

Diagnosis of Intra-amniotic Infection by Proteomic Profiling and Identification of Novel Biomarkers

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DESPITE IMPROVEMENTS IN PRE-natal care, preterm birth occurs in 11.8% of births in the United States and remains the major obstetrical problem in developed countries.¹ Recently, intra-amniotic infection (IAI) has been implicated as a major cause of preterm birth. Intra-amniotic infections cause more than 50% of very low-birth-weight neonates born before 30 weeks of gestation and account for the highest number of neonatal deaths, the most serious complications, and a disproportionate share of aggregate perinatal health care costs (estimated in the United States to be \$4 billion to \$6 billion annually).^{2,3}

Current evidence indicates that if a microorganism causes preterm birth, it usually does so by infection of the chorioamnion and the amniotic fluid or fetus. Infection of the amniotic fluid or the fe-

Context Intra-amniotic infection (IAI) is commonly associated with preterm birth and adverse neonatal sequelae. Early diagnosis of IAI, however, has been hindered by insensitive or nonspecific tests.

Objective To identify unique protein signatures in rhesus monkeys with experimental IAI, a proteomics-based analysis of amniotic fluid was used to develop diagnostic biomarkers for subclinical IAI in amniotic fluid and blood of women with preterm labor.

Design, Setting, and Participants Surface-enhanced laser desorption-ionization/time-of-flight mass spectrometry, gel electrophoresis, and tandem mass spectrometry were used to characterize amniotic fluid peptides in 19 chronically instrumented pregnant rhesus monkeys before and after experimental IAI. Candidate biomarkers were determined by liquid chromatography–tandem mass spectrometry. Polyclonal antibodies were generated from synthetic peptides for validation of biomarkers of IAI. Amniotic fluid peptide profiles identified in experimental IAI were subsequently tested in a cohort of 33 women admitted to Seattle, Wash, hospitals between June 25, 1991, and June 30, 1997, with preterm delivery at 35 weeks or earlier associated with subclinical IAI (n=11), preterm delivery at 35 weeks or earlier without IAI (n=11), and preterm contractions with subsequent term delivery at later than 35 weeks (n=11).

Main Outcome Measures Identification of peptide biomarkers for occult IAI.

Results Protein expression profiles in amniotic fluid showed unique signatures of over-expression of polypeptides in the 3- to 5-kDa and 10- to 12-kDa molecular weight ranges in all animals after infection and in no animal prior to infection. In women, the 10- to 12-kDa signature was identified in all 11 patients with subclinical IAI, in 2 of 11 with preterm delivery without IAI, and in 0 of 11 with preterm labor and term delivery without infection ($P<.001$). Peptide fragment analysis of the diagnostic peak in amniotic fluid identified calgranulin B and a unique fragment of insulinlike growth factor binding protein 1, which were also expressed in maternal serum. Mapping of other amniotic fluid proteins differentially expressed in IAI identified several immunoregulators not previously described in amniotic fluid.

Conclusions This proteomics-based characterization of the differential expression of amniotic fluid proteins in IAI identified a distinct proteomic profile in an experimental primate chorioamnionitis model that detected subclinical IAI in a human cohort with preterm labor. These diagnostic protein expression signatures, complemented by immunodetection of specific biomarkers in amniotic fluid and in maternal serum, might have application in the early detection of IAI.

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tal membranes (ie, chorioamnionitis) may be acute or chronic depending on specific virulence factors, the microbial inoculum size, and host defense mechanisms; thus, the timing and severity of the clinical presentation vary widely. Most women in preterm labor with intact membranes and a positive amniotic fluid microbial culture are refractory to standard tocolytic therapy and rapidly deliver compared with women in preterm labor with sterile amniotic fluid.⁴ Moreover, antibiotic therapy has not prevented preterm delivery in most studies, possibly because the patient subgroup with early IAI that might benefit from antibiotic treatment is identified too late or not at all.^{3,5} Improved diagnostic methods are needed to identify women who may benefit from specific interventions, such as antibiotics or anti-inflammatory agents.⁶

Recent advances in proteomics present a new opportunity to examine the global expression of proteins in tissues and fluids. The proteins or peptides that are preferentially expressed and identified in a disease or pathologic state are well suited for the development of convenient, rapid, sensitive, and specific diagnostic assays. The objective of this study was to discover novel biomarkers for subclinical or occult IAI by proteomic profiling methods. We used surface-enhanced laser desorption-ionization/time-of-flight (SELDI-TOF) mass spectrometry, gel electrophoresis, and tandem mass spectrometry to characterize amniotic fluid peptides in pregnant rhesus monkeys with experimental IAI. The proteome profile that was identified in infected monkeys was then tested for its ability to detect subclinical IAI among women in preterm labor.

METHODS

Experimental IAI in Nonhuman Primates

The protocol was approved by the Institutional Animal Care and Utilization Committee of the Oregon National Primate Research Center and followed humane animal care guidelines. Nineteen pregnant rhesus mon-

keys (*Macaca mulatta*) with timed gestations were chronically catheterized and maintained postoperatively, as previously described.⁷ Each animal served as its own control for proteomic analysis by serially sampling blood and amniotic fluid before and after infection. Experimental IAI was established between days 126 and 138 of gestation (term is 167 days) by intra-amniotic inoculation of (1) 10^6 colony-forming units (CFU) of group B *Streptococcus*, type III, suspended in saline (n=7); (2) 10^7 CFU of *Ureaplasma parvum* (formerly *Ureaplasma urealyticum*, serovar 1) suspended in 10B media (n=6); or (3) 10^5 to 10^7 CFU of *Mycoplasma hominis* (n=6) suspended in phosphate-buffered saline with 10% sucrose and 2% endotoxin-free fetal calf serum.

The group B *Streptococcus* isolate was originally recovered from a neonate with meningitis. The *U parvum* was from a low-passaged clinical isolate from placenta and membranes of a patient with chorioamnionitis and whose neonate had sepsis. The *M hominis* was an endometrial isolate from a patient with postpartum endomyometritis. Amniotic fluid samples were collected serially from all animals during the study period for quantitative microbial cultures, white blood cell analysis, cytokine and prostaglandin measurements (as previously reported⁷⁻⁹), and proteomic analysis. Uterine activity was expressed as the hourly contraction area in millimeters of mercury \times seconds per hour. After delivery, usually cesarean delivery, fetal, decidual, placental, and intermembrane bacterial cultures were obtained to confirm infection. Histopathologic studies were performed to document chorioamnionitis.

Subclinical IAI in Women

The study population was drawn from 309 women admitted in premature labor at 22 to 34 weeks of gestation with intact fetal membranes to the University of Washington Medical Center or associated hospitals in Seattle between June 25, 1991, and June 30, 1997, as previously reported.¹⁰ These women represented 27% of eligible women and did

not differ from nonparticipants in maternal age, race, parity, gravidity, prior preterm delivery, or gestational age at delivery. Informed consent was obtained and the protocol approved by the respective institutional review boards.

Transabdominal amniocentesis was performed and maternal venous blood samples were collected by venipuncture in all study participants at the time of enrollment. Amniotic fluid microbial cultures were performed for facultative and anaerobic bacteria and for genital mycoplasma (*M hominis*, *U parvum*), as previously described.¹⁰ Preterm labor was defined as regular uterine contractions at a frequency of 10 minutes or less with either documented cervical change or a cervical dilatation of more than 1 cm or effacement greater than 50%.

Subclinical IAI was defined by a positive amniotic fluid microbial culture and/or an amniotic fluid interleukin 6 (IL-6) concentration greater than 2 ng/mL, histologic evidence for chorioamnionitis (defined as presence of ≥ 10 polymorphonuclear leukocytes per $400\times$ field in 10 nonadjacent fields¹¹ and absence of uterine tenderness or fever). We have previously reported that an amniotic fluid IL-6 concentration of more than 2 ng/mL was at or above the 75th percentile for the entire study population and was associated with the detection of bacteria by polymerase chain reaction.¹² Women with cervical dilatation of more than 4 cm or ruptured membranes at admission were excluded.

A subset of patients was identified from this study population for proteomic analysis as reported herein. This subset included 3 groups of 11 patients each. Group 1 included all patients with evidence of subclinical IAI as defined herein for whom amniotic fluid and maternal serum was available; group 2 was a randomly selected subset of patients without documented intrauterine infection but with preterm birth at 35 weeks of gestation or earlier; and group 3 consisted of randomly selected patients without infection and with preterm labor responsive to tocolytic therapy who subsequently delivered near term (>35 weeks

of gestation). Samples were stored at -20°C until analysis and not thawed more than twice. Repeated thawing did not affect SELDI-TOF profiles or protein identification.

SELDI-Mass Spectrometry Profiling of Amniotic Fluid

A total of 0.5 to 3.0 μg of unfractionated protein from amniotic fluid was analyzed on 3 different protein chip arrays, a normal-phase NP2 0 (SiO_2 surface), a reverse-phase H4 (hydrophobic surface), and immobilized nickel (IMAC) chips (CIPHERGEN Biosystems Inc, Fremont, Calif). Chips were incubated for 1 hour with the sample followed by a 5- μL water wash. After drying, a saturated solution of sinapinic acid in 50% acetonitrile (vol/vol) and 0.5% trifluoroacetic acid (vol/vol) was added before reading the arrays on a SELDI-TOF instrument (CIPHERGEN Protein Biology System II, CIPHERGEN Biosystems).^{13,14} Spectra were calibrated using internal standards and analyzed using Protein Chip software, version 3.0 (CIPHERGEN Biosystems).

Protein Identification by Liquid Chromatography (LC)-Tandem Mass Spectrometry Analysis

One hundred micrograms of amniotic fluid protein from control and infected samples was reduced with iodoacetamide and resolved on a 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).¹⁵ The gel was stained with Coomassie blue R-250, and distinct bands from each lane were cut from the gel, destained, and digested in-gel with trypsin for 24 hours at 37°C using the method of Courchesne and Patterson.¹⁶ Peptides were then extracted with 0.1% trifluoroacetic acid and purified using Zip-Tip c18 pipette tips from Millipore. After in-gel digestion, samples were analyzed on a Q-ToF-2 mass spectrometer (Micromass, Manchester, England) coupled to a CapLC (Waters Inc, Milford, Mass) and/or on an ion trap (LCQ, ThermoFinnigan, San Jose, Calif) coupled to a 1100 Capillary LC System (Agilent Technologies, Foster City, Calif). Reverse-phase separation was performed using an Integrafrit

C1875- μm ID \times 15-cm fused silica capillary column (Q-TOF2) and Zorbax C-18 0.5-mm \times 150-mm microbore column using a 10 $\mu\text{L min}^{-1}$ flow rate and a gradient of 0% to 40% (75% acetonitrile in water) over 1 hour (LCQ). For Q-ToF-2 analysis, masses of 400 to 1500 Da were scanned for the mass spectrometry survey and masses of 50 to 1900 Da were scanned for tandem mass spectrometry. Tandem mass spectrometry spectra were acquired in an automated mode using standard LCQ software.

Statistical Analysis

Proteomic Analysis. Analysis of mass spectrometry/mass spectrometry spectra was performed using SEQUEST software, version 3.1 (ThermoFinnigan) and DTASelect (Scripps Research Institute, La Jolla, Calif) as described.^{17,18} Searches were run with the default parameters using a combined, indexed, nonredundant database of protein sequences obtained from the Protein Information Resource and SwissProt (<http://www.expasy.org>). S-carboxyamidated cysteine was the only considered modification. Spectra from the LCQ mass spectrometer were filtered with a cross-correlation score cutoff of 2.4 for the doubly charged ions. Each spectra and proposed sequence pair selected by DTASelect were visually inspected and the final results were input into a Microsoft Access database (Microsoft, Redmond, Wash).

Study Population/Outcomes. The 3 groups of women were compared using 1-way analysis of variance for continuous data (*P* values from *F* statistics are presented) and by the Pearson χ^2 or 2-tailed Fisher exact test for categorical data. For each analysis, overall differences were assessed; no pairwise comparisons were evaluated because of the small sample size. All analyses were performed using SAS, version 8 (SAS Institute Inc, Cary, NC).

Using the Pearson χ^2 statistic with 2 degrees of freedom and 33 patients allocated equally into the 3 patient groups, the power to detect an effect size of 0.54 was calculated (NCSS and PASS Number Cruncher Statistical Systems,

Kaysville, Utah). As an example, an effect of this size corresponds approximately to correctly classifying 9 of 11 in group 1, 7 of 11 in group 2, and 9 of 11 in group 3. Thus, the sample size was adequate to detect effects that would be observed in evaluating currently available diagnostic tests that have sensitivity of 80% and a specificity of 80%.

Polyclonal Antibodies and Western Immunoblotting

Immunogenic peptides from corresponding proteins were used to generate rabbit polyclonal antibodies (DSL Laboratories, Webster, Tex). Affinity-purified antibodies were then used for Western blots. One hundred micrograms of amniotic fluid protein was resolved on 4% to 20% SDS-PAGE and transferred to polyvinylidene difluoride membranes. From maternal serum, 300 μg of protein was used for immunoprecipitation using insulinlike growth factor binding protein 1 (IGFBP-1) monoclonal antibody (DSL Laboratories) and products underwent Western blot analysis. For detection of calgranulin B in maternal serum, 150 μg of albumin-depleted maternal serum was used for Western blot. The membranes were blocked with 5% fat-free milk in phosphate-buffered saline-tris for 45 minutes at room temperature and incubated with 1 $\mu\text{g/mL}$ of primary antibody (IGFBP-1, azurocidin, vitamin D binding protein from DSL Laboratories, and calgranulin B from Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C . After 3 washes with TBST tris-buffered saline containing 0.1% (vol/vol) Tween-20, the membrane was incubated with IgG-HRP secondary antibody (Sigma Chemical Co, St Louis, Mo) and visualized with enhanced chemiluminescence.

RESULTS

Experimental IAI in Nonhuman Primates

Infection was rapidly established following intra-amniotic inoculation. Uterine contractility, which increased from basal levels of 100 mm Hg \times s/h to greater than 10000 mm Hg \times s/h, oc-

curred at a mean (SD) of 33 (9) hours after inoculation with group B *Streptococcus*; a mean (SD) of 43 (14) hours after inoculation with *U parvum*; and a mean (SD) of 62 (14) hours after inoculation with *M hominis*. Uterine contractions led to progressive cervical dilatation and effacement in all instances. Increases in uterine contractility were preceded by significant elevations in the proinflammatory cytokines tumor necrosis factor α , IL-1 β , IL-6, and IL-8 and prostaglandins E₂ and F_{2 α} , as previously reported.⁷⁻⁹ No animal demonstrated clinical signs of IAI prior to onset of labor. Chorioamnionitis was confirmed histologically in all cases.

Subclinical Chorioamnionitis in Women

Demographic and delivery data for the patients are summarized in TABLE 1. There were no differences in maternal age, race/ethnicity, or parity among the 3 groups. Patients with subclinical IAI had a somewhat earlier gestational age at enrollment and delivered at a significantly earlier gestational age than patients with preterm delivery without infection or those with term delivery. Ninety-one percent of patients with occult IAI delivered within 7 days of enrollment in this study.

Four of 11 patients with occult IAI had microorganisms recovered (2 with *Escherichia coli*, 1 with *Candida albicans*, and 1 with mixed anaerobes). Neither *U parvum* nor *M hominis* was

isolated. The other 7 patients with infection were identified on the basis of markedly elevated amniotic fluid IL-6 concentrations (>2 ng/mL). The mean (SD) amniotic fluid concentration of IL-6 was 27.7 (7.8) ng/mL among these patients compared with 0.68 (0.20) ng/mL among those with preterm delivery without infection and 0.25 (0.13) ng/mL among those with preterm contractions but term delivery ($P < .001$).

Identification of Diagnostic Protein Profiles by SELDI-TOF Mass Spectrometry

Whole-spectrum SELDI-TOF mass spectrometry analyses collected at 235 laser intensity of amniotic fluid extracts bound to chemically defined normal-phase chip arrays revealed several peak intensity differences in 3- to 5-kDa and 11- to 12-kDa regions between infected and noninfected primate and human amniotic fluid (FIGURE 1). The 11-kDa cluster was differentially expressed between control and infected in all cases ($P = .004$). Longitudinal sampling following group B *Streptococcus* inoculation revealed that the 11-kDa peak was first noted as early as 12 hours after inoculation and preceded increases in hourly contraction area in infected animals.

As shown in FIGURE 2, the 11-kDa peak was also detected in experimental IAI induced by the other 2 microbes. In the human cohort, all 11 patients with confirmed occult IAI and

none of the 11 patients who subsequently delivered at term had the SELDI-TOF mass spectrometry profile consistent with IAI and characterized by the presence of the 11-kDa peak. This profile was also present in 2 of 11 patients in whom infection was not confirmed but who delivered prematurely (Table 1). These differences in the proteome profile could not be attributed to differences in gestational age.

Global Analysis of Amniotic Fluid Proteins in IAI Using LC-Tandem Mass Spectrometry

Global analysis of primate and human infected and noninfected amniotic fluid using 1-dimensional gel electrophoresis separation and in-gel digestion of specific bands identified expression of various immunoregulatory proteins not described previously in IAI (TABLE 2). Liquid chromatography-tandem mass spectrometry analysis of the approximately 11-kDa band from 1-dimensional gel identified several polypeptides derived from calgranulins and a unique fragment of IGFBP-1.

Immunodetection of IAI Biomarkers

To validate the differential expression of proteins identified in IAI, we selected 2 markers identified from the 11-kDa SELDI-TOF mass spectrometry profile (calgranulin B and IGFBP-1), 1 immunoregulatory molecule (azurocidin), and an unregulated protein (vitamin D bind-

Table 1. Identification of Subclinical Intra-amniotic Infection in Humans Using Surface-Enhanced Laser Desorption-Ionization Time-of-Flight Mass Spectrometry Profiling*

Characteristic	Preterm Delivery (≤ 35 wk)		Term Delivery (>35 wk) After Preterm Labor (n = 11)	P Value
	Intra-amniotic Infection (n = 11)	No Intra-amniotic Infection (n = 11)		
Maternal age, y	24.5 (5.4)	26.6 (9.0)	25.6 (6.0)	.93†
White race, No. (%)	6 (55)	4 (36)	6 (55)	.62‡
Parity	1.9 (1.6)	1.9 (1.5)	3.0 (2.5)	.15†
Gestational age, wk				
Enrollment	26.9 (1.1)	28.6 (1.1)	30.3 (1.1)	.10†
Delivery	27.3 (0.9)	29.8 (1.0)	37.0 (0.9)	<.001†
Time from enrollment to delivery, d	2.1 (5.6)	8.4 (6.3)	46.9 (5.6)	<.001†
Diagnostic protein profile, No./total No. (%)	11/11 (100)	2/11 (18)	0	<.001§

*Values are expressed as mean (SD) unless otherwise indicated.

†One-way analysis of variance.

‡Pearson χ^2 test.

§Fisher exact test.

ing protein) identified in global protein expression analysis. As shown in FIGURE 3, Western blot analysis confirmed all 3 biomarkers showing differential expression, consistent with the protein identification experiments performed on IAI amniotic fluid.

Using specific antibodies developed against IGFBP-1 and calgranulin B, we investigated whether differentially expressed proteins in amniotic fluid could be identified in pooled maternal serum from a limited number of patients (n=5) for whom serum samples were avail-

able. In this limited analysis, the 11-kDa proteolytic fragment of IGFBP-1 and of calgranulin B, corresponding to the 11-kDa peak that was differentially present in infected vs control amniotic fluid, was also detected in maternal serum in response to IAI (Figure 3B) and in nonhuman primates following experimental IAI (data not shown). Azurocidin was not detected in serum.

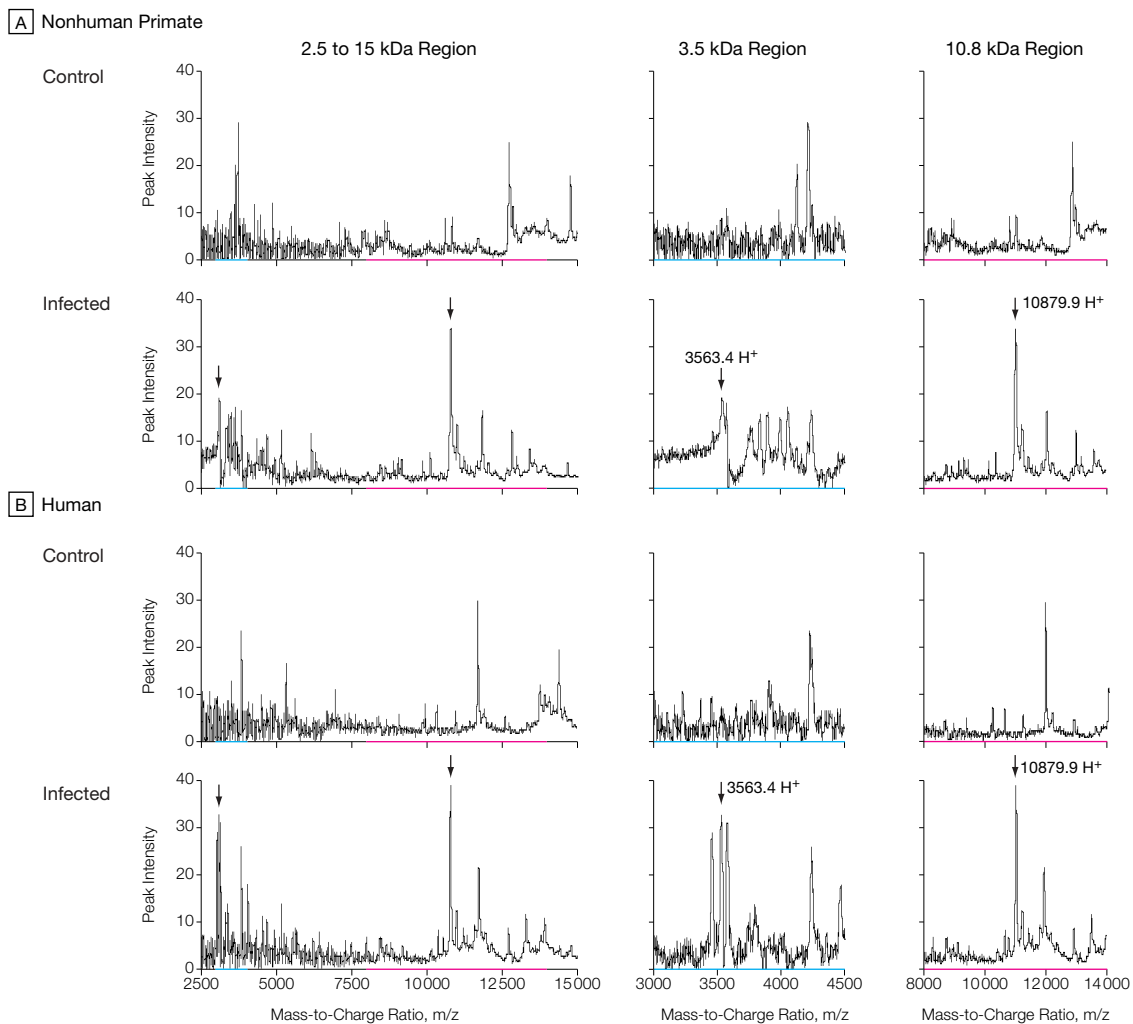
COMMENT

Intra-amniotic infection is an important and potentially preventable cause

of premature births, neonatal sepsis, periventricular leukomalacia/cerebral palsy, and maternal febrile morbidity.^{2,3,19-21} Overt or subclinical IAI is present in at least 50% of extremely premature births; an inverse relationship has been demonstrated between gestational age at birth and both the frequency of microorganisms recovered from the chorioamnion and histologic chorioamnionitis.^{22,11}

Despite growing evidence that IAI is responsible for a substantial proportion of premature births in the United

Figure 1. SELDI-TOF Mass Spectrometry Analysis of Amniotic Fluid in Control and Group B *Streptococcus*-Infected Samples



SELDI-TOF indicates surface-enhanced laser desorption/ionization time-of-flight. Group B *Streptococcus* infection-induced differential protein expression in nonhuman primate (A) and human (B) amniotic fluid samples. SELDI-TOF-mass spectrometry spectrum of amniotic fluid extracts bound to chemically defined Normal Phase Protein chip arrays. Spectrum from 2.5 to 15 kDa collected at 235 laser intensity. Detailed spectra show increased expression of the 3.5-kDa and 10.8-kDa peaks between control and infected. Arrows indicate the unique peaks represented by polypeptides overexpressed in infection.

States, antibiotic treatment has had limited success in treating patients with preterm labor and intact fetal membranes or in preventing preterm delivery.^{3,5} This dilemma may be attributable in part to misclassification and patient selection bias, whereby the subgroup of women with occult IAI and intact fetal membranes that might benefit from antibiotics is not reliably distinguished from women without infection. Accurate and early diagnosis of IAI would facilitate early and appropriate interventions as well as enhance the design of therapeutic trials. Early diagnosis of IAI is problematic, however, because clinical signs and symptoms (including preterm labor) tend to be late manifestations of this condition. Furthermore, the available noninvasive diagnostic tests have limited predictive value, or, as in the case of measurement of IL-6, polymerase chain reaction tests, or microbial cultures, the results are often delayed and amniocentesis is required.^{12,22}

We previously demonstrated in a nonhuman primate model the causal relationship between IAI with group B *Streptococcus*, *U parvum*, or *M hominis* and preterm labor, which resembles the clinical progression of events that is observed in women with IAI.⁷⁻⁹ These microorganisms are also frequently isolated from the amniotic fluid and chorioamnion in women with preterm labor and delivery.^{11,23,24} In the rhesus monkey, as in women, histologic appearance of the fetal membranes is consistent with subacute chorioamnionitis.

Therefore, we used this experimental model to search for novel amniotic fluid protein and peptide biomarkers for IAI with the expectation that they could similarly be found in human amniotic fluid and would provide the basis for the subsequent development of sensitive and specific assays to detect subclinical IAI. We identified a unique amniotic fluid peptide profile in IAI by SELDI-TOF that appeared within 12 hours after intra-amniotic inoculation of microorganisms and was reliably present before the onset of labor or other clinical signs or symptoms of infection. We validated our

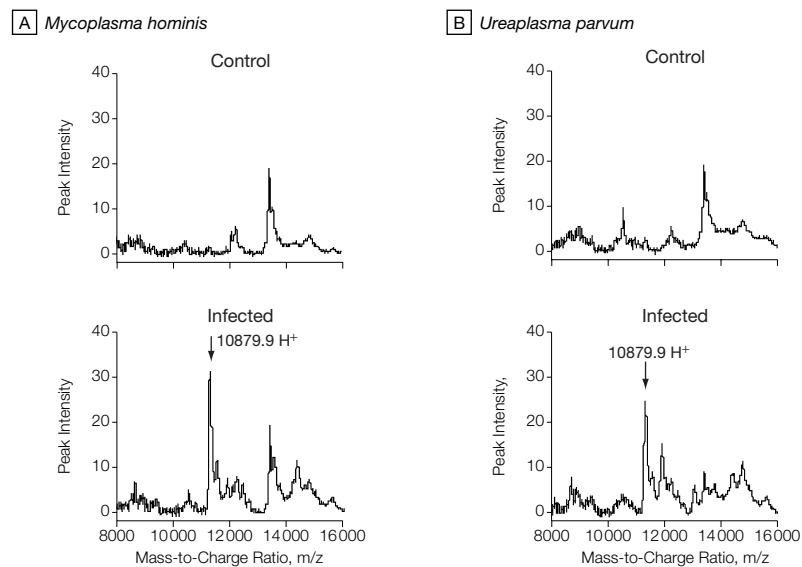
observations in a cohort of women in preterm labor by microbiologic, biochemical, or histologic evidence of chorioamnionitis and in a control group without evidence of infection.

We used 2 distinct proteomic approaches: a low-resolution rapid protein fingerprinting approach (SELDI-TOF mass spectrometry) that generates distinct expression profiles and is amenable to developing rapid

screening assays, together with a high-throughput protein identification approach (LC-tandem mass spectrometry) that provides the identity of the biomarkers suitable for identification by conventional immunoassays.

The detection of a differentially expressed 11-kDa peak in SELDI-TOF mass spectrometry in the setting of IAI in the experimental primate model, which used clinically relevant microor-

Figure 2. Detection of Intra-amniotic Infection Resulting From Different Microorganisms



Surface-enhanced laser desorption/ionization time-of-flight analysis of nonhuman primate amniotic fluid before and after infection using Normal Phase Protein chip arrays. The 10.8-kDa candidate biomarker is detected in amniotic fluid infected with *Mycoplasma hominis* and *Ureaplasma parvum*. Detection in group B *Streptococcus* is shown in Figure 1.

Table 2. Immunoregulatory Proteins and Polypeptides Detected Using Tandem Mass Spectrometry and de Novo Sequencing Analysis of Human Amniotic Fluid

GenBank Account No.	Protein ID	Protein Name
U12026	CAPG_HUMAN	Macrophage capping protein*
X83006	NGAL_HUMAN	Neutrophil gelatinase-associated lipocalin*
M19507	PERM_HUMAN	Myeloperoxidase precursor*
M22300	PLSL_HUMAN	L-plastin (lymphocyte cytosolic protein 1)
NM001700	AZU1_HUMAN	Azurocidin*†
Z38026	FA39_HUMAN	Antibacterial protein FALL-39 precursor*
AF159456	Q9UKJ4	Gp-340 variant protein
AL355392	Q9H4V6	Novel protein similar to mouse von Ebner salivary gland protein, isoform 2
M93056	ILEU_HUMAN	Leukocyte elastase inhibitor*
Y00278	S108_HUMAN	Calgranulin A
X06233	S109_HUMAN	Calgranulin B*†

*Peptides representing these proteins are more abundantly or uniquely detected in infected amniotic fluid.
†Proteins also shown to be differentially expressed by immunoassays.

ganisms and in women infected with different microorganisms, confirms this signature for IAI caused by a broad range of pathogens. Peptides within the differentially expressed peak may represent a basic intrauterine immune response to infection since one set of proteins identified in this unique cluster, ie, the calgranulins, are members of the S-100 calcium-binding protein family, expressed by macrophages and by epithelial cells in acutely inflamed tissues.^{25,26} The second candidate from this cluster, a specific proteolytic fragment of IGFBP-1, indicates a potential protease-related mechanism in response to infection. Intact IGFBP-1 is the major IGFBP found in amniotic fluid and is synthesized by both fetal membranes and maternal decidua.²⁷ Although the proteolytic cleavage of IGFBPs is a well-characterized phenomenon,²⁸ the particular fragment described in the current study has not been previously described.

In the second approach, characterization of proteins expressed in amniotic fluid in control and IAI using LC-tandem mass spectrometry identified a significant number of infection and immune response-related molecules in IAI

for the first time. Macrophage-capping protein is a Ca²⁺-sensitive protein that modulates actin filaments and is involved in inflammatory processes.²⁹ Leukocyte elastase and neutrophil gelatinase-associated lipocalin are involved in bacteriostatic and bacterolytic mechanisms.³⁰

In addition to the above immunomodulators, the detection of antibacterial proteins Fall-39 and azurocidin in amniotic fluid in response to infection provides new insights into intrauterine immune responses. Antibacterial protein Fall-39 (LL-37) binds to bacterial lipopolysaccharides, is a potent chemotactic factor for mast cells, and serves as a first-line defense to prevent local infection and systemic invasion of microbes.³¹ Azurocidin (CAP37) is a cationic antimicrobial protein isolated from human neutrophils with important implications in host defense and inflammation.³² Glycoprotein-340 variant protein is a scavenger receptor previously identified in lung that binds to bacteria.³³ Identification of these proteins complements the sensitive proteomic approaches used to identify biomarkers for IAI. Since our experimental

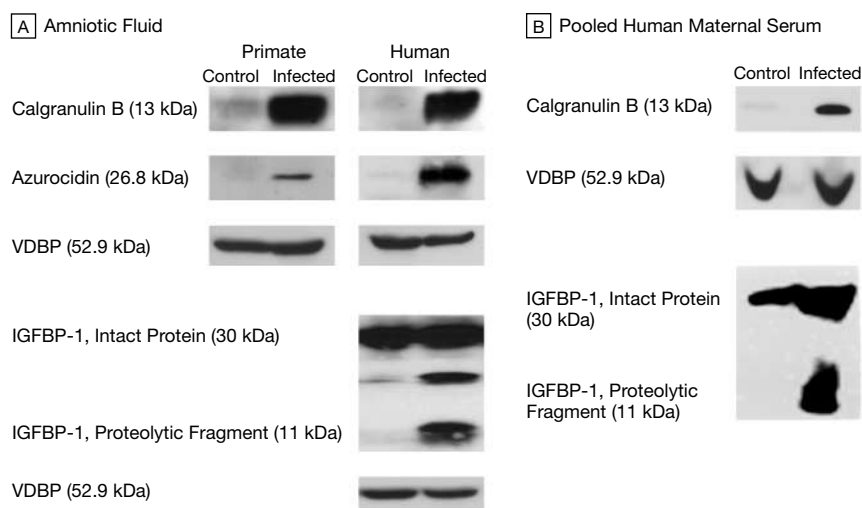
objectives were focused on the discovery of novel biomarkers unique to IAI, we did not look for the peptide profiles of cytokines such as IL-6.

Currently available laboratory tests used to confirm the diagnosis of IAI include measurement of maternal C-reactive protein, direct examination of amniotic fluid for leukocytes or bacteria on Gram stain, amniotic fluid microbial culture or polymerase chain reaction, measurement of amniotic fluid glucose and IL-6 concentrations, and detection of amniotic fluid leukocyte esterase. Only microbial cultures and broad-spectrum bacterial recombinant DNA polymerase chain reaction of amniotic fluid appears to be sensitive and specific for IAI.^{12,34} However, microbial cultures for genital mycoplasmas or polymerase chain reaction-based tests are not widely available and amniocentesis is required to obtain amniotic fluid.

One advantage of a proteomics-based approach is that candidate peptide biomarkers lend themselves to the development of rapid point-of-service and cost-effective diagnostic immunoassays that can be completed within hours. By analogy with other amniotic fluid and serum screening tests (eg, α -fetoprotein for neural tube defects), we predicted that peptides identified within infected amniotic fluid could also be detected in maternal serum and might lead to the development of noninvasive diagnostic tests.³⁵ Our preliminary results using pooled maternal serum samples suggest the feasibility of this approach, in that IGFBP-1-restrictive fragment and calgranulin B are 2 such candidate biomarkers that appear in both amniotic fluid and maternal blood during IAI but not in the absence of infection (Figure 3B). In contrast, elevated IL-6 concentrations have not been reported in maternal serum in association with intra-amniotic infection.

In summary, we used a proteomics-based analysis of amniotic fluid to identify biomarkers for IAI in both an experimental nonhuman primate model and a cohort of women with preterm labor and occult IAI. Diagnostic protein expression profiles were identified by

Figure 3. Immunodetection of Potential Biomarkers for Intra-amniotic Infection



A, Nonhuman primate control and infected amniotic fluid and human control and infected amniotic fluid. VDBP indicates vitamin D binding protein, an unregulated control marker. Insulinlike growth factor binding protein 1 (IGFBP-1) bands represent the intact protein (approximately 30 kDa) and proteolytic fragment (approximately 11 kDa). B, Detection of calgranulin B and IGFBP-1 in pooled human maternal serum from immunoprecipitates.

SELDI-TOF mass spectrometry profiling that was both sensitive and specific in detecting IAI. High-throughput analysis of expressed proteins in amniotic fluid identified expression of several immunoregulatory molecules for the first time. Immunodetection of proteins detected in the diagnostic 11-kDa peak (IGFBP-1 and calgranulin B) confirmed the presence and differential expression of these biomarkers in amniotic fluid and in maternal blood during IAI.

Our findings hold promise for the future development of rapid and noninvasive assays to detect occult IAI during pregnancy. This would be an important breakthrough since it would allow clinicians and investigators to target a particular subgroup of women at high risk of premature delivery for specific interventions and in therapeutic trials. Finally, these studies demonstrate the potential utility of proteomics-based approaches to identify specific biomarkers and diagnostic profiles for infectious and inflammatory processes and in pathophysiologic conditions of pregnancy.

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Author Contributions: Dr Gravett had full and complete access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Acquisition of data: Gravett, Rosenfeld, Reddy, Jacob, Turner, McCormack, Hitti, Eschenbach, Roberts, Nagalla.

Drafting of the manuscript: Gravett, Novy, Rosenfeld, Hitti, Eschenbach, Nagalla.

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