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Fetal Inflammatory Response in Women with Proteomic Biomarkers Characteristic of Intra-Amniotic Inflammation and Preterm Birth

Catalin S. Buhimschi, M.D.¹, Antonette T. Dulay, M.D.¹, Sonya Abdel-Razek, M.D.¹, Guomao Zhao, B.S.¹, Sarah Lee, M.D.¹, Eric J. Hodgson, M.D.¹, Vineet Bhandari, M.D.², and Irina A. Buhimschi, M.D.¹

¹Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, CT 06520

²Department of Pediatrics, Division of Perinatal Medicine, Yale University School of Medicine, New Haven, CT 06520

Abstract

Objective—To determine the relationship between presence of amniotic fluid (AF) biomarkers characteristic of inflammation (defensins 2 and 1, calgranulins C and A) and fetal inflammatory status at birth.

Design—Prospective observational cohort.

Setting—Tertiary referral University hospital

Population—132 consecutive mothers (gestational age, median [interquartile range]: 29.6 [24.1–33.6] weeks), who had a clinically indicated amniocentesis to rule-out infection and their newborns.

Methods—Intra-amniotic inflammation was diagnosed by mass spectrometry SELDI-TOF. The AF proteomic fingerprint [Mass Restricted (MR) score] ranges from 0–4 (none to all biomarkers present). The intensity of intra-amniotic inflammation was graded based on the number of proteomic biomarkers: MR score 0: “no” inflammation; MR score 1–2: “minimal” inflammation; MR score 3–4: “severe” inflammation. At birth, cord blood was obtained for all cases. Severity of histological chorioamnionitis (HCA) and early onset neonatal sepsis (EONS) was based on established histological and hematological criteria. Interleukin-6 (IL-6) levels were measured by sensitive immunoassays. The cord blood-to-AF IL-6 ratio was used as an indicator of the differential inflammatory response in the fetal versus the AF compartment.

Main Outcome Measures—to relate proteomic biomarkers of intra-amniotic infection to cord blood IL-6 and to use the latter as the primary marker of fetal inflammatory response.

Address and correspondence to: Dr. Catalin S. Buhimschi, Director Perinatal Research, Yale University, Department of Obstetrics, Gynecology & Reprod. Sci., 333 Cedar Street, LLCI 804, New Haven, CT 06520, Telephone: 203-785-6164, Fax: 203-737-2327, catalin.buhimschi@yale.edu.

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Contribution to Authorship: CSB and IAB designed the study, collected, analyzed, and interpreted the data, and drafted the manuscript. VB collected, analyzed, and interpreted the neonatal data. ATD and GZ conducted ELISA assays and collected part of the proteomics data. CSB, ATD, SAR, SL, EJH recruited patients, collected biological specimens prospectively, and reviewed the data analysis. All the co-authors participated with aspects of study design, critical interpretation of the data, contributed to writing of the paper and have reviewed and approved the final version.

Results—Women with intra-amniotic inflammation delivered at an earlier gestational age (ANOVA, $P<0.001$) and had higher AF IL-6 levels ($P<0.001$). At birth, neonates of women with “severe” intra-amniotic inflammation had higher cord blood IL-6 levels ($P=0.002$) and a higher frequency of EONS ($P=0.002$). EONS was characterized by significantly elevated cord blood IL-6 levels ($P<0.001$). Out of the 39 neonates delivered by mothers with “minimal” intra-amniotic inflammation, 15 (39%) had umbilical cord blood IL-6 levels above the mean for the group, and 2 neonates had confirmed sepsis. The severity of the neutrophilic infiltrate in the chorionic plate ($P<0.001$), choriodecidua ($P=0.002$), umbilical cord ($P<0.001$), but not amnion ($P>0.05$) was an independent predictor of the cord blood-to-AF IL-6 ratio. Relationships were maintained following correction for gestational age, birthweight, amniocentesis-to delivery interval, cesarean delivery, status of the membranes, race, MR score, antibiotics and steroid exposure.

Conclusion—We provide evidence that presence in the AF of proteomic biomarkers characteristic of inflammation is associated with an increased inflammatory status of the fetus at birth. Neonates mount an increased inflammatory status and have positive blood cultures even in the context of “minimal” intra-amniotic inflammation.

Keywords

Proteomics; biomarkers; amniotic fluid; inflammation; defensin; calgranulin; umbilical cord blood; interleukin-6

Introduction

Premature birth (PTB) remains a significant public health problem worldwide. In 2006, the prevalence of PTB rose to an unprecedented 12.8%.¹ A persistent theme of modern perinatology is that inflammatory intrauterine processes adversely affect the fetus prior to birth.^{2,3,4,5} This notion is based on studies demonstrating that complications of immature organ systems (respiratory, gastrointestinal, immune, central nervous system), which are characteristic of early onset neonatal sepsis (EONS), are at least partially the result of a fetal inflammatory response *in utero* and one of the primary causes of the high neonatal morbidity and mortality attendant PTB.^{5,6,7}

Fetal inflammation represents a highly orchestrated process designed to combat infection and tissue injury.^{8,9} However, while chemokines and pro-inflammatory cytokines are essential for fetal defense against infection, excessive influx of activated leukocytes coupled with exaggerated production of pro-inflammatory mediators can have deleterious consequences for the host.¹⁰ One such pro-inflammatory cytokine, interleukin-6 (IL-6), is traditionally considered an activator of acute phase responses and of lymphocytes.¹¹ Previous studies have shown that elevated fetal IL-6 levels at birth are a risk factor for sepsis-induced disseminated intravascular coagulation, pneumonia and cerebral palsy.^{12,13,14,15} Therefore, IL-6 is postulated to be an inflammatory marker of EONS, particularly in the preterm neonate.^{16,17}

The most recent advances in proteomics have generated novel research and diagnostic applications that have only begun to impact on the diagnosis of intra-amniotic inflammation.^{18,19,20,21} However, development of targeted therapies aimed to change neonatal outcome is impossible without a better understanding of the fetal inflammatory response to intra-amniotic infection/inflammation. We have previously shown that proteomic mapping of the amniotic fluid (AF) reveals a proteomic profile [Mass Restricted (MR) score], that is highly characteristic of intra-amniotic inflammation.^{18,19} Recently, we have demonstrated that presence in the AF of 4 inflammatory proteomic biomarkers (neutrophil defensins 2 and 1, calgranulins C and A) is highly predictive of histological chorioamnionitis (HCA), funisitis and EONS.^{22,23} Given our results we have concluded that

proteomic analysis of the AF provides an opportunity for recognition of HCA *in-utero* and that this methodology may in the future identify candidates for antenatal therapeutic interventions.²³ The fetal inflammatory response in women displaying a proteomic fingerprint characteristic of inflammation remains unknown. The degree of placental HCA is an important factor in modulating cytokine levels in the fetuses of women whose pregnancies are complicated by intra-amniotic inflammation.¹⁰ Therefore, in this study, we sought to determine the relationship between presence in the AF of biomarkers characteristic of inflammation, HCA and cord blood IL-6, and to use the latter as the primary marker of fetal inflammatory status at birth.

Methods

Study population and research design

We studied prospectively 132 consecutive preterm singleton neonates born to mothers who had an amniocentesis to rule out infection. Women were recruited following admission to Labor and Birth or to the High Risk antepartum units at Yale New Haven Hospital (YNHH). Amniocentesis was indicated independent of our study protocol. The Human Investigation Committee of Yale University approved our study.

Amniotic fluid was collected by ultrasound-guided amniocentesis for all the participants and each woman was followed prospectively to the point of delivery. Inclusion criteria included: a singleton fetus, gestational age ≥ 33.1 weeks, symptoms of preterm labor, preterm premature rupture of membranes (PPROM), advanced cervical dilatation (>3 cm), or uterine contractions intractable to tocolysis. Women with anhydramnios, human immunodeficiency or hepatitis viral infections were not enrolled. Gestational age was determined based on last menstrual period and ultrasound information obtained prior to 20 weeks of gestation.²⁴ Preterm labor was defined as the presence of regular uterine contractions and documented cervical effacement and/or dilatation in patients <37 weeks gestational age. The diagnosis of PPRM was confirmed by vaginal AF “pooling”, “nitrazine”, “ferning” or by an amniocentesis-dye positive test. In the absence of infection PPRM was managed expectantly, and no digital exams were permitted. Patients received corticosteroids for lung maturity if <32 weeks gestational age and antibiotic therapy as clinically indicated.²⁵ The neonatology resuscitation team was present at the time of delivery for all cases.

Laboratory tests performed for the purpose of diagnosing infection/inflammation were: glucose, lactate dehydrogenase (LDH), Gram stain and white blood cell (WBC) count. For clinical management, an AF glucose cut-off of ≥ 15 mg/dL and LDH levels ≥ 419 U/L were considered suggestive of intra-amniotic infection.^{26,27} Amniotic fluid not used for clinical purposes was spun at $3000 \times g$ at 4°C for 20 min., aliquoted and immediately stored at -80°C until IL-6 levels were measured by a specific and sensitive immunoassay.

Microbiological analysis of AFs by culture

Amniotic fluid was retrieved using sterile techniques and examined immediately for the presence of microorganisms using the traditional culturing method. Briefly, AF was centrifuged and the sediment re-suspended in 0.25 ml of supernatant. The concentrated AF was cultured for anaerobic bacteria, *Ureaplasma* and *Mycoplasma spp* using laboratory media such as Chocolate, Martin Lewis, MacConkey, Azido benzoic acid, Thioglycollate, Bacteroides Bile Esculin/Laked Blood Kanamycin Vancomycin (BBE/LKV) and Columbia CNA agar, *Ureaplasma* broth and agar. In addition the AF was cultured in an anaerobic chamber (Forma Anaerobic System, Thermo Electron Co, Waltham, MA) at 37°C . The results of the microbiological tests were available for case management and were reported as final after 5 days of culturing. Presumptive bacterial identification was based on standard

microbiological criteria of colonial morphology, medium reaction, Gram stain and the use of a VITEK 2 automated card system (bioMérieux, Hazelwood, MO, <http://www.biomerieux-usa.com>) for bacterial identification based on biochemical tests and antibiotic susceptibility.

MR score

Fresh AF was used to generate the MR score. The method for generation of the MR score has been previously described.¹⁸ The MR score ranges from 0 to 4, depending upon the presence or absence of each of the 4 protein biomarkers.¹⁸ A value of 1 was assigned if a biomarker peak was present and 0 if absent. Based on our previous results, we stratified the study population based on the “severity” of inflammation (MR 0: “no” inflammation; MR 1-2: “minimal” inflammation; MR 3-4: “severe” inflammation).¹⁹ All SELDI assays and scorings of the AF samples were performed by one investigator (IAB) without knowledge of the maternal clinical outcome, the results of the placental histological examination, AF and umbilical cord IL-6 levels and EONS.

Umbilical cord blood

Umbilical cord blood was obtained by aseptic puncture of the clamped umbilical vein at the time of delivery. Immediately following collection, the cord blood was centrifuged at 1000g for 15 minutes. Serum was aliquoted in sterile polypropylene tubes and stored at -80°C until IL-6 levels were examined.

Immunoassays for the IL-6

Enzyme-Linked ImmunoSorbent Assay for human IL-6 (Pierce-Endogen, Rockford, IL) was performed in duplicate according to manufacturers' instructions by investigators unaware of the AF and umbilical cord blood sample origin. The minimal detectable concentration was 1-pg/mL and the inter- and intra-assay coefficients of variation <10%.

Histological evaluation of the placenta and diagnosis of inflammation

In all 132 cases, hematoxylin and eosin- stained sections of extraplacental membranes (amnion and chorio-decidua), chorionic plate, chorio-decidua and umbilical cord were examined systematically for inflammation. Three histological stages of chorioamnionitis²⁸ (stage I: intervillitis, stage II: chorionic inflammation, and stage III: full-thickness inflammation of both chorion and amnion) were complemented by a previously described histological grading system that includes 4 grades of inflammation of the amnion, chorio-decidua and umbilical cord.²⁹

Evaluation of EONS

Neonatal hematological indices and sepsis categorization were assessed from blood specimens and cultures obtained within 2 hours from the time of birth by an investigator (VB) unaware of the results of the umbilical cord IL-6 levels, proteomic profiling of the AF or histological evaluation of the placenta.^{19,22} All 132 neonates were admitted to the Yale Newborn Special Care Unit. Neonatal sepsis was defined as the presence of confirmed or suspected sepsis at 72 hours after birth. Confirmed sepsis represented the presence of a positive blood culture. A diagnosis of EONS was based on clinical symptoms corroborated with hematological laboratory results.^{30,31} The following hematological criteria were used as indicators of EONS: 1). absolute neutrophil count of < 7500 or $> 14,500$ cells/mm³; 2). Absolute band count > 1500 cells/mm³; 3). Immature/total neutrophil (I:T) ratio > 0.16 ; 4). Platelet count $< 150,000$ cells/mm³.³² Sepsis was suspected in the presence of 2 hematological criteria in the absence of a positive blood culture. Early-onset neonatal sepsis was dichotomized into present (when sepsis was either confirmed or suspected) or absent.

All neonates with confirmed or suspected sepsis received antibiotic therapy, per institutional protocol.

The results of the AF IL-6 levels, histological examination of the placenta and presence or absence of EONS for 79 women were previously used in studies aimed to explore the maternal and fetal response to intrauterine infection/inflammation.^{19,22,23,54} The relationship between the MR score and levels of cord blood IL-6, the results of the AF and cord blood IL-6 levels, histological examination of the placenta and specific hematological indices suggestive of EONS for 53 subjects included in the current submission have not been reported before. The relationship between cord blood IL-6 levels, EONS and absolute neutrophil count as well as the results of the relationship between cord blood IL-6 to AF IL-6 ratio and histological examination of the placenta for all 132 cases included in the present analysis are novel.

Statistical analysis

Statistical analyses were performed with Sigma Stat, version 2.03 (SPSS Inc., Chicago, IL) and MedCalc (Broekstraat, Belgium) statistical softwares. Normality testing was performed using the Kolmogorov-Smirnov test. Data were compared with one-way ANOVA followed by Dunnett's tests (parametric) or Kruskal-Wallis on ranks followed by Dunn's tests (non-parametric), to adjust for multiple comparisons as appropriate. The IL-6 concentrations were presented as arithmetic means with inter-quartile range. Statistical analysis was completed before (Kruskal-Wallis ANOVA) or after (One-Way ANOVA) logarithmic transformation of data. Pearson correlations were used to measure co-linearity between the selected independent variables as well as other relevant relationships between dependent and independent variables. Comparisons between proportions were done with Chi-square tests. Stepwise multivariable regression analysis was used to determine concurrent relationships between variables and to correct for possible influences of gestational age and birthweight. A *p* value of <0.05 was considered significant throughout the analysis.

Results

In Table 1 we present the clinical characteristics of the study subjects at the time of enrollment and their pregnancy outcomes. In this cohort, women of African American descent were more frequently diagnosed with “severe” (MR score 3-4) intra-amniotic inflammation. Women with MR scores 3-4 were of lower gestational age and more often had symptoms of clinical chorioamnionitis compared with the group of women with “no” (MR score 0) or “minimal” inflammation (MR score 1-2). Similarly, women with “severe” inflammation had a shorter amniocentesis to delivery interval, and consequently, gave birth at an earlier gestational age compared to the other 2 groups. At birth, neonates of mothers with “severe” inflammation had lower birthweight and Apgar scores. Women with “severe” inflammation of the AF more often underwent Cesarean section delivery.

We present the results of the AF analysis in Table 2. Women with “severe” intra-amniotic inflammation had lower AF glucose, higher LDH and WBC counts compared to the women with “no” or “minimal” inflammation. This finding was consistent with a higher prevalence of a positive Gram stain and a positive microbial culture of the AF in the group of women with “severe” inflammation. There was a significant inverse correlation between gestational age at enrollment and intra-amniotic infection as determined by a positive microbial culture ($R = -0.407$, $P < 0.001$). Pathological examination of the placenta demonstrated that “severe” inflammation of the AF was associated with a higher incidence and grades/stages of amniochorion, chorionic plate, and umbilical cord histological inflammation (Table 3).

We found that neonates delivered by mothers with MR scores 3-4 were more often anemic and had leukocytosis (Table 4). These neonates were lymphopenic, had bandemia and a higher I:T neutrophil ratio. Overall, 6 neonates had confirmed sepsis documented by a positive blood culture. Two of the neonates were delivered by mothers with negative AF cultures but had MR scores of 2. All mothers with MR scores 3-4 who delivered neonates with proven sepsis (n=4) had positive AF cultures [*Escherichia coli* (n=3); mixed flora: *Staphylococcus aureus* and *Corynebacterium* (n=1)]. Neonates delivered by mothers with AF MR score 3-4, more often had a hematological work-up suggestive of EONS compared to neonates delivered by mothers with MR score 0 or MR score 1-2. This relationship persisted after adjusting for gestational age at birth. The overall prevalence of EONS in our cohort was 26% (34/132) of which 56% of instances occurred in the context of PPROM. Interestingly, 5 of the 6 cases with proven sepsis documented by positive blood cultures occurred in the context of intact membranes.

The AF IL-6 levels of women with MR scores 3-4 were significantly higher compared to the level of women with “no” or “minimal” inflammation (median [interquartile range], **MR score 0:** 0.3 [0.1 - 0.7] vs. **MR score 1-2:** 1.3 [0.5 - 3.9] vs. **MR score 3-4:** 22.1 [8.5 - 58.2] ng/ml, Kruskal Wallis ANOVA, $P < 0.001$) (Figure 1A). Neonates delivered by mothers with MR scores 3-4 had higher umbilical cord IL-6, compared to neonates delivered by mothers with “no” or “minimal” inflammation (**MR score 0:** 5.9 [5.5 - 8.6] vs. **MR score 1-2:** 6.9 [5.4 - 97.6] vs. **MR score 3-4:** 28.9 [8.5 - 161.3] pg/ml, $P < 0.001$) (Figure 1B).

Out of the 39 neonates delivered by mothers with MR score 1-2, 15 (39%) had umbilical cord blood IL-6 levels above the mean for the group. Two neonates had confirmed sepsis. The two neonates with positive blood cultures delivered by mothers with MR score 1-2 had umbilical cord blood IL-6 levels considerably above the mean for their group (IL-6 absolute values: 49,834 and 1,533 pg/ml, respectively). This occurred in the context of low AF IL-6 levels (IL-6 absolute values: 0.134 and 3.9 ng/mL). Both neonates had an amniocentesis to delivery interval less than 4 hours. Evaluation of white matter disorders showed that both neonates developed periventricular leucomalacia. In addition, the neuro-developmental outcome assessment at 18 months of age showed that both children had cerebral palsy. Two of the 4 neonates delivered by mothers with MR score 3-4 who had documented positive blood cultures expired soon after delivery (umbilical cord IL-6 absolute levels: 19,817 and 77.2 pg/ml, respectively). The other 2 neonates did not develop periventricular leucomalacia (umbilical cord IL-6 absolute levels: 90.2 and 460.6 pg/ml, respectively). The results of the neuro-developmental outcome at 18 months of age for the first infant showed mild gross motor delay for which the infant is currently getting therapy. The results of the neuro-developmental outcome for the second infant were not available at the time of this report. The amniocentesis to delivery interval was less than 10 hours for all 4 neonates.

Of all the hematological indices that comprise EONS criteria, cord blood IL-6 correlated best with the I:T neutrophil ratio ($R = 0.501$, $P < 0.001$) and the absolute band count ($R = 0.330$, $P < 0.001$). Cord blood IL-6 correlated inversely with the degree of anemia as measured by neonatal hemoglobin concentration ($R = -0.337$, $P < 0.001$) and hematocrit ($R = -0.300$, $P < 0.001$). Neonates diagnosed with EONS had significantly higher cord blood IL-6 levels (**No EONS:** 7.9 [5.8-37.2] vs. **Yes EONS:** 83.7 [17.3 - 361.6] pg/ml, $P < 0.001$) (Figure 2A). At birth, there was a significant inverse correlation between the absolute neutrophil count and cord blood IL-6 levels, which was maintained following correction for gestational age and birthweight ($R = -0.439$, $P < 0.001$) (Figure 2B).

Overall, there was a significant correlation between the AF and cord blood IL-6 levels ($R = 0.415$, $P < 0.001$) as shown in Figure 3. However, in 94% of the cases, (124/132) the absolute value AF IL-6 concentration was higher than that measured in the umbilical cord blood.

When separated by severity of intra-amniotic inflammation as depicted by the MR score, a disproportionate increase in AF IL-6 level compared to the fetal compartment was seen in cases complicated by severe AF inflammation. This led to an apparent decrease in the proportion of cord blood IL-6 relative to that in AF (cord blood-to-AF IL-6 ratio: **MR score 0:** 2.8% [1.0-5.2] vs. **MR score 1-2:** 1.4% [0.4-6.4] vs. **MR score 3-4:** 0.2% [0.1-1.2], $P<0.001$).

Because of the variability in the fetal response to the severity of intra-amniotic inflammation, we searched for factors that may impact on the degree to which AF inflammation leads to an increase in cord blood IL-6 levels. Multivariate stepwise analysis was conducted, with the cord blood-to-AF IL-6 ratio as the dependent variable and parameters such as HCA and funisitis, gestational age at delivery, birthweight, amniocentesis-to delivery interval, cesarean delivery, status of the membranes, race, MR score, antibiotics and steroid exposure as independent variables. We found out that the intensity of intra-amniotic inflammation as determined by the MR score in conjunction with the degree of both HCA and funisitis predicted the cord blood-to-AF IL-6 ratio (F ratio: 16.6, $R=0.540$, $P<0.001$). This suggests that the degree of placental inflammation is an important factor in modulating cytokine levels in the fetuses of women whose pregnancies are complicated by intra-amniotic inflammation. To further explore this possibility, the relationship between the extent of the inflammatory infiltrate in the amnion, chorio-decidua, chorionic plate blood vessels (stages of histological chorioamnionitis) and umbilical cord (grades of funisitis) was examined, yielding a significant increase in cord blood-to-AF IL-6 ratio in the context of stages II and III neutrophil infiltration of the chorionic plate (Figure 4A, ANOVA, $P<0.001$), grades 3-4 choriodecidualitis (Figure 4B, $P=0.002$) and grades 2-4 funisitis (Figure 4C, $P<0.001$). Conversely, the cord blood-to-AF IL-6 ratio was not influenced by the degree of amnionitis ($P>0.05$).

Discussion

In this study we sought to determine the relationship between the presence of 4 AF proteomic biomarkers (human neutrophil defensins 2 and 1, calgranulins C and A) characteristic of intra-amniotic inflammation and the likelihood and severity of the fetal inflammatory response. We determined that although there is a direct correlation between the AF and cord blood IL-6 levels, the intensity of the fetal inflammatory response is not always directly correlated with the severity of intra-amniotic inflammation. This conclusion is based on our analysis showing that only neonates delivered in the context of “severe” intra-amniotic inflammation have significantly elevated cord blood IL-6 levels, but not those delivered by women with “no” or “minimal” inflammation. Similar to other studies, we have shown that neonates with hematological indices suggestive of EONS have higher umbilical cord IL-6 concentrations, which correlates best with the I:T ratio.^{14,30,33,34,35,36,37} We further provide evidence that a significant inverse correlation between the absolute neutrophil count and umbilical cord IL-6 levels exists. Lastly, we demonstrated that the cord blood-to-AF IL-6 ratio (an indicator of the differential inflammatory response in the fetal versus the AF compartment) is highly correlated with the MR score and highly dependent on the severity of histological inflammation of the chorionic plate, chorio-decidua and funisitis, but not that of the amnion.

In prior studies we described that the components of a given MR score have an order in appearance (first defensins followed by calgranulins).¹⁹ Our approach allowed us to objectively stratify the intensity of intra-amniotic inflammation and simultaneously assess the degree of fetal inflammation. In the present study, we reveal that the fetal inflammatory status in women with MR score 1-2 (defensin stage) is frequently minimal. Because the strength of the fetal inflammatory response is highly associated with the degree of placental

HCA we can conclude that at the stage of “minimal” AF inflammation the fetus is most commonly protected. However, detection of a positive blood culture and umbilical cord IL-6 levels well above the mean in several neonates delivered by women with MR scores 1-2, show that this protection is not always complete. Several explanations may be possible.

Experimental data indicate that the inflammatory process of the placenta and its membranes is generally regarded as a continuum.³⁸ It is believed that organisms first colonize and spread along the chorio-amniotic structure. Thus, the initial inflammatory response is thought to be maternal in its origin.^{39,40} The next step involves infiltration of the membranous chorio-amnion followed by widespread inflammation within the AF cavity and development of necrotizing chorioamnionitis. This view holds true in light of studies which genotyped the amniochorion neutrophils and demonstrated that these cells matched the maternal genotype in all cases.⁴¹ Lastly, placental lesions related to alterations in the uteroplacental vascular pathology of the chorionic plate (chorionitis, vasculitis, and moderate to severe villous edema) are regarded as an advanced stage of placental inflammation with mixed maternal and fetal origin.^{41,42,43,44} That the above is consistent with experimental data in animals, it stands to reason that the hematogenous infection of the human fetus and mounting of a fetal inflammatory response, in the absence of concurrent AF colonization and inflammation, is possible.^{45,46,47,48}

Another possible explanation for the differential maternal and fetal inflammatory response may rest with our genetic makeup.⁴⁹ Deciphering the human genome has provided partial explanation for the extent to which the genetic make-up differs from person to person. The working model for inflammation induced PTB suggests that mothers and fetuses have a genetic predisposition toward either a hyper- or a hypo-reactive immune system.⁵⁰ The implications of this dissimilarity make a strong case for the variant maternal and fetal inflammatory response observed in this study and may provide clarification for our finding that at birth, several fetuses had positive blood cultures and an overwhelming inflammatory response in the context of “minimal” AF inflammation.

The results of the microbiological studies pointed out that in approximately 30% of the cases the bacterial cultures were negative, although proteomic analysis of the AF implied “severe” intra-amniotic inflammation. This should not come as a surprise since factors other than infection (i.e bleeding) may be responsible for triggering intra-amniotic inflammation.⁵¹ Lastly, although microbial cultures of the AF are highly regarded as the “gold standard” for detection of intra-amniotic infection, “difficult-to-cultivate” or “uncultivated” bacteria may not be found if the clinicians are relying on culture conditions alone.⁵² The recent development in DNA-based culture-independent technology (16S rRNA) enables detection of previously unidentified uncultivated or difficult-to-cultivate bacterial species and may replace in the future the microbiological cultures to allow for a highly accurate diagnosis of intra-amniotic infection.⁵²

The presence of molecular pattern recognition receptors, notably Toll-like receptors (TLR) 2 and 4 on human trophoblast, and the existence of the S100A12/ENRAGE in the placental vascular tissue proves that the placenta is prepared to respond to a bacterial attack through an outpouring of inflammatory cytokines.^{53,54} Our results imply that in the initial phase of the inflammatory response, the placenta plays an important protective role for the fetus. Yet, in a more advanced stage of inflammation, the placenta becomes an important regulator of the differential immune response observed in the AF versus the fetal compartment. Though the activity of Tolllike receptors seems to exhibit genetic control, the literature continues to be conflicted in regard to the existence of placental-fetal or placental-AF cytokine trafficking.^{55,56,57} Although it has been demonstrated that radiolabeled IL-6 crosses the rat fetus in mid gestation, studies showed that in vitro there is either minimal or no transfer of

proinflammatory cytokines across the human placenta.^{57,58} If this hypothesis is true our data seem to suggest that this transfer is overwhelmingly directed toward the AF and not the fetal compartment.

Compelling evidence suggests that intra-amniotic inflammation and HCA are risk factors for perinatal injury that includes white matter injury and development of periventricular leukomalacia.^{2,59,60} Because a significant number of fetuses exposed to intra-amniotic inflammation and elevated AF cytokine levels do not develop cerebral palsy it appears reasonable to assume that along with genetic predisposition, several other factors may be involved in fetal defense. Still, the decision to actively intervene and deliver a fetus subjected to a hostile intra-uterine inflammatory process should take into account the risks and benefits for both the mother and for its very preterm neonate.

A major clinical concern remains early and accurate recognition of the fetuses with positive blood cultures who have already mounted a robust inflammatory response in the context of “minimal” intra-amniotic inflammation. Unfortunately, neither the biophysical profile nor evaluation of the fetal heart rate reliably provide early identification and therefore, need for immediate delivery of a sick fetus.^{61,62,63} Yet, ultrasound evaluation of the fetal adrenal gland volume has been recently proposed as a useful clinical marker for early recognition of an increased fetal inflammatory status *in utero*.⁶⁴ A rapid intervention based on this marker (i.e delivery versus anti-inflammatory treatment) may prove beneficial in the future for prevention of cerebral palsy. Until its clinical usefulness is proven in large randomized studies, or other clinically relevant genetic markers identified, for women with “severe” intra-amniotic inflammation, a reasonable approach may involve removing the fetus from a septic environment if birth in the very short term appears most likely.²⁵

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Figure 1. Relationship between presence in the amniotic fluid of biomarkers characteristic of intra-amniotic inflammation and amniotic fluid (AF) and umbilical cord blood (CB) interleukin-6 (IL-6) levels

(A). Amniotic fluid interleukin-6 (IL-6) levels in women with and without intra-amniotic inflammation. (B). Umbilical cord blood IL-6 levels in neonates delivered by women with and without intra-amniotic inflammation. The MR score ranges from 0 to 4. MR score 0 (zero) indicates “no” inflammation; MR score 1-2 indicates “minimal” inflammation; MR score 3-4 indicates “severe” inflammation. Data presented in logarithmic format. (pg=picograms; mL=milliliters)

Figure 2. Relationship between the umbilical cord blood (CB) interleukin-6 (IL-6) levels, early onset neonatal sepsis (EONS) and absolute neutrophil count
 (A). Umbilical cord blood IL-6 levels in neonates diagnosed with or without EONS. (B). Distribution of cord blood interleukin-6 levels on the Y axis in relationship to the absolute neutrophil count on the X axis. Data presented in logarithmic format. (pg=picograms; mL=milliliters, EONS=early onset neonatal sepsis)

Figure 3. Relationship between the amniotic fluid (AF) and umbilical cord (CB) blood interleukin-6 (IL-6) levels
 Distribution of the AF (Y axis) in relationship to the cord blood IL-6 levels on the X axis.
 Data presented in logarithmic format. (pg=picograms; mL=milliliters)

Figure 4. Relationship between the umbilical cord blood (CB) interleukin-6 (IL-6)/amniotic fluid (AF) interleukin-6 ratio and histological inflammation of the placenta

(A). CB IL-6/AF IL-6 ratio in women with stages I - III histological inflammation of the chorionic plate; (B). CB IL-6/AF IL-6 ratio in women with grades 0-4 choriodecidualitis; (C). CB IL-6/AF IL-6 ratio in women with grades 0-4 funistis. Data for the CB and AF IL-6 levels are presented in logarithmic format.

Table 1
Demographic and clinical characteristics of women and their newborns

Variable	MR SCORE			P value
	MR 0 n=26	MR 1-2 n=39	MR 3-4 n=67	
Maternal characteristics at enrollment and hospital course				
Age, years ‡	28 [24 - 32]	29 [22 - 32]	28 [22 - 36]	0.737
Gravidity ‡	2 [2 - 3]	2 [1 - 4]	2 [2 - 5]	0.411
Parity ‡	1 [0 - 1]	0 [0 - 1]	1 [0 - 2]	0.269
Race §				
Caucasian	14 (55)	26 (66)	15 (23)	< 0.001
African-American	4 (15)	8 (21)	34 (51)	< 0.001
Hispanic	4 (15)	5 (13)	13 (19)	0.060
Other	4 (15)	0 (0)	5 (7)	0.052
Gestational age, weeks ‡	30.8 [29.5 - 32.4]	29.6 [25.7 - 32.1]	27.4 [24.7 - 30.1]	< 0.001
Ruptured membranes §	21 (81)	23 (59)	38 (57)	0.089
Uterine contractions §	10 (63)	16 (70)	33 (49)	0.553
Clinical chorioamnionitis §	0 (0)	0 (0)	11 (16)	0.003
History of preterm birth §	10 (63)	7 (18)	20 (30)	0.176
Cervical dilatation ‡	0 [0 - 2]	1 [0 - 3]	2 [0 - 4]	0.101
Steroid exposure during pregnancy §	22 (85)	34 (87)	61 (91)	0.250
Prenatal antibiotic treatment §	23 (88)	32 (82)	57 (85)	0.777
Outcome characteristics				
Amniocentesis-to-delivery, hours ‡	53 [16 - 144]	46 [11 - 135]	8 [4 - 19]	< 0.001
Amniocentesis-to-delivery - % delivery within 72 hours §	16 (62)	21 (54)	62 (92)	< 0.001
Gestational age at delivery, weeks †	32.1 [30.1 - 33.2]	30.6 [27.5 - 32.5]	27.5 [25.1 - 30.1]	< 0.001
Birthweight, grams ‡	1802 [1,400 - 2,145]	1630 [1,135 - 2,815]	1030 [772 - 1,492]	< 0.001
Cesarean delivery §	5 (19)	19 (49)	30 (45)	0.040
Apgar score at 1 minute ‡	8 [6 - 9]	7 [5 - 8]	6 [3 - 8]	0.011

Variable	MR SCORE			P value
	MR 0 n=26	MR 1-2 n=39	MR 3-4 n=67	
Apgar score at 5 minutes [‡]	9 [8 - 9]	9 [8 - 9]	8 [6 - 9]	0.019

[‡]Data presented as median [interquartile range] and analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks test.

[§]Data presented as n (%) and analyzed by Chi square tests. The MR score ranges from 0 to 4. MR score 0 (zero) indicates “no” inflammation; MR score 1-2 indicates “minimal” inflammation; MR score 3-4 indicates “severe” inflammation.

Table 2
Amniotic fluid analysis

Variable	MR SCORE			P value
	MR 0 n=26	MR 1-2 n=39	MR 3-4 n=67	
Amniotic fluid				
Glucose, mg/dL ‡	27 [19 - 40]	25 [15 - 36]	4 [2 - 15]	<0.001
LDH, U/L ‡	122 [104 - 198]	198 [150 - 266]	718 [441 - 1,145]	<0.001
WBC, cells/mm³ ‡	4 [2 -10]	8 [2 - 25]	756 [123 - 1,570]	<0.001
Positive Gram stain §	1 (4)	4 (11)	34 (51)	<0.001
Positive cultures §	1 (4)	5 (13)	45 (67)	<0.001

[‡]Data presented as median [interquartile range] and analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks test.

[§]Data presented as n (%) and analyzed by Chi square tests. Mg= milligrams; U=units; L=liter; WBC= white blood cell count. The MR score ranges from 0 to 4. MR score 0 (zero) indicates “no” inflammation; MR score 1-2 indicates “minimal” inflammation; MR score 3-4 indicates “severe” inflammation.

Table 3
Placental pathology

Variable	MR SCORE			P value
	MR 0 n=26	MR 1-2 n=39	MR 3-4 n=67	
<i>Presence and degree of placental inflammation</i>				
Chorionic plate inflammation, stage $\ddagger$	0 [0-0]	1 [0-2]	3 [2-3]	< 0.001
Chorionic plate inflammation (stage 2-3) $\S$	6 (23)	12 (31)	54 (81)	< 0.001
Amnionitis, grade $\ddagger$	0 [0-0]	0 [0-1]	3 [1-3]	< 0.001
Amnionitis (grade 2-4) $\S$	2 (8)	9 (23)	49 (73)	< 0.001
Choriodecidualitis, grade $\ddagger$	0 [0-2]	1.5 [0-3]	3 [3-3]	< 0.001
Choriodecidualitis (grade 2-4) $\S$	9 (35)	19 (49)	61 (91)	< 0.001
Funisitis, grade $\ddagger$	0 [0-0]	0 [0-1]	2 [0-4]	< 0.001
Funisitis (grade 1-4) $\S$	3 (12)	11 (28)	39 (58)	< 0.001

§ Data presented as n (%) and analyzed by Chi square tests;

‡ Data presented as median [interquartile range] and analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks test. The MR score ranges from 0 to 4. MR score 0 (zero) indicates “no” inflammation; MR score 1-2 indicates “minimal” inflammation; MR score 3-4 indicates “severe” inflammation.

Table 4
Results of neonatal haematological indices

Variable	MR SCORE			P value
	MR 0 n=26	MR 1-2 n=39	MR 3-4 n=67	
<i>Hematological indices</i>				
Hematocrit % $\ddagger$	48 [44 - 54]	48 [44 - 53]	44 [40 - 49]	0.030
Hemoglobin, g/dL $\ddagger$	16 [14 - 17]	15 [14 - 17]	14 [13 - 16]	0.005
WBC, cells $\times 1000/mm^3$ $\ddagger$	9 [7 -12]	10 [7 -12]	13 [2-38]	0.061
Platelets, cells $\times 1000/mm^3$ $\ddagger$	246 [209 - 300]	250 [212 - 293]	261 [222 - 332]	0.698
Segmented, % $\ddagger$	32 [26 - 45]	31 [22 - 38]	33 [24 - 42]	0.455
Lymphocytes, % $\ddagger$	45 [32 -59]	49 [44 - 59]	33 [24 - 47]	<0.001
ANC, cells/mm ³ $\ddagger$	3,054 [2,185 - 4,818]	3,317 [2,023 - 4,113]	4,030 [2,016 -7,040]	0.234
ABC, cells/mm ³ $\ddagger$	186 [0 - 592]	316 [80 -677]	668 [266 - 2,592]	<0.001
I:T ratio, % $\ddagger$	5 [2 - 9]	5 [2 - 7]	9 [4 - 19]	0.017
Positive blood cultures $\S$	0 (0)	2 (5)	4 (6)	0.453
EONS, $\S$	4 (18)	4 (11)	26 (39)	0.002

[‡]Data presented as median [interquartile range] and analyzed by Kruskal-Wallis One Way Analysis of Variance on Ranks test.

[§]Data presented as n (%) and analyzed by Chi square tests. WBC= white blood cell count; ANC= absolute neutrophil count; ABC= absolute neutrophil count; I:T= immature/total neutrophil count; EONS= early onset neonatal sepsis. The MR score ranges from 0 to 4. MR score 0 (zero) indicates “no” inflammation; MR score 1-2 indicates “minimal” inflammation; MR score 3-4 indicates “severe” inflammation.