Lee J, Chaemsaithong P, Chaiyasit N, Yoon BH. An elevated amniotic fluid prostaglandin F2alpha concentration is associated with intra-amniotic inflammation/infection, and clinical and histologic chorioamnionitis, as well as impending preterm delivery in patients with preterm labor and intact membranes. *J Matern Fetal Neonatal Med*. 2016;29:2563-2572.

135. Romero R, Chaemsaithong P, Chaiyasit N, et al. CXCL10 and IL-6: markers of two different forms of intra-amniotic inflammation in preterm labor. *Am J Reprod Immunol*. 2017;78:e12685.

136. Oh KJ, Kim SM, Hong JS, et al. Twenty-four percent of patients with clinical chorioamnionitis in preterm gestations have no evidence of either culture-proven intraamniotic infection or intraamniotic inflammation. *Am J Obstet Gynecol*. 2017;216:604 e601-604 e611.

137. Chaiyasit N, Romero R, Chaemsaithong P, et al. Clinical chorioamnionitis at term VIII: a rapid MMP-8 test for the identification of intra-amniotic inflammation. *J Perinat Med*. 2017;45:539-550.

138. Pacora P, Romero R, Erez O, et al. The diagnostic performance of the beta-glucan assay in the detection of intra-amniotic infection with Candida species. *J Matern Fetal Neonatal Med*. 2017;1-18.

139. Tricomi V, Hall JE, Bittar A, Chambers D. Arborization test for the detection of ruptured fetal membranes. Clinical evaluation. *Obstet Gynecol*. 1966;27:275-279.

140. Friedman ML, McElin TW. Diagnosis of ruptured fetal membranes. Clinical study and review of the literature. *Am J Obstet Gynecol*. 1969;104:544-550.

141. Bennett SL, Cullen JB, Sherer DM, Woods JR Jr. The ferning and nitrazine tests of amniotic fluid between 12 and 41 weeks gestation. *Am J Perinatol*. 1993;10:101-104.

142. Tchirikov M, Schlabritz-Loutsevitch N, Maher J, et al. Midtrimester preterm premature rupture of membranes (PPROM): etiology, diagnosis, classification, international recommendations of treatment options and outcome. *J Perinat Med*. 2017;46:465-488.

143. Romero R, Jimenez C, Lohda AK, et al. Amniotic fluid glucose concentration: a rapid and simple method for the detection of intraamniotic infection in preterm labor. *Am J Obstet Gynecol*. 1990;163:968-974.

144. Romero R, Emamian M, Quintero R, et al. The value and limitations of the Gram stain examination in the diagnosis of intraamniotic infection. *Am J Obstet Gynecol*. 1988;159:114-119.

145. Iavazzo C, Tassis K, Gourgiotis D, et al. The role of human beta defensins 2 and 3 in the second trimester amniotic fluid in predicting

preterm labor and premature rupture of membranes. *Arch Gynecol Obstet*. 2010;281:793-799.

146. Starner TD, Agerberth B, Gudmundsson GH, McCray PB Jr. Expression and activity of beta-defensins and LL-37 in the developing human lung. *J Immunol*. 2005;174:1608-1615.

147. Zaga-Clavellina V, Martha RV, Flores-Espinosa P. In vitro secretion profile of pro-inflammatory cytokines IL-1beta, TNF-alpha, IL-6, and of human beta-defensins (HBD)-1, HBD-2, and HBD-3 from human chorioamniotic membranes after selective stimulation with Gardnerella vaginalis. *Am J Reprod Immunol*. 2012;67:34-43.

148. Boldenow E, Jones S, Lieberman RW, et al. Antimicrobial peptide response to group B Streptococcus in human extraplacental membranes in culture. *Placenta*. 2013;34:480-485.

149. Kai-Larsen Y, Gudmundsson GH, Agerberth B. A review of the innate immune defence of the human foetus and newborn, with the emphasis on antimicrobial peptides. *Acta Paediatr*. 2014;103:1000-1008.

150. Boldenow E, Hogan KA, Chames MC, Aronoff DM, Xi C, Loch-Caruso R. Role of cytokine signaling in group B Streptococcus-stimulated expression of human beta defensin-2 in human extraplacental membranes. *Am J Reprod Immunol*. 2015;73:263-272.

151. Young A, Thomson AJ, Ledingham M, Jordan F, Greer IA, Norman JE. Immunolocalization of proinflammatory cytokines in myometrium, cervix, and fetal membranes during human parturition at term. *Biol Reprod*. 2002;66:445-449.

152. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel LA, Nien JK. Inflammation in preterm and term labour and delivery. *Semin Fetal Neonatal Med*. 2006;11:317-326.

153. Haddad R, Tromp G, Kuivaniemi H, et al. Human spontaneous labor without histologic chorioamnionitis is characterized by an acute inflammation gene expression signature. *Am J Obstet Gynecol*. 2006;195(394):e391-324.

154. Hassan SS, Romero R, Haddad R, et al. The transcriptome of the uterine cervix before and after spontaneous term parturition. *Am J Obstet Gynecol*. 2006;195:778-786.

155. Romero R, Gotsch F, Pineles B, Kusanovic JP. Inflammation in pregnancy: its roles in reproductive physiology, obstetrical complications, and fetal injury. *Nutr Rev*. 2007;65:S194-S202.

156. Norman JE, Bollapragada S, Yuan M, Nelson SM. Inflammatory pathways in the mechanism of parturition. *BMC Pregnancy Childbirth*. 2007;7(Suppl 1):S7.

157. Gomez-Lopez N, Estrada-Gutierrez G, Jimenez-Zamudio L, Vega-Sanchez R, Vadillo-Ortega F. Fetal membranes exhibit selective leukocyte chemotaxic activity during human labor. *J Reprod Immunol*. 2009;80:122-131.

158. Yuan M, Jordan F, McInnes IB, Harnett MM, Norman JE. Leukocytes are primed in peripheral blood for activation during term and preterm labour. *Mol Hum Reprod*. 2009;15:713-724.

159. Bollapragada S, Youssef R, Jordan F, Greer I, Norman J, Nelson S. Term labor is associated with a core inflammatory response in human fetal membranes, myometrium, and cervix. *Am J Obstet Gynecol*. 2009;200(104):e101-e111.

160. Mittal P, Romero R, Tarca AL, et al. Characterization of the myometrial transcriptome and biological pathways of spontaneous human labor at term. *J Perinat Med*. 2010;38:617-643.

161. Nhan-Chang CL, Romero R, Tarca AL, et al. Characterization of the transcriptome of chorioamniotic membranes at the site of rupture in spontaneous labor at term. *Am J Obstet Gynecol*. 2010;202(462):e461-441.

162. Gomez-Lopez N, Vega-Sanchez R, Castillo-Castrejon M, Romero R, Cubeiro-Arreola K, Vadillo-Ortega F. Evidence for a role for the adaptive immune response in human term parturition. *Am J Reprod Immunol*. 2013;69:212-230.

163. Ibrahim SA, Ackerman W, Summerfield TL, Lockwood CJ, Schatz F, Kniss DA. Inflammatory gene networks in term human decidual cells define a potential signature for cytokine-mediated parturition. *Am J Obstet Gynecol*. 2016;214:284 e281-284 e247.

164. Gomez-Lopez N, Romero R, Xu Y, et al. Inflammasome assembly in the chorioamniotic membranes during spontaneous labor at term. *Am J Reprod Immunol*. 2017;77:e12648.

165. Herrera CA, Stoerker J, Carlquist J, et al. Cell-free DNA, inflammation, and the initiation of spontaneous term labor. *Am J Obstet Gynecol*. 2017;217:583 e581-583 e588.

166. Romero R, Xu Y, Plazyo O, et al. A role for the inflammasome in spontaneous labor at term. *Am J Reprod Immunol*. 2018;79:e12440.

167. Panaitescu B, Romero R, Gomez-Lopez N, et al. In vivo evidence of inflammasome activation during spontaneous labor at term. *J Matern Fetal Neonatal Med*. 2018:1-14.

168. Zaga-Clavellina V, Ruiz M, Flores-Espinosa P, et al. Tissue-specific human beta-defensins (HBD)-1, HBD-2 and HBD-3 secretion profile from human amniochorionic membranes stimulated with Candida albicans in a two-compartment tissue culture system. *Reprod Biol Endocrinol*. 2012;10:70.

169. Vora P, Youdim A, Thomas LS, et al. Beta-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *J Immunol*. 2004;173:5398-5405.

170. Beghini J, Giraldo PC, Eleuterio J Jr, Amaral RL, Polpeta NC, Goncalves AK. Vaginal inflammation: association between leukocyte concentration and levels of immune mediators. *Am J Reprod Immunol*. 2016;75:126-133.

171. Samejima T, Nagamatsu T, Schust DJ, et al. Elevated concentration of secretory leukocyte protease inhibitor in the cervical mucus before delivery. *Am J Obstet Gynecol*. 2016;214(741):e741-e747.

172. Mukura LR, Hickey DK, Rodriguez-Garcia M, Fahey JV, Wira CR. *Chlamydia trachomatis* regulates innate immune barrier integrity and mediates cytokine and antimicrobial responses in human uterine ECC-1 epithelial cells. *Am J Reprod Immunol*. 2017;78:e12764.

173. Modi BP, Teves ME, Pearson LN, et al. Mutations in fetal genes involved in innate immunity and host defense against microbes increase risk of preterm premature rupture of membranes (PPROM). *Mol Genet Genomic Med*. 2017;5:720-729.

174. Strauss JF 3rd, Romero R, Gomez-Lopez N, et al. Spontaneous preterm birth: advances toward the discovery of genetic predisposition. *Am J Obstet Gynecol*. 2018;218:294-314 e292.

175. Gomez-Lopez N, Romero R, Plazyo O, et al. Intra-amniotic administration of HMGB1 induces spontaneous preterm labor and birth. *Am J Reprod Immunol*. 2016;75:3-7.

176. Plazyo O, Romero R, Unkel R, et al. HMGB1 induces an inflammatory response in the chorioamniotic membranes that is partially mediated by the inflammasome. *Biol Reprod*. 2016;95:130.

177. Behnia F, Sheller S, Menon R. Mechanistic differences leading to infectious and sterile inflammation. *Am J Reprod Immunol*. 2016;75:505-518.

178. Gomez-Lopez N, Romero R, Xu Y, et al. A role for the inflammasome in spontaneous preterm labor with acute histologic chorioamnionitis. *Reprod Sci*. 2017;24:1382-1401.

179. Gomez-Lopez N, Romero R, Plazyo O, et al. Preterm labor in the absence of acute histologic chorioamnionitis is characterized by cellular senescence of the chorioamniotic membranes. *Am J Obstet Gynecol*. 2017;217:592 e591-592 e517.

180. Vardar-Sengul S, Demirci T, Sen BH, Erkizan V, Kurulgan E, Baylas H. Human beta defensin-1 and -2 expression in the gingiva of patients with specific periodontal diseases. *J Periodontal Res*. 2007;42:429-437.

181. Fang XM, Shu Q, Chen QX, et al. Differential expression of alpha- and beta-defensins in human peripheral blood. *Eur J Clin Invest*. 2003;33:82-87.

182. Mercer BM, Miodovnik M, Thurnau GR, et al. Antibiotic therapy for reduction of infant morbidity after preterm premature rupture of the membranes. A randomized controlled trial. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. *JAMA*. 1997;278:989-995.

183. Mercer BM, Crouse DT, Goldenberg RL, et al. The antibiotic treatment of PPROM study: systemic maternal and fetal markers and perinatal outcomes. *Am J Obstet Gynecol*. 2012;206(145):e141 -e149.

184. Schumann A, Nutten S, Donnicola D, et al. Neonatal antibiotic treatment alters gastrointestinal tract developmental gene expression and intestinal barrier transcriptome. *Physiol Genomics*. 2005;23:235-245.

185. Clarke TB, Davis KM, Lysenko ES, Zhou AY, Yu Y, Weiser JN. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat Med*. 2010;16:228-231.