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Microbial invasion of the amniotic cavity in pregnancies with small-for-gestational-age fetuses

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Abstract

Objective—Microbial invasion of the amniotic cavity (MIAC) has been detected in women with preterm labor, preterm prelabor rupture of membranes (PROM), and in patients at term with PROM or in spontaneous labor. Intrauterine infection is recognized as a potential cause of fetal growth restriction; yet, the frequency of MIAC in pregnancies with small-for-gestational-age (SGA) fetuses is unknown. The aim of this study was to determine the frequency, diversity and relative abundance of microbes in amniotic fluid of women with an SGA neonate using a combination of culture and molecular methods.

Method—Amniotic fluid from 52 subjects with an SGA neonate was analyzed with both cultivation and molecular methods in a retrospective cohort study. Broad-range and group-specific PCR assays targeted small subunit rDNA, or other gene sequences, from bacteria, fungi and archaea. Results of microbiologic studies were correlated with indices of the host inflammatory response.

Results—1) All amniotic fluid samples (n=52) were negative for microorganisms based on cultivation techniques, whereas 6% (3/52) were positive based on PCR; and 2) intra-amniotic inflammation was detected in one of the three patients with a positive PCR result, as compared with 3 patients (6.1%) of the 49 with both a negative culture and a negative PCR (p=0.2).

Conclusion—Microbial invasion of the amniotic cavity is detected by PCR in some patients with an SGA fetus who were not in labor at the time of amniotic fluid collection.

Keywords

16S rRNA; chorioamnionitis; cytokines; FIRS; IL-6; intra-amniotic infection; intra-amniotic inflammation; molecular microbiology; PCR; pregnancy; SGA

INTRODUCTION

A small-for-gestational-age (SGA) neonate is usually defined as one whose birth weight is below the 10th percentile for gestational age.[1,24,71] An SGA newborn may be constitutionally small or the consequence of several mechanisms of disease, such as uteroplacental insufficiency, chromosomal abnormalities, congenital infection, genetic syndromes, etc.[76] Therefore, SGA is considered one of the “great obstetrical syndromes” because it has multiple etiologies, a long preclinical phase and the other criteria that define these syndromes.[15,55,56]

Proposed mechanisms of disease of SGA include endothelial cell dysfunction,[5] an anti-angiogenic state,[9,10,18,25,62,74] inadequate physiologic transformation of the spiral arteries [7,22] and a maternal intravascular exaggerated inflammatory response.[29,34,46,70,72] Perinatal infections, mainly of viral or parasitic origin (i.e., cytomegalovirus, rubella, herpes, toxoplasmosis, etc)[26,28,33,37,48,51,52,73], have also been implicated as a cause of SGA.

Experimental studies have demonstrated that chronic infection/inflammation during pregnancy may result in an SGA fetus in hamsters[11,12] and mice[38,78]. In humans, maternal microbial infections during pregnancy have been associated with impaired fetal growth.[3,13,19,21,42,44,45]. However, it is unknown if microbial invasion of the amniotic cavity (MIAC) with bacteria or fungi could be associated with SGA neonates in humans. A literature search in Pubmed performed in March 2010 using different combinations of the key words: “small-for-gestational age”, “SGA”, “intra-uterine growth retardation”, “IUGR”, “infection”, and “amniotic fluid” limited to humans and published in English did not reveal any study addressing this question.

The objectives of this study were to determine the frequency, taxonomic diversity and relative abundance of microbes in amniotic fluid of women with an SGA neonate using a combination of cultivation and molecular methods.

METHODS

Study population

A retrospective cohort study was conducted of patients with an SGA neonate (defined below) who met the following inclusion criteria: 1) singleton gestation; 2) gestational age between 24 and 42 weeks; and 3) amniocentesis with microbiological studies of amniotic fluid. Exclusion criteria were: 1) active term or preterm labor; 2) ruptured membranes; 3) preeclampsia; or 4) a major fetal chromosomal and/or congenital anomaly. Patients in labor and/or with rupture of membranes were excluded because these conditions have been associated with a high rate of MIAC and could confound the research question of this study.

All women provided written informed consent prior to the collection of biological samples. The utilization of samples and clinical data for research purposes was approved by the Institutional Review Boards of Sotero del Rio Hospital, Azienda Ospedaliera of Padova, Wayne State University, the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD/NIH/DHHS), and Stanford University.

Definitions

An SGA neonate was defined by sonographic estimated fetal weight below the 10th percentile for gestational age[1,24] and confirmed by neonatal birthweight. Histologic chorioamnionitis was diagnosed based on the presence of inflammatory cells in the chorionic plate and/or chorioamniotic membranes.[32,53] Acute funisitis was diagnosed by the presence of neutrophils in the wall of the umbilical vessels and/or Wharton's jelly using criteria previously described.[49] Intra-amniotic inflammation was defined by an amniotic-fluid interleukin (IL)-6 concentration >2.6 ng/mL.[81]

Sampling procedures

Patients with an SGA fetus were offered amniocentesis for genetic indications, to assess the microbial status of the amniotic cavity and to assess fetal lung maturity. In patients undergoing cesarean delivery, amniotic fluid was retrieved intra-operatively. Amniotic fluid was transported in a capped sterile syringe to the clinical laboratory where it was cultured for aerobic and anaerobic bacteria, including genital mycoplasmas, as described previously.[16] A white blood cell (WBC) count[64] and Gram stain[58] of amniotic fluid were also performed shortly after collection using methods previously described. Shortly after the amniocentesis, amniotic fluid not required for clinical assessment was centrifuged at $1300 \times g$ for 10 minutes at 4°C, and the supernatant was aliquoted into gamma-irradiated nonpyrogenic DNase/RNase-free cryovials (Corning, Acton, MA, USA), and immediately frozen at -70°C. Amniotic fluid IL-6 and matrix metalloproteinase (MMP)-8 concentrations were determined using a specific and sensitive immunoassay which had been validated for amniotic fluid.[43] IL-6 and MMP-8 determinations were performed after all patients were delivered and were not used in clinical management.

Genomic DNA extraction

Amniotic fluid that was not required for clinical purposes ($\approx 200 \mu\text{l}$ of each amniotic fluid sample) was shipped on dry ice to Stanford, CA, where genomic DNA was extracted as described previously.[17] Extracted DNA was eluted into a final volume of 100 μl of QIAamp® AE buffer and stored at -20°C or colder until thawing for molecular analyses. Strategies to prevent, detect and neutralize potential contamination were implemented at critical steps,[4] according to a previously described protocol. This included mock extraction blanks (sterile water processed in parallel, and in the same manner as amniotic fluid samples) to monitor potential contamination (at least one mock was included per 17 processed samples).[16]

Qualitative analysis by end-point PCR

DNA from each amniotic fluid sample was analyzed by end-point PCR using broad-range bacterial 16S ribosomal DNA (rDNA) primers, and by group-specific end-point PCR using primers specific for six taxonomic groups, including *Candida* sp. (Table 1[6,14,36,50,77,82]. PCR reactions, screening of PCR products by gel electrophoresis, and purification and cloning of amplicons from broad-range PCR were performed as described.[17] Sequencing of amplicons directly from group-specific PCR, and of recombinant clones from broad-range PCRs (up to 10 clones per reaction) was performed as described.[16]

Sequence alignment and phylogenetic analysis

Forward and reverse sequence reads were assembled into contigs as described.[16] Assembled sequences from group-specific PCR were queried against NCBI's GenBank database using a basic local alignment search tool (BLAST) algorithm[2] to confirm specificity. Assembled sequences from broad-range end-point PCR were aligned and subjected to phylogenetic analysis as described.[16] After removal of vector, human, and poor-quality sequences from the alignment, a neighbor-joining tree was generated based on Felsenstein correction and 682

unambiguous filter positions. Phylotypes were defined using a 99% sequence similarity threshold, which approximates a species-level classification.

Quantitative analysis by real-time PCR

DNA from each sample was analyzed by means of two real-time PCR assays, each of which was designed to amplify in a specific manner and quantify 16S rDNA of domain *Bacteria* or domain *Archaea* (Table 1). Reactions were carried out as described.[17]

Statistical analysis

Comparison between continuous variables was performed with the Mann-Whitney *U*-test. Comparison of proportions was performed using Fisher's exact tests. A *p*-value <0.05 was considered statistically significant. Analysis was performed with SPSS, version 12 (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographic and clinical characteristics of the 52 patients enrolled in the study are presented in Table 2.

Microbial invasion of the amniotic cavity in SGA

All samples were negative for MIAC based on cultivation methods whereas 5.8% (3/52) of samples were positive for MIAC based on PCR methods. Two of the three PCR-positive samples were detected by broad-range PCR: one sample had evidence of *Streptococcus agalactiae* (10 clones; 100% identity to type strain ATCC 13813^T), and one had evidence of *Staphylococcus epidermidis* (2 clones; 100% identity to type strain ATCC 14990^T). The other sample with molecular evidence of MIAC was positive by group-specific PCR for *Candida* species. In addition, group-specific PCR for *Streptococcus agalactiae* was also positive in the sample that yielded this species by broad-range PCR. This was also the only sample with a high microbial rDNA abundance (e.g., >500 genes per µl AF) based on broad-range real-time bacterial PCR, which estimated 16S rDNA abundance in this sample to be approximately 10⁵ genes per µL of amniotic fluid. Table 3 displays the clinical information of the cases that were positive by PCR.

Assessment of the intra-amniotic inflammatory response

Intra-amniotic inflammation was detected in one of the three patients with a positive PCR (Table 3). Among the 49 patients with both a negative amniotic fluid culture and a negative PCR, 3 cases (6.1%) had intra-amniotic inflammation (*p*=0.2). The median concentrations of the different markers of intra-amniotic infection/inflammation (i.e. WBC count and glucose, IL-6 and MMP-8 concentration) were not significantly different between patients with a positive PCR and those with negative cultures and negative PCR ((AF WBC: *p*=0.4, glucose: *p*=0.1, IL-6: *p*=0.1, and MMP-8: *p*=0.4).

Short-term neonatal outcome

In the case that was PCR-positive for *Candida* species, the neonate had an elevated C-reactive protein (CRP) in the first day of life; he received antibiotics (ampicillin and gentamicin) for 4 days and the CRP concentration subsequently normalized and blood cultures were negative. In the case with *Staphylococcus epidermidis*, the neonatal WBC count and differential were normal and blood cultures were negative. Of note, the managing physicians were not aware of the results of PCR which was performed later.

In the case that was PCR-positive for *Streptococcus agalactiae*, the neonate had an uneventful outcome and was discharged home with the mother.

DISCUSSION

Principal findings of the study

Using cultivation techniques, none of the patients had microorganisms detected in the amniotic fluid; however by including molecular methods in our approach, we found MIAC in approximately 6% of patients with an SGA neonate.

Detection of microbial invasion of the amniotic cavity

The amniotic fluid in normal pregnancy is considered sterile in the majority of cases. However, MIAC has been demonstrated in 18% of patients in spontaneous labor at term with intact membranes,[63] 34% of women with prelabor rupture of membranes (PROM) at term,[61] 13% of women presenting with an episode of preterm labor,[23] 32% of women with preterm PROM, [23] and 9% of women with a short cervix.[27] Among women with cervical insufficiency, the prevalence of MIAC is about 50%.[59] However, all these estimates are based upon cultivation techniques and rely on the ability to provide adequate conditions required for the growth of microorganisms in the laboratory.

Molecular methods offer a cultivation-independent approach to microbial detection, and various types of molecular assays provide relative advantages. For example, broad-range PCR assays that target rDNA with universal primers enable detection and characterization of diverse microbial taxa, including previously-unknown species.[54] On the other hand, group-specific PCR assays that amplify gene sequences unique to smaller groups of related taxa are often more sensitive; however, the specific microbial groups must be suspected in advance. Both approaches yielded positive findings in the current study.

Our group previously reported that specific PCR assays for *Ureaplasma urealyticum* are more sensitive than cultivation for this species in amniotic fluid of patients with preterm labor and intact membranes,[80] preterm PROM,[79] and cervical insufficiency.[8] We have also employed a combination of broad-range and specific PCR assays for bacteria and fungi, and have demonstrated that the combination of culture and molecular methods allows improved detection of MIAC.[16,17] Importantly, an intrauterine inflammatory response is associated with the presence of microbial DNA in the amniotic fluid, even in the settings of a negative culture.[16,17,31,79,80] Such findings provide evidence that a positive PCR-based assay has biological significance.[20]

MIAC in patients with SGA

We have not been able to identify any prior study that has systematically examined MIAC in SGA with cultivation or molecular methods. Most studies have focused on the presence of selected viruses, such as Cytomegalovirus, or specific microorganisms such as *Toxoplasma gondii*. [26,28,37,48,51,52,73]

Our findings suggest that ~6% of women with SGA neonates have MIAC detected by molecular techniques, and that these cases escape detection by cultivation techniques routinely employed in a clinical laboratory supporting an obstetrical service. The organisms identified included *Streptococcus agalactiae* (group B streptococci), *Candida* species and *Staphylococcus epidermidis*.

One interesting sample (containing *Streptococcus agalactiae*) was positive both by broad-range PCR and by group-specific PCR, and was found to have a high microbial burden based

on 16S rDNA copy number, as measured by real-time PCR. This case had evidence of a robust immune response, and was associated with the highest concentrations of both IL-6 (44.8 ng/mL), and MMP-8 (32.6 ng/mL) in our study population (the next highest measurements of each marker were 8.78 ng/mL for IL-6, and 1.03 ng/mL for MMP-8). These two parameters have been associated with the presence of intra-amniotic infection in previous studies.[39–41,57,66,81] In addition, our prior studies found microbial rDNA levels to be inversely correlated with gestational age at delivery.[16,17]

Two other samples were positive by PCR for a single taxon each: one for *Candida* sp., and one for *Staphylococcus epidermidis*. In these cases, the concentrations of IL-6 and MMP-8 were not elevated; therefore, it is possible that PCR detected MIAC at an early stage prior to the development of a significant host response, or that one or more of these taxa represent contamination, despite the rigorous method of amniotic fluid collection. These samples were collected in the Operating Room at the time of cesarean delivery, and the patients were not in labor.

Implications of the findings

Our results suggest that a small group of SGA fetuses have subclinical MIAC, and that in some instances, this is associated with an intra-amniotic inflammatory response as determined by the amniotic fluid concentrations of IL-6 and MMP-8. It is also interesting that the patient with a high microbial burden and a robust response was not in labor; however, a cesarean section was performed at term. It seems that not all cases of MIAC are associated with the spontaneous onset of labor, even though the natural history of the patient was interrupted by a cesarean section. Whether microorganisms may exist in the amniotic cavity for a period of weeks without eliciting an inflammatory response remains to be determined. Similarly, whether microorganisms can multiply in amniotic fluid, eliciting an inflammatory response, but not lead to the initiation of labor or rupture of membranes is also possible.

Strengths and limitations of the study

This is the first study to examine, in a systematic manner, amniotic fluid from pregnancies with SGA neonates to determine the presence or absence of microbial invasion using both cultivation and molecular methods. We also examined indices of the intra-amniotic inflammatory response (IL-6 and MMP-8).

One limitation of our study was its sample size (n=52). However, prior to this study, there was no estimate of the rate of MIAC in this clinical phenotype. The conventional view has been that MIAC is associated with spontaneous preterm labor (with intact or ruptured membranes), [60,61,65,67–69,80] cervical insufficiency,[35,47,59] or short cervix,[27,75] but not with indicated causes of preterm delivery such as SGA and preeclampsia.

Conclusions

MIAC was detected using molecular techniques, but not cultivation techniques, in association with approximately 6% of SGA neonates. The detection in one case of *Streptococcus agalactiae* (group B streptococci) was associated with a demonstrable amniotic fluid inflammatory response. The role of microbes in the pathophysiology of SGA requires further study. Studies to determine the frequency, diversity, and relative abundance of microorganisms in amniotic fluid from normal pregnant women in the mid-trimester of pregnancy and from women at term not in labor are in progress. We believe that such studies will assist in placing the information presented in this study in context.

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Reference List

- Alexander GR, Himes JH, Kaufman RB, Mor J, Kogan M. A United States national reference for fetal growth. *Obstet Gynecol* 1996;87:163–168. [PubMed: 8559516]
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403–410. [PubMed: 2231712]
- Avar Z, Gero G, Hajagos E. Effect of pyelonephritis during pregnancy on mother and fetus. *Acta Chir Acad Sci Hung* 1980;21:203–211. [PubMed: 7324716]
- Borst A, Box AT, Fluit AC. False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy. *Eur J Clin Microbiol Infect Dis* 2004;23:289–299. [PubMed: 15015033]
- Bretelle F, Sabatier F, Blann A, D'Ercole C, Boutiere B, Mutin M, et al. Maternal endothelial soluble cell adhesion molecules with isolated small for gestational age fetuses: comparison with pre-eclampsia. *BJOG* 2001;108:1277–1282. [PubMed: 11843391]
- Brinig MM, Lepp PW, Ouverney CC, Armitage GC, Relman DA. Prevalence of bacteria of division TM7 in human subgingival plaque and their association with disease. *Appl Environ Microbiol* 2003;69:1687–1694. [PubMed: 12620860]
- Brosens I, Dixon HG, Robertson WB. Fetal growth retardation and the arteries of the placental bed. *Br J Obstet Gynaecol* 1977;84:656–663. [PubMed: 911717]
- Bujold E, Morency AM, Rallu F, Ferland S, Tetu A, Duperron L, et al. Bacteriology of amniotic fluid in women with suspected cervical insufficiency. *J Obstet Gynaecol Can* 2008;30:882–887. [PubMed: 19038071]
- Chaiworapongsa T, Espinoza J, Gotsch F, Kim YM, Kim GJ, Goncalves LF, et al. The maternal plasma soluble vascular endothelial growth factor receptor-1 concentration is elevated in SGA and the magnitude of the increase relates to Doppler abnormalities in the maternal and fetal circulation. *J Matern Fetal Neonatal Med* 2008;21:25–40. [PubMed: 18175242]
- Chaiworapongsa T, Romero R, Gotsch F, Espinoza J, Nien JK, Goncalves L, et al. Low maternal concentrations of soluble vascular endothelial growth factor receptor-2 in preeclampsia and small for gestational age. *J Matern Fetal Neonatal Med* 2008;21:41–52. [PubMed: 18175243]
- Collins JG, Smith MA, Arnold RR, Offenbacher S. Effects of *Escherichia coli* and *Porphyromonas gingivalis* lipopolysaccharide on pregnancy outcome in the golden hamster. *Infect Immun* 1994;62:4652–4655. [PubMed: 7927735]
- Collins JG, Windley HW III, Arnold RR, Offenbacher S. Effects of a *Porphyromonas gingivalis* infection on inflammatory mediator response and pregnancy outcome in hamsters. *Infect Immun* 1994;62:4356–4361. [PubMed: 7927695]
- Dasanayake AP, Boyd D, Madianos PN, Offenbacher S, Hills E. The association between *Porphyromonas gingivalis*-specific maternal serum IgG and low birth weight. *J Periodontol* 2001;72:1491–1497. [PubMed: 11759860]
- DeLong EF. Archaea in coastal marine environments. *Proc Natl Acad Sci US A* 1992;89:5685–5689.
- Di Renzo GC. The great obstetrical syndromes. *J Matern Fetal Neonatal Med* 2009;22:633–635. [PubMed: 19736613]
- DiGiulio DB, Romero R, Amogan HP, Kusanovic JP, Bik EM, Gotsch F, et al. Microbial prevalence, diversity and abundance in amniotic fluid during preterm labor: a molecular and culture-based investigation. *PLoS One* 2008;3:e3056. [PubMed: 18725970]

17. DiGiulio DB, Romero R, Kusanovic JP, Gomez R, Kim CJ, Seok K, et al. Prevalence and Diversity of Microbes in the Amniotic Fluid, the Fetal Inflammatory Response, and Pregnancy Outcome in Women with Preterm Pre-Labor Rupture of Membranes. *Am J Reprod Immunol.* 2010
18. Erez O, Romero R, Espinoza J, Fu W, Todem D, Kusanovic JP, et al. The change in concentrations of angiogenic and anti-angiogenic factors in maternal plasma between the first and second trimesters in risk assessment for the subsequent development of preeclampsia and small-for-gestational age. *J Matern Fetal Neonatal Med* 2008;21:279–287. [PubMed: 18446652]
19. Eslick GD, Yan P, Xia HH, Murray H, Spurrett B, Talley NJ. Foetal intrauterine growth restrictions with *Helicobacter pylori* infection. *Aliment Pharmacol Ther* 2002;16:1677–1682. [PubMed: 12197848]
20. Fredericks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clin Microbiol Rev* 1996;9:18–33. [PubMed: 8665474]
21. Gerards LJ, Cats BP, Hoogkamp-Korstanje JA. The influence of group B streptococcal-carriership on pregnancy outcome. *J Perinat Med* 1982;10:279–285. [PubMed: 6761428]
22. Gerretsen G, Huisjes HJ, Elema JD. Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation. *Br J Obstet Gynaecol* 1981;88:876–881. [PubMed: 7272259]
23. Goncalves LF, Chaiworapongsa T, Romero R. Intrauterine infection and prematurity. *Ment Retard Dev Disabil Res Rev* 2002;8:3–13. [PubMed: 11921380]
24. Gonzalez RP, Gomez RM, Castro RS, Nien JK, Merino PO, Etchegaray AB, et al. A national birth weight distribution curve according to gestational age in Chile from 1993 to 2000. *Rev Med Chil* 2004;132:1155–1165. [PubMed: 15631202]
25. Gotsch F, Romero R, Kusanovic JP, Chaiworapongsa T, Dombrowski M, Erez O, et al. Preeclampsia and small-for-gestational age are associated with decreased concentrations of a factor involved in angiogenesis: soluble Tie-2. *J Matern Fetal Neonatal Med* 2008;21:389–402. [PubMed: 18570117]
26. Gulmezoglu M, de Onis M, Villar J. Effectiveness of interventions to prevent or treat impaired fetal growth. *Obstet Gynecol Surv* 1997;52:139–149. [PubMed: 9027913]
27. Hassan S, Romero R, Hendler I, Gomez R, Khalek N, Espinoza J, et al. A sonographic short cervix as the only clinical manifestation of intra-amniotic infection. *J Perinat Med.* 2005
28. Hollier LM, Grissom H. Human herpes viruses in pregnancy: cytomegalovirus, Epstein-Barr virus, and varicella zoster virus. *Clin Perinatol* 2005;32:671–696. [PubMed: 16085026]
29. Johnston TA, Greer IA, Dawes J, Calder AA. Neutrophil activation in small for gestational age pregnancies. *Br J Obstet Gynaecol* 1991;98:105–106. [PubMed: 1998619]
30. Ke D, Menard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, et al. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. *Clin Chem* 2000;46:324–331. [PubMed: 10702518]
31. Kim M, Kim G, Romero R, Shim SS, Kim EC, Yoon BH. Biovar diversity of *Ureaplasma urealyticum* in amniotic fluid: distribution, intrauterine inflammatory response and pregnancy outcomes. *J Perinat Med* 2003;31:146–152. [PubMed: 12747231]
32. Kim MJ, Romero R, Gervasi MT, Kim JS, Yoo W, Lee DC, et al. Widespread microbial invasion of the chorioamniotic membranes is a consequence and not a cause of intra-amniotic infection. *Lab Invest* 2009;89:924–936. [PubMed: 19506551]
33. Kobayashi K, Tajima M, Toishi S, Fujimori K, Suzuki Y, Udagawa H. Fetal growth restriction associated with measles virus infection during pregnancy. *J Perinat Med* 2005;33:67–68. [PubMed: 15841617]
34. Kusanovic JP, Romero R, Hassan SS, Gotsch F, Edwin S, Chaiworapongsa T, et al. Maternal serum soluble CD30 is increased in normal pregnancy, but decreased in preeclampsia and small for gestational age pregnancies. *J Matern Fetal Neonatal Med* 2007;20:867–878. [PubMed: 17853188]
35. Lee SE, Romero R, Park CW, Jun JK, Yoon BH. The frequency and significance of intraamniotic inflammation in patients with cervical insufficiency. *Am J Obstet Gynecol* 2008;198:633–638. [PubMed: 18342290]
36. Lepp PW, Brinig MM, Ouverney CC, Palm K, Armitage GC, Relman DA. Methanogenic Archaea and human periodontal disease. *Proc Natl Acad Sci US A* 2004;101:6176–6181.

37. Lin CC, Santolaya-Forgas J. Current concepts of fetal growth restriction: part I. Causes, classification, and pathophysiology. *Obstet Gynecol* 1998;92:1044–1055. [PubMed: 9840574]
38. Lin D, Smith MA, Champagne C, Elter J, Beck J, Offenbacher S. *Porphyromonas gingivalis* infection during pregnancy increases maternal tumor necrosis factor alpha, suppresses maternal interleukin-10, and enhances fetal growth restriction and resorption in mice. *Infect Immun* 2003;71:5156–5162. [PubMed: 12933859]
39. Maymon E, Romero R, Chaiworapongsa T, Berman S, Conoscenti G, Gomez R, et al. Amniotic fluid matrix metalloproteinase-8 in preterm labor with intact membranes. *Am J Obstet Gynecol* 2001;185:1149–1155. [PubMed: 11717649]
40. Maymon E, Romero R, Chaiworapongsa T, Kim JC, Berman S, Gomez R, et al. Value of amniotic fluid neutrophil collagenase concentrations in preterm premature rupture of membranes. *Am J Obstet Gynecol* 2001;185:1143–1148. [PubMed: 11717648]
41. Maymon E, Romero R, Pacora P, Gomez R, Athayde N, Edwin S, 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. [PubMed: 10920315]
42. Mazor-Dray E, Levy A, Schlaeffer F, Sheiner E. Maternal urinary tract infection: is it independently associated with adverse pregnancy outcome? *J Matern. Fetal Neonatal Med* 2009;22:124–128.
43. Nien JK, Yoon BH, Espinoza J, Kusanovic JP, Erez O, Soto E, et al. A rapid MMP-8 bedside test for the detection of intra-amniotic inflammation identifies patients at risk for imminent preterm delivery. *Am J Obstet Gynecol* 2006;195:1025–1030. [PubMed: 17000236]
44. Offenbacher S, Katz V, Fertik G, Collins J, Boyd D, Maynor G, et al. Periodontal infection as a possible risk factor for preterm low birth weight. *J Periodontol* 1996;67:1103–1113. [PubMed: 8910829]
45. Offenbacher S, Lieff S, Boggess KA, Murtha AP, Madianos PN, Champagne CM, et al. Maternal periodontitis and prematurity. Part I: Obstetric outcome of prematurity and growth restriction. *Ann Periodontol* 2001;6:164–174. [PubMed: 11887460]
46. Ogge G, Romero R, Chaiworapongsa T, Gervasi MT, Pacora P, Erez O, et al. Leukocytes of pregnant women with small-for-gestational age neonates have a different phenotypic and metabolic activity from those of women with preeclampsia. *J Matern Fetal Neonatal Med*. 2009;10.3109/1476050903216033
47. Oh KJ, Lee SE, Jung H, Kim G, Romero R, Yoon BH. Detection of ureaplasmas by the polymerase chain reaction in the amniotic fluid of patients with cervical insufficiency. *J Perinat Med* 2010;38:261–8. [PubMed: 20192887]
48. Ornoy A. The effects of Cytomegalic virus (CMV) infection during pregnancy on the developing human fetus. *Harefuah* 2002;141:565–8. 577. [PubMed: 12119775]
49. Pacora P, Chaiworapongsa T, Maymon E, Kim YM, Gomez R, Yoon BH, et al. Funisitis and chorionic vasculitis: The histological counterpart of the fetal inflammatory response syndrome. *J Matern Fetal Neonatal Med* 2002;11:18–25. [PubMed: 12380603]
50. Palmer C, Bik EM, DiGiulio DB, Relman DA, Brown PO. Development of the human infant intestinal microbiota. *PLoS Biol* 2007;5:e177. [PubMed: 17594176]
51. Pergam SA, Wang CC, Gardella CM, Sandison TG, Phipps WT, Hawes SE. Pregnancy complications associated with hepatitis C: data from a 2003–2005 Washington state birth cohort. *Am J Obstet Gynecol* 2008;199:38–39. [PubMed: 18486089]
52. Pollack RN, Divon MY. Intrauterine growth retardation: definition, classification, and etiology. *Clin Obstet Gynecol* 1992;35:99–107. [PubMed: 1544253]
53. Redline RW, Faye-Petersen O, Heller D, Qureshi F, Savell V, Vogler C. Amniotic infection syndrome: nosology and reproducibility of placental reaction patterns. *Pediatr Dev Pathol* 2003;6:435–448. [PubMed: 14708737]
54. Relman D, Loutit J, Schmidt T, Falkow S, Tompkins L. The agent of bacillary angiomatosis: An approach to the identification of uncultured pathogens. *N Engl J Med* 1990;323:1573–1580. [PubMed: 2233945]
55. Romero R. Prenatal Medicine: the child is the father of the man. *Prenatal and Neonatal Medicine* 1996;1:8–11.

56. Romero R. Prenatal medicine: the child is the father of the man. 1996. *J Matern Fetal Neonatal Med* 2009;22:636–639. [PubMed: 19736614]
57. Romero R, Avila C, Santhanam U, Sehgal PB. Amniotic fluid interleukin 6 in preterm labor. Association with infection. *J Clin Invest* 1990;85:1392–1400. [PubMed: 2332497]
58. Romero R, Emamian M, Quintero R, Wan M, Hobbins JC, Mazor M, 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. [PubMed: 2456013]
59. Romero R, Gonzalez R, Sepulveda W, Brandt F, Ramirez M, Sorokin Y, et al. Infection and labor. VIII. Microbial invasion of the amniotic cavity in patients with suspected cervical incompetence: prevalence and clinical significance. *Am J Obstet Gynecol* 1992;167:1086–1091. [PubMed: 1415396]
60. Romero R, Mazor M. Infection and preterm labor. *Clin Obstet Gynecol* 1988;31:553–584. [PubMed: 3066544]
61. Romero R, Mazor M, Morretti 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. [PubMed: 1301006]
62. Romero R, Nien JK, Espinoza J, Todem D, Fu W, Chung H, et al. A longitudinal study of angiogenic (placental growth factor) and anti-angiogenic (soluble endoglin and soluble vascular endothelial growth factor receptor-1) factors in normal pregnancy and patients destined to develop preeclampsia and deliver a small for gestational age neonate. *J Matern Fetal Neonatal Med* 2008;21:9–23. [PubMed: 18175241]
63. Romero R, Nores J, Mazor M, Sepulveda W, Oyarzun E, Parra M, et al. Microbial invasion of the amniotic cavity during term labor. Prevalence and clinical significance. *J Reprod Med* 1993;38:543–548. [PubMed: 8410850]
64. Romero R, Quintero R, Nores J, Avila C, Mazor M, Hanaoka S, 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. [PubMed: 1951538]
65. Romero R, Quintero R, Oyarzun E, Wu YK, Sabo V, Mazor M, et al. Intraamniotic infection and the onset of labor in preterm premature rupture of the membranes. *Am J Obstet Gynecol* 1988;159:661–666. [PubMed: 3421266]
66. Romero R, Sepulveda W, Kenney JS, Archer LE, Allison AC, Sehgal PB. Interleukin 6 determination in the detection of microbial invasion of the amniotic cavity. *Ciba Found Symp* 1992;167:205–20. discussion 220–3. [PubMed: 1425014]
67. Romero R, Shamma F, Avila C, Jimenez C, Callahan R, Nores J, 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. [PubMed: 2403156]
68. Romero R, Sirtori M, Oyarzun E, Avila C, Mazor M, Callahan R, 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. [PubMed: 2675611]
69. Romero, R.; Yoon, BH.; Goncalves, LF., et al. The clinical significance of microbial invasion of the amniotic cavity with mycoplasmas in patients with preterm PROM. *Scientific Abstr; Fortieth Annual Meeting of the Society for Gynecologic Investigation; March 31–April 3; 1993. p. S4*
70. Schiff E, Friedman SA, Baumann P, Sibai BM, Romero R. Tumor necrosis factor-alpha in pregnancies associated with preeclampsia or small-for-gestational-age newborns. *Am J Obstet Gynecol* 1994;170:1224–1229. [PubMed: 8178841]
71. Seeds JW. Impaired fetal growth: definition and clinical diagnosis. *Obstet Gynecol* 1984;64:303–310. [PubMed: 6379528]
72. Selvaggi L, Lucivero G, Iannone A, dell’Osso A, Loverro G, Antonaci S, et al. Analysis of mononuclear cell subsets in pregnancies with intrauterine growth retardation. Evidence of chronic B-lymphocyte activation. *J Perinat Med* 1983;11:213–217. [PubMed: 6604801]
73. Stagno S, Whitley RJ. Herpesvirus infections of pregnancy. Part I: Cytomegalovirus and Epstein-Barr virus infections. *N Engl J Med* 1985;313:1270–1274. [PubMed: 2997607]

74. Tsatsaris V, Goffin F, Munaut C, Brichant JF, Pignon MR, Noel A, et al. Overexpression of the soluble vascular endothelial growth factor receptor in preeclamptic patients: pathophysiological consequences. *J Clin Endocrinol Metab* 2003;88:5555–5563. [PubMed: 14602804]
75. Vaisbuch E, Hassan SS, Mazaki-Tovi S, Nhan-Chang CL, Kusanovic JP, Chaiworapongsa T, et al. Patients with an asymptomatic short cervix (≤ 15 mm) have a high rate of subclinical intra-amniotic inflammation: implications for patient counseling. *Am J Obstet Gynecol* 2010;202:433, e1–8. [PubMed: 20452483]
76. Vrachnis N, Botsis D, Iliodromiti Z. The fetus that is small for gestational age. *Ann NY Acad Sci* 2006;1092:304–9. 304–309. [PubMed: 17308155]
77. Wilson KH, Blitchington R, Frothingham R, Wilson JA. Phylogeny of the Whipple's-disease-associated bacterium. *Lancet* 1991;338:474–475. [PubMed: 1714530]
78. Xu DX, Chen YH, Wang H, Zhao L, Wang JP, Wei W. Tumor necrosis factor alpha partially contributes to lipopolysaccharide-induced intra-uterine fetal growth restriction and skeletal development retardation in mice. *Toxicol Lett* 2006;163:20–29. [PubMed: 16263228]
79. Yoon BH, Romero R, Kim M, Kim EC, Kim T, Park JS, et al. Clinical implications of detection of *Ureaplasma urealyticum* in the amniotic cavity with the polymerase chain reaction. *Am J Obstet Gynecol* 2000;183:1130–1137. [PubMed: 11084554]
80. Yoon BH, Romero R, Lim JH, Shim SS, Hong JS, Shim JY, et al. The clinical significance of detecting *Ureaplasma urealyticum* by the polymerase chain reaction in the amniotic fluid of patients with preterm labor. *Am J Obstet Gynecol* 2003;189:919–924. [PubMed: 14586326]
81. Yoon BH, Romero R, Moon JB, Shim SS, Kim M, Kim G, et al. Clinical significance of intra-amniotic inflammation in patients with preterm labor and intact membranes. *Am J Obstet Gynecol* 2001;185:1130–1136. [PubMed: 11717646]
82. Zariffard MR, Saifuddin M, Sha BE, Spear GT. Detection of bacterial vaginosis-related organisms by real-time PCR for *Lactobacilli*, *Gardnerella vaginalis* and *Mycoplasma hominis*. *FEMS Immunol Med Microbiol* 2002;34:277–281. [PubMed: 12443827]

Table 1

PCR assays used in this study.

Approximate taxonomic level	End-point PCR taxonomic specificity	Lower detection limit (gene copies per μ L)	Oligonucleotide name	Use	Sequence (5' $\rightarrow$ 3')	Gene target	Reference
domain	<i>Bacteria</i>	100	Bact-8FM	FP	AGAGTTTGATCMTGGCTCAG	16S rDNA	[50]
			Bact-806R	RP	GGACTACGAGGTATCTAAT		[77]
genus	<i>Ureaplasma</i>	10	Urease185F	FP	GCTGCTGACGTTTCAAGAAAG	urease gene	[17]
			Urease756R	RP	CTCCTGGTTCAAAACGAATAGC		[17]
genus	<i>Fusobacterium</i>	100	Fuso-422F	FP	CGGAA TGTAAAAGTGCTTTC	16S rDNA	[17]
			Fuso-710R	RP	CCCATCGGCATTCCTAC		[17]
genus	<i>Sneathia/Leptorrichia</i>	10	SsLa-140F	FP	TAGACTGGGATAACAGAGG	16S rDNA	[17]
			SsLa-406R	RP	AGTCCTAAAACCTTCTTCACAC		[17]
species	<i>Streptococcus agalactiae</i>	10	Sag059F	FP	TTTCAACAGCTGTATTAGAAGTA	<i>cfb</i>	[30]
			Sag190R	RP	GTTCCCTGAACATTATCTTTGAT		[30]
species	<i>Mycoplasma hominis</i>	10	Mh-148F	FP	CAATGGCTAATGCCGGGATACG	16S rDNA	mod. from [82]
			Mh-463R	RP	GGTACCGTCAGTCTGCAATC		mod. from [82]
genus	<i>Candida</i>	10	Cand-ITS2-42F	FP	GGGTTTGCTTGAAAGACGGTA	ITS2	[17]
			Cand-ITS2-125R	RP	TTGAAGATATACGTGTRGACGTTA		[17]
domain	<i>Bacteria</i>	15 to 1e8	Bact-8FM	FP	AGAGTTTGATCMTGGCTCAG	16S rDNA	[50]
			Bact-338K*	Probe	CCAKACTCCTACGGGAGGCAGCAG		[50]
domain	<i>Archaea</i>	100 to 1e8	Bact-515R	RP	TTACCGCGGCKGCTGCAC	16S rDNA	[6]
			Arch333F	FP	TCCAGGCCCTACGGG		[36]
domain	<i>Archaea</i>	100 to 1e8	Univ-515F*	Probe	GTGCCAGCMGCCGCGGTAA	16S rDNA	[6]
			Arch958R	RP	YCCGGCGTTGAMTCCAATT		[14]

FP = forward primer, RP = reverse primer, Probe = TaqMan probe

* Conjugated on the 5' end to 6-carboxyfluorescein, and on the 3' end to 6-carboxy-tetramethylrhodamine

Table 2

Demographic and clinical characteristics of the study population.

Variable	Patients with SGA (n=52)
Maternal age (years)	30 (23–34)
Ethnicity	
African American	26 (50)
Caucasian	20 (28.5)
Hispanic	6 (11.5)
Pre-pregnancy BMI (kg/m ²)	22.3 (20.3–27.1)
Gestational age at amniocentesis (weeks)	36.9 (34.5–39)
Gestational age at delivery (weeks)	37.5 (34.6–39)
Birthweight (grams)	2245 (1690–2587)

Data presented as median (interquartile range) or number (percentage)

BMI – body mass index

Table 3

Clinical characteristics of the three cases with a positive amniotic fluid PCR

Patient	Microbe	GA at AC (weeks)	AF WBC (cell/mL)	AF glucose (mg/dl)	AF IL-6 (ng/mL)	AF MMP-8 (ng/mm ³)	Placental pathology	GA at delivery (weeks)	Birthweight (grams)
#1	<i>Candida</i> species	32.3	7	41	0.64	3.81	No inflammation	32.3	1370
#2	<i>Staphylococcus epidermidis</i>	35.3	4	44	0.41	1.51	No inflammation	35.3	1730
#3	<i>Streptococcus agalactiae</i>	39.9	4	50	44.81	32.65	Alterations villous tree	39.9	2670

GA - gestational age; AC- amniocentesis; AF – amniotic fluid; WBC – white blood cell;