의학박사 학위논문

조기진통 및 조기양막파열 산모에서 양수배양 음성인 조직학적 융모양막염의 임상적 의미 및 조직학적 융모양막염의 비침습적 예측지표 개발

Clinical significance of histologic chorioamnionitis with a negative amniotic fluid culture and non-invasive predictors for histologic chorioamnionitis in patients with preterm labor and premature membrane rupture

2018 년 2 월

서울대학교 대학원

의학과 산부인과학 전공

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지도교수 박 교 훈

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Clinical significance of histologic chorioamnionitis with a negative amniotic fluid culture and non-invasive predictors for histologic chorioamnionitis in patients with preterm labor and premature membrane rupture

by Jeong Woo Park, M.D.

(Directed by Kyo Hoon Park, M.D., Ph.D.)

A thesis submitted to the Department of Obstetrics & Gynecology in Partial Fullfillment of the Requirements for the Degree of Doctor of Philosophy in Medicine at the Seoul National University College of Medicine

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Approved by thesis committee:

Professor_____Chairman
Professor_____Vice chairman
Professor_____
Professor_____
Professor_____

Abstract

Clinical significance of histologic chorioamnionitis with a negative amniotic fluid culture and non-invasive predictors for histologic chorioamnionitis in patients with preterm labor and premature membrane rupture

Jeong Woo Park

Obstetrics & Gynecology

The Graduate School

Seoul National University

Objectives: (1) To evaluate the effect of histologic chorioamnionitis (HCA) with a negative amniotic fluid (AF) culture on adverse pregnancy and neonatal outcomes and inflammatory status in the AF compartment in women with preterm labor (PTL) or preterm premature rupture of membranes (PPROM) and (2) to identify novel immunoregulatory proteins in maternal plasma associated with

HCA by using a membrane-based human cytokine microarray technology in women with PTL and PPROM.

Methods: This study consisted of two phases. (1) In the first phase of the study, 153 consecutive women diagnosed as having a PTL or PPROM (20–34 weeks) who delivered singleton gestations within 48 hours of amniocentesis participated. AF obtained through amniocentesis was cultured, and interleukin (IL)-6, IL-8, and metalloproteinase-9 (MMP-9) levels were determined. The placentas were examined histologically. (2) In the second phase of the study, a nested case-control study was conducted to identify novel plasma biomarkers associated with HCA. The second phase consisted of two stages. Firstly, plasma samples were obtained < 96 h before delivery from 14 cases with HCA and 14 control subjects (without HCA) in women with PTL. Discovery work using by membrane-based protein microarray was performed to compare the profiles of immunoregulatory proteins in the maternal plasma. Secondly, validation of selected candidate biomarkers was done by ELISA in the final cohort (n=74) with additional 46 plasma samples in women with PTL. Membrane-based microarray analysis (n=28) and validation (n=82) with additional 54 plasma samples in women with PPROM was performed in the same manner as in PTL patients. Receiver operating characteristic curves were generated to compare the diagnostic accuracy to predict HCA between serum C-reactive protein which has been in clinical use and the candidate proteins.

Results: (1) In the first phase of the study, the prevalence of HCA with negative AF culture was 23.5% (36/153). The women with HCA but with a negative AF culture (group 2) and those with a positive AF culture (group 3) had a significantly lower mean gestational age at amniocentesis and delivery than those with a negative AF culture and without HCA (group 1). Women in group 3 had the highest levels of AF IL-6, IL-8, and MMP-9, followed by those in group 2, and those in group 1. Composite

neonatal morbidity was significantly higher in groups 2 and 3 than in group 1, but this was no longer significant after adjusting for confounders caused mainly by the impact of gestational age.

(2) In the second phase of the study, differentially expressed proteins (12 proteins in PTL and 14 proteins in PPROM) were identified by membrane-based protein microarray analysis. In women with PTL (n=74), validation by ELISA confirmed significantly higher levels of S100 A8/A9 in women with HCA, compared with control subjects. However, this significance was not remained after adjusting for gestational age at sampling. In women with PPROM (n=82), ELISA validation found S100 A8/A9, MMP-9, and IL-6 in maternal plasma to have significantly higher levels in women with HCA, compared with those without HCA. After adjusting for gestational age at sampling, use of tocolytics, and corticosteroids administration, increased plasma MMP-9 was significantly associated with HCA in women with PPROM. However, its diagnostic indices were not superior to those of serum C-reactive protein in predicting HCA.

Conclusion: (1) In women who delivered preterm neonates, HCA with a negative AF culture was associated with increased risks of preterm birth, intense intra-amniotic inflammatory response, and prematurity-associated composite neonatal morbidity, and its risks are similar to the risk posed by positive AF culture. (2) The protein expression pattern in the maternal plasma is significantly altered between women with and without HCA. Although not found in women with PTL using by membrane-based protein microarray and ELISA validation, in our cohort of PPROM patients, increased levels of MMP-9 in maternal plasma can be a potentially novel candidate biomarker for predicting HCA non-invasively and antenatally.

Key words: Amniotic fluid culture, histological chorioamnionitis, intra-amniotic inflammation, neonatal morbidity, preterm birth, non-invasive predictor, membrane-based protein microarray.

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목 차

List of Tables

List of Figures

Introduction

Histologic chorioamnionitis (HCA) complicates as many as 30–80% of preterm births with preterm labor (PTL) or preterm premature rupture of membranes (PPROM), and has been shown to be a risk factor for adverse maternal and neonatal outcomes, including earlier gestational age at delivery and neonatal brain and lung injuries.[1-4] Moreover, HCA is associated with the presence of microbial invasion of the amniotic cavity (MIAC), serving as a route for ascending infection, when detected in preterm placenta.[5-7]

Most of the previous studies that demonstrated a strong association between HCA and documented poor neonatal and pregnancy outcomes included mixed cases of HCA with and without MIAC.[2, 4, 7, 8] However, the maternal and neonatal outcomes of women with HCA alone may differ from those of women who had both MIAC and HCA because MIAC is the advanced stage of ascending intrauterine infection, and infection and inflammation with microorganisms in the amniotic fluid (AF) may result in direct invasion of the fetus, contributing to clinical disease in the neonatal period.[3, 6] By contrast, HCA alone is the early stage (localized inflammatory processes detected in the membranes), as proposed by Romero et al.[3, 6] Nevertheless, to date, to what extent or whether HCA alone may have adverse effects on maternal and neonatal outcomes and intense inflammatory responses in the AF is unclear. Indeed, a substantial number of women with PTL or PPROM have HCA but have negative AF culture results.[5, 7, 9, 10]

In terms of the potential association between HCA and clinical outcomes, therefore, a more accurate and earlier prenatal diagnosis of subclinical HCA, especially by using non-invasive methods, is clinically important for deciding on the treatment strategy and for counseling patients with either PTL or PPROM. However, placental pathology cannot be evaluated before delivery. Traditionally, investigators have considered inflammatory biomarkers in AF as useful for the prediction of subclinical HCA.[7, 10-13] But its clinical use is, at present, limited by requirement of invasive AF sampling. In this regard, alternative non-invasive approaches such as measurement of these proteins in

a maternal blood sample could be a means to replace traditional invasive amniocentesis because acute inflammatory processes of the placenta are of maternal origin[14] and thus may be reflected in the maternal blood compartment. In fact, C-reactive protein (CRP), interleukin (IL)-6, granulocyte-colony stimulating factor, white blood cell (WBC) count, and neutrophil to lymphocyte ratio in maternal blood were previously reported to be a predictor of HCA.[7, 15-19] However, none of these biomarkers alone measured in maternal blood have demonstrated to have sufficient sensitivity and specificity for clinical use. This, in part, may be related to the complexity of inflammatory process, multiple causes for acute HCA, and variations in the individual innate immune system function. Moreover, these studies included already known limited range of serum markers.

To compensate this weakness that each study investigated only a few biomarkers for predicting HCA, a relatively new technology of membrane-based protein microarray has been used to find specific risk factors or biomarkers for certain diseases.[20-23] Antibody microarray system (RayBio® Custom Human Cytokine Array) is an antibody-based protein array technology (RayBiotech, Inc., Norcross, GA) developed recently to simultaneously scrutinize 507 cytokines, chemokines, growth factors, and other molecules. This technology could even be used to identify chemokine or cytokine biomarkers for HCA antenatally.

The purposes of this study were as follows: (1) to determine the frequency and impact of HCA with a negative AF culture on adverse pregnancy and neonatal outcomes, and inflammatory status in the AF compartment in women with PTL or PPROM, and (2) to compare the profiles of immunoregulatory proteins in the plasma of women with HCA and those without HCA in PTL or PPROM patients, to validate selected candidate proteins in a larger cohort, and to identify novel plasma biomarkers associated with HCA.

Materials and methods

Study design

1-1. Study population in the first phase of the study

The study population consisted of consecutive women diagnosed as having either PTL or PPROM who were admitted to Seoul National University Bundang Hospital (Seongnam, Republic of Korea) from June 2004 through August 2013 and underwent transabdominal amniocentesis. The patients were retrospectively identified by searching our perinatal database according to the following criteria: (1) singleton gestation; (2) amniocentesis performed; (3) delivery at a gestational age between 20+0 and 34+6 weeks; (4) delivery within 48 hours of amniocentesis; and (5) histopathological examination of the placenta. The exclusion criteria were as follows: (1) multiple gestations; (2) a time interval of >48 hours from amniocentesis to delivery (used to maintain a meaningful temporal relationship between the AF culture and the placental histological examination results); and (3) major congenital anomalies. The data of 58 patients were previously reported in a previous retrospective study that evaluated the predictive value of intra-amniotic and serum markers for inflammatory lesions of preterm placenta.[7] The primary outcome measures were composite neonatal morbidity and mortality, inflammatory status in the AF, and the gestational age at which the clinical symptom and delivery occurred. The local ethics committee of Seoul National University Bundang Hospital (project No. B-1105/128-102) approved this study. All the women provided written informed consent to undergo the amniocentesis procedure and for the use of AF samples for research purposes prior to the amniocentesis.

In women who were admitted under the diagnosis of either PTL or PPROM, and delivered a preterm neonate at our institution, amniocentesis for the assessment of microbiological and inflammatory statuses of the amniotic cavity was recommended. In addition, placentas were routinely sent for histopathological examination. Based on the results of the placental histological examination and AF culture, the subjects were divided into 3 patient groups as follows: (1) no placental

inflammation with a negative AF culture result (group 1, n=64), (2) placental chorioamnionitis with a negative AF culture result (group 2, n=36), and a positive AF culture result (group 3, n=53).

1-2. Sample collection and processing

AF was aseptically obtained by performing abdominal amniocentesis under sonographic guidance. AF was analyzed for WBC counts and cultured for aerobic and anaerobic bacteria, as well as genital mycoplasmas (*Mycoplasma hominis* and *Ureaplasma species*), according to previously described methods.[24] The remaining AF that was not required for clinical assessment was centrifuged at 1,500*g* and 4°C for 10 minutes. The supernatant was aliquoted and stored at −70°C until assayed. The samples were not subjected to freeze-thaw cycles before assay. To evaluate AF inflammatory status, we selected IL-6, IL-8, and metalloproteinase-9 (MMP-9) because previous studies of ours and others demonstrated the association between these cytokines and placental inflammatory lesions or intra-amniotic infection.[7, 10, 19, 25-30] The levels of IL-6, IL-8, and MMP-9 in stored AF were measured by using an enzyme-linked immunosorbent assay (ELISA) human DuoSet Kit (R&D System, Minneapolis, MN, USA). The ranges of the IL-6, IL-8, and MMP-9 standard curves were 7.8–600, 31.2–2000, and 31.2–2000 pg/ml, respectively. All the samples were assayed in duplicate. The intra- and inter-assay coefficients of variation were each <10%. The AF culture results and WBC counts, but not AF IL-6, IL-8, and MMP-9 levels, were available to the managing obstetricians and neonatologists. Immediately after amniocentesis, WBC count and CRP level in maternal blood were measured, and details of these measurements were previously described.[24]

2-1. Study population in the second phase of the study

In the second phase of the study, we performed a nested case-control study and included singleton pregnant women at 23 to 34 weeks of gestation diagnosed with either PTL or PPROM who were

admitted to the Seoul National University Bundang Hospital (Seongnamsi, Republic of Korea) from June 2004 through July 2015 in PTL and through May 2016 in PPROM. The ethics committee at Seoul National University Bundang Hospital approved the study (IRB no. B-1105/128-102). The inclusion criteria were as follows: (1) a live fetus; (2) an aliquot of maternal plasma available for analysis; (3) available results of placental histopathological examination; and (4) delivered within 96 hours of blood sampling (used to maintain a meaningful temporal relationship between the immunoregulatory proteins measured in maternal plasma and placental histological examination results). The exclusion criteria were: multiple pregnancies, major congenital anomalies, transfer to another hospital after sampling, a time interval of >96 hours from sampling to delivery, and evidence of clinical chorioamnionitis.

For discovery work, we initially performed a nested case-control study including 28 plasma samples which were selected randomly from 14 women with PTL and HCA (case subjects) and 14 women with PTL without HCA (control subjects matched by gestational age) using by membrane-based human cytokine antibody microarray. The discovery work in women with PPROM was performed in the same manner as in PTL patients. And then we performed the validation work using by ELISA in the final cohorts with additional 46 plasma samples in women with PTL and additional 54 plasma samples in those with PPROM. A total of 74 women with PTL and 82 women with PPROM were recruited for validation work of the second phase of this study.

2-2. Plasma sample collection and processing

At the time of recruitment, maternal blood samples were obtained <96 h before delivery and collected into EDTA tubes. The samples were immediately centrifuged at 1500 g at 4°C for 10 minutes and the supernatant was aliquoted and stored at -70°C until assayed. The samples of blood were collected before administering the medications such as antibiotics, tocolytics and corticosteroids.

2-3. Membrane-based human antibody array in the second phase of the study: discovery work

RayBio® Biotin Label-based Human Antibody Array L-series Membrane Kit (Catalog No: AAH-BLM-1000-4; RayBiotech Inc., Norcross, GA, USA) was used to compare the differences in protein expression levels in the plasma of women with HCA versus the controls. This antibody array membrane is marked with 507 specific antibodies against various proteins including cytokines, chemokines, protease, or other immunoregulatory proteins. The assay was performed in duplicate according to the manufacturer's protocol, and its methods have been previously described in detail.[21] To compare the profiles of immunoregulatory proteins in the plasma of women with HCA and those without HCA, an equivalent protein load of individual plasma samples was pooled in the HCA (n=14) and non-HCA (n=14) groups in PTL and PPROM patients, respectively. A volume of plasma corresponding to 35.7 ㎍ was separated from each sample. Separated plasmas from each group (500 ㎍ per group) were pooled, mixed, and incubated with Biotin-labeling reagent at room temperature for 30 min on a rotator. The tube was tapped gently to mix the reaction solution every 5 min. After incubation, 5 ㎕ stop solution was added into the reaction solution. The samples were then centrifuged at 1000 g for 3 min using a spin column to remove any unbound biotin and the filtered samples were collected. Each array membrane in the tray was blocked with 8 ㎖ of blocking buffer and incubated at room temperature with gentle shaking for 1 hr. After aspirating the blocking buffer from each tray, 10 ㎖ of diluted sample onto each membrane was added and incubated at 4℃ on the shaker overnight. The membranes were washed 3 times with a washing butter at room temperature on the shaker for 5 min per wash and then incubated with 10 ㎖ of HRP-Conjugated Streptavidin at room temperature for 2 hr. After incubation, the membranes were washed 3 times and 4 ㎖ of detection buffer was pipetted onto each membrane and incubated at room temperature for 2 min with gentle shaking. The membrane was placed on a plastic film and covered with another plastic film. The spot signal of the membrane was detected directly using chemiluminescence imaging system (Bio-Rad ChemiDoc XRS™ Systems; Bio-Rad Laboratories, Inc.) and quantified using

chemiluminescence image analysis (Bio-Rad Quantity 4.6.7; Bio-Rad Laboratories, Inc.). The measured densities were exported to a Microsoft Excel spreadsheet. The background intensity was subducted before analysis and spot signal intensities were normalized as a percentage of positive controls on each membrane. The spot selection criteria were as follows: (1) the spots in which the quantitative values of the two groups differ by 1.9 times or more were selected, and (2) the spots were confirmed again by the naked eye.

2-4. ELISA validation

Commercially available ELISA kits (DuoSet, R&D System, Minneapolis, MN, USA) were used to further quantify selected target proteins showing different signal intensities in 28 plasma samples of the discovery work and additional 46 plasma samples in women with PTL (44 women with HCA versus 30 women without HCA). In women with PPROM, validation was performed in 28 plasma samples of the discovery work and additional 54 plasma samples (35 women with HCA versus 47 without HCA). The ranges of the MMP-9, TIMP-1, IL-6, angiopoietin-2, and S100 A8/A9 standard curves were 31.2–2000 pg/mL, 15.6–1000 pg/mL, 0.156-5 pg/mL, 93.8-6000 pg/mL, and 93.8-6000 pg/mL, respectively. Prior to the measurement of these proteins, the plasma samples were diluted using the ratio 1:500 for MMP-9 and S100 A8/A9, 1:100 for TIMP-1, 1:5 for IL-6, and 1:4 for angiopoietin 2. All the samples were assayed in duplicate. The intra- and inter-assay coefficients of variation were <10% for all analyzed proteins.

Definitions of various factors

Gestational age was calculated based on the last menstrual period and the first trimester or second trimester (≤20 weeks) ultrasound results, when available. The diagnostic criteria for preterm labor and PPROM were previously described in detail elsewhere.[24, 31] Management of PTL, PPROM, and

clinical chorioamnionitis has been previously described in detail elsewhere.[24, 31] MIAC was defined as the presence of microorganisms in an AF culture. Acute HCA was diagnosed when acute inflammation was observed in any placental tissue samples (amnion, chorion-decidua, umbilical cord, and chorionic plate). The presence of acute inflammation was noted and classified as grade 1 or 2 (Table 1) according to previously published criteria.[10] Funisitis was diagnosed when neutrophil infiltration was observed in the umbilical vessel walls or Wharton's jelly. Intra-amniotic inflammation was defined as elevated AF levels of IL-6 and/or IL-8 ($\geq$1.5 and/or $\geq$1.3 ng/mL, respectively).[32] Clinical chorioamnionitis was diagnosed based on the criteria proposed by Gibbs et al.[33] Early-onset neonatal sepsis, respiratory distress syndrome (RDS), bronchopulmonary dysplasia (BPD), intraventricular hemorrhage (IVH) of grade II or higher, periventricular leukomalacia (PVL), and necrotizing enterocolitis (NEC) were diagnosed according to the definitions previously described in detail.[34] Composite morbidity was defined as the presence of any of these complications. Of these criteria, a description about RDS, BPD, and NEC was specifically shown as follows: (1) RDS was diagnosed in the presence of respiratory grunting and retracting, an increased oxygen requirement (forced inspiratory oxygen capacity >0.4), and diagnostic radiographic and laboratory findings in the absence of evidence of other causes of respiratory disease;[10] (2) BPD was diagnosed according to the National Institutes of Health workshop consensus definition,[35] which uses oxygen dependency at 36 weeks postmenstrual age (plus a total oxygen duration of $\geq$28 days), delineates severity (e.g. mild, moderate or severe), and distinguishes between infants less than or more than 32 weeks' gestation in terms of diagnostic criteria; (3) NEC was defined in infants with stage II or higher according to the modified Bell's staging criteria.[36]

Histopathological examination of the placentas

Placental examination was performed in both phases of the study as follows: A full-thickness section of the placenta, including the maternal and fetal surfaces, a membrane roll, and a section of the

umblilical cord which was sectioned at 1 cm intervals were obtained for histologic evaluation. Tissue samples of the placenta were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of the tissue blocks were stained with hematoxylin and eosin. Histopathological examination was performed by experienced board-certificated pathologists. The results of the protein assay in the plasma were not available to the pathologists and clinicians.

Statistical analyses

All statistical analyses were performed by using SPSS version 20.0 for Windows (IBM SPSS Statistics, Chicago, IL, USA).

In the first phase of the study, continuous data are presented as mean ± standard deviation; and dichotomous data, as frequency and associated percentage. Shapiro-Wilk and Kolmogorov-Smirnov tests were used to evaluate whether the data are normally distributed. As the continuous data were not normally distributed, non-parametric tests were used for the analyses. The Kruskal-Wallis test was used to test for comparison of continuous data among 3 groups. Multiple comparisons between the groups were performed by using the Mann-Whitney U test for continuous data and the χ^2 or Fisher exact test for categorical data, adjusted with Bonferroni correction ($P<0.0167$). Multivariable logistic regression analysis was used to examine the relationship between the HCA with a negative AF culture, neonatal outcome, and inflammatory status in AF after adjusting for compounding factors (the effect of gestational age and fetal membrane status).

In the second phase of the study, demographic and clinical characteristics and candidate biomarkers of interest were compared using the Student's *t*-test or Mann-Whitney U test for continuous data and Fisher's exact test or χ^2-test for categorical data, as appropriate. Continuous data are expressed as the mean and standard deviation, whereas categorical are presented as the percentage and number of patients in the groups. In terms of comparison of ELISA data between HCA and non-HCA groups, a logistic regression analysis was further performed to examine the independent

relationship of candidate biomarkers levels of interest in the plasma to the HCA after controlling for potential confounding factors. Receiver operating characteristic (ROC) curves based on the HCA were generated for each candidate protein and serum CRP and used to identify the optimal cut-off value (maximizing the sum of sensitivity and specificity) and the best biomarkers in the maternal plasma. The areas under the ROC curves (AUCs) for the candidate proteins and serum CRP were measured, and compared using the method by DeLong et al.[37] All reported *P* values are two-sided, and *P* values <0.05 were considered statistically significant.

Table 1. Histological grade for acute intrauterine inflammation

Amnion and chorion-decidua
Grade 1: at least one focus of > 5 neutrophils
Grade 2: diffuse neutrophilic infiltration
Umbilical cord
Grade 1: neutrophilic infiltration confined to umbilical vessel wall
Grade 2: extension of neutrophilic infiltration into Wharton's jelly
Chorionic plate
Grade 1: > 1 focus of at least 10 neutrophilic collections or diffuse inflammation in subchorionic plate
Grade 2: diffuse and dense inflammation, neutrophilic infiltration into connective tissue of placental plate, or placental vasculitis

Results

1. The first phase of the study

1-1. Clinical characteristics and pregnancy outcomes

During the study period, 159 consecutive women with preterm labor (n=83) and PPROM (n=76) who met the inclusion criteria were identified. Of the 159 women, 5 had no available AF samples for retrospective analysis of IL-6, IL-8, and MMP-9 levels and one had no available placental pathological report. Thus, 153 women (95%) were included in the final analysis. Among the 153 women, 50% (76/153) had HCA and 20% (31/153) had funisitis. The prevalence of a positive AF culture was 35% (53/153). Of the 53 women with a positive AF culture, 13 (25%) had no evidence of HCA, of whom 12 had PPROM and one had preterm labor. The microorganisms that were isolated from the amniotic cavity included *Ureaplasma urealyticum* (n=38), *Mycoplasma hominis* (n=30), *Streptococcus viridans* (n=5), *Streptococcus agalactiae* (n=2), *Staphylococcus aureus* (n=2), *Staphylococcus mitis* (n=1), *Staphylococcus oralis* (n=1), *Escherichia coli* (n=1), *Lactobacillus* species (n=1), unidentified gram-positive cocci (n=3), unidentified gram-negative rod (n=1), and unidentified gram-positive rod (n=1). Polymicrobial invasion was present in 31 cases (58%, 31/53). HCA was present in 36% (36/100) of cases with a negative AF culture and in 75% (40/53) of those with a positive AF culture.

Table 2 shows the clinical characteristics and pregnancy outcomes of the study population according to the results of the placental histological examination and AF culture. Women with HCA but a negative AF culture (group 2) and those with a positive AF culture (group 3) had a significantly lower mean gestational age at amniocentesis and delivery, a higher mean maternal serum CRP level and WBC count, and a higher rate of funisitis than those with a negative AF culture without HCA (group 1). However, no significant differences in these independent parameters were found between the women in group 2 and those in group 3. AF IL-6, IL-8, and MMP-9 levels, and the rate of intra-amniotic inflammation were highest in group 3, followed by group 2, and lowest in group 1 (Fig 1). In

terms of AF IL-6 level and the rate of intra-amniotic inflammation, these results were not changed after adjusting for potential confounders such as gestational age at amniocentesis and the state of fetal membranes, showing significant differences among the 3 groups. When compared with the women in group 1, the women in group 3 had significantly more frequent clinical chorioamnionitis, but not those in group 2. However, the mean maternal age and rates of nulliparity, PPROM, antibiotic administration, corticosteroid treatment, and cesarean delivery did not differ among the 3 groups of women.

1-2. Neonatal outcomes

Measures of neonatal outcomes for the 3 groups based on the results of the placental histological examination and AF culture are shown in Table 3. Of a total of 153 neonates, sixteen who could not be evaluated were excluded from the analysis because of fetal death in utero (n=1) and extreme prematurity before viability (n=15). Of these 16, 4 were in group 1, 4 in group 2, and 8 in group 3, respectively. Composite neonatal morbidity was significantly higher among the women in groups 2 and 3 than among those in group 1. However, this was no longer significant after adjusting for confounders caused mainly by the impact of gestational age. The incidence rates of early-onset neonatal sepsis, RDS, BPD, IVH, PVL, NEC, and death showed only slight differences between neonates born to women in groups 1, 2, and 3, except that the incidence rates of RDS in group 3 and BPD in group 2 were significantly increased compared with those in group 1. However, these were also insignificant increases after adjusting for confounders (gestational age and the state of fetal membranes).

2. The second phase of the study

2-1. Membrane-based protein microarray profiles: discovery work

To assess differences in the maternal plasma protein profile between women with and without HCA in PTL patients, plasma proteins of 28 women were analysed firstly by membrane-based microarray assay. Plasma samples from 14 women with HCA (cases) were pooled against 14 pooled plasma samples from 14 women without HCA (controls). As a result of matching, there were no significant differences between the groups (Table 4). The final analysis revealed that twelve human proteins, including angiopoietin-2, angiopoietin-like 2, angiostatin, BMP-2, BMP-4, BMPR-1A/ALK-3, BMPR-1B/ALK-6, BMPR-II, CXCR4 (fusin), GDF3, M-CSF, and S100 A8/A9, were detected at higher densities in patients with HCA as compared to the control group (Fig. 2). The density ratios between groups were 1.9, 2.3, 2.2, 2.3, 1.9, 2.1, 2.1, 2.6, 2.2, 2.3, 2.1, and 2.3, respectively.

In patients with PPROM, plasma samples from 14 women with HCA (cases) were pooled against 14 pooled plasma samples from 14 women without HCA (controls). Table 5 describes baseline demographic and clinical characteristics of 28 women with PPROM involved in the discovery work. No factors were different between the groups (matched by gestational age at sampling). Fourteen proteins, including 6Ckine, BMP-2, CCR4, CD40/TNFRSF5, CXCR5/BLR-1, CXCR6, IL-6, LIF R alpha, MMP-9, Neuropilin-2, Pref-1, S100 A8/A9, TIMP-1, and TSG-6, were detected at higher densities in subjects with HCA as compared to the controls (Fig. 3). The density ratios between groups were 5.7, 2.9, 3.4, 3.3, 3.4, 3.4, 2.2, 3.1, 3.9, 3.0, 2.9, 2.0, 3.0 and 3.5, respectively.

2-2. Validation of target proteins by ELISA

Of these various molecules, we selected S100 A8/A9 and angiopoietin-2 in PTL group and S100 A8/A9, MMP-9, TIMP-1, and IL-6 in PPROM group to validate and compare the levels of candidate proteins between women with HCA and the controls using by ELISA kits. Selected proteins, which were available in our laboratory and thus chosen, were validated in women with PTL (n=74) with

additional 46 plasma samples and in women with PPROM (n=82) with additional 54 plasma samples, adhering to subjects eligibility criteria described above.

The demographic and clinical characteristics and plasma concentration of selected candidate proteins in women with PTL according to the presence or absence of HCA were described in Table 6. Women with HCA in PTL group had a significantly lower mean gestational age at sampling and higher rate of positive AF culture result and mean AF IL-6, serum CRP, and plasma S100 A8/A9 levels than did those in PTL group without HCA. However, the plasma levels of angiopoietin-2 was not significantly different between the groups.

Table 7 shows the demographic and clinical characteristics and plasma protein levels of women presenting with PPROM according to the presence or absence of HCA. Women with HCA in PPROM group had a significantly lower mean gestational age at sampling and higher rates of tocolytic therapy, administration of corticosteroids, and positive AF culture result than those without HCA. The mean plasma levels of S100 A8/A9, MMP-9 and IL-6, but not TIMP-1, were significantly higher in women with HCA, compared with those without HCA.

Although several candidate biomarkers as above were revealed to have differences between women with and without HCA, gestational ages at sampling were significantly different between the groups in PTL and PPROM and thus multivariate logistic regression analysis were performed whether this significance was maintained or not after adjusting for potential confounding factors. Any target molecules were not remained significant after adjusting for the confounding factor such as gestational age at sampling in women with PTL (Table 8). However, elevated levels of maternal plasma MMP-9, but not S100 A8/A9 and IL-6, was still significantly associated with HCA in women with PPROM after adjusting for gestational age at sampling, and use of tocolytics and corticosteroids (Table 9).

ROC curves based on HCA were generated for plasma MMP-9 and serum CRP in Figure 4. Table 10 presents diagnostic indices of serum CRP and plasma MMP-9 to predict HCA in women

with PPROM. The AUCs, sensitivities, and specificities were not significantly different between serum CRP and plasma MMP-9 for predicting HCA in women with PPROM (P >0.05).

Table 2. Clinical characteristics and pregnancy outcome of the study population according to the results of placental histological examination and amniotic fluid (AF) culture[a]

	Negative AF culture						Positive AF culture		
Characteristic	HCA negative (group 1; n=64)	*P* value[b] Un-adjusted	*P* value[b] Adjusted[e]	HCA positive (group 2; n=36)	*P* value[c] Un-adjusted	*P* value[c] Adjusted[e]	Group 3 (n=53)	*P* value[d] Un-adjusted	*P* value[d] Adjusted[e]
Maternal age (years)	30.8 ± 3.4	.252	.226	31.7 ± 4.4	.737	.854	31.4 ± 3.2	.363	.072
Nulliparity	50% (32)	.423	.549	42% (15)	.871	.819	43% (23)	.476	.389
Membrane status									
Intact membranes	48% (31)	.021	-	72% (26)	**.007**	-	43% (23)	.586	-
Preterm PROM	52% (33)			28% (10)			57% (30)		
Gestational age at amniocentesis (weeks)[f]	32.0 ± 3.7	**.001**	-	30.1 ± 3.8	.469	-	29.3 ± 4.3	**< .001**	-

Gestational age at delivery (weeks)[f]	32.1 ± 3.7	**.001**	-	30.2 ± 3.8	.462	-	29.4 ± 4.3	**< .001**	-
Antibiotics	68% (43)	.321	.651	58% (21)	.019	.321	81% (43)	.115	.424
Corticosteroids	48% (31)	.223	.405	61% (22)	.771	.507	64% (34)	.089	.021
Cesarean delivery	44% (28)	.946	.770	44% (16)	.169	.141	30% (16)	.132	.714
Maternal WBC (10^3/mm³)[g]	10.7 ± 3.7	**.003**	.072	12.6 ± 3.3	.767	.820	12.6 ± 3.8	**.003**	.033
Maternal CRP (mg/dL)[f]	0.6 ± 1.0	**< .001**	.031	1.6 ± 1.8	.201	.139	2.2 ± 2.1	**< .001**	**.001**
AF WBC counts (10^2/mm³)[f]	0.5 ± 2.3	.306	.037	5.4 ± 13.4	**< .001**	.037	32.9 ± 103.4	**< .001**	**.001**
AF IL-6 (ng/ml)[f]	1.4 ± 2.1	**< .001**	**.005**	8.0 ± 0.01	**.004**	**.003**	28.3 ± 0.03	**< .001**	**.001**
AF IL-8 (ng/ml)[f]	1.0 ± 2.0	**< .001**	.032	6.4 ± 12.6	**< .001**	**.001**	30.4 ± 32.2	**< .001**	**< .001**
AF MMP-9 (ng/ml)[f]	3.8 ± 11.8	**.001**	.301	208.8 ± 500.7	**< .001**	**.005**	627.7 ± 734.4	**< .001**	**< .001**
Intraamniotic inflammation[h]	25% (16)	**< .001**	**.002**	67% (24)	.043	**.005**	85% (45)	**< .001**	**< .001**
Funisitis	0% (0)	**.001**	**.008**	22% (8)	.040	.081	43% (23)	**< .001**	**.001**

Clinical chorioamnionitis	2% (1)	> .999	.764	3% (1)	.025	.099	19% (10)	**.002**	.047

AF, amniotic fluid; HCA, histologic chorioamnionitis; PROM, premature rupture of membranes; WBC, white blood cell; CRP, C-reactive protein; IL, interleukin; MMP, matrix metalloproteinase.

Data are mean ± standard deviation, analyzed by Mann-Whitey *U* test among the 3 groups; and % (n), analyzed by χ^2 test or Fisher's exact test among the 3 groups.

[a]Significant findings ($p < 0.0167$) after Bonferroni correction are presented in bold letters.

[b]Comparison between group 1 and 2.

[c]Comparison between group 2 and 3.

[d]Comparison between group 3 and 1.

[e]Adjusted for gestational age at amniocentesis and the state of fetal membranes (logistic regression analysis).

[f]$P < .001$, by Kruskal-Wallis analysis of variance test.

[g]$P < .01$, by Kruskal-Wallis analysis of variance test.

[h]Intra-amniotic inflammation was defined as elevated AF levels of IL-6 (≥1.5 ng/mL) and/or IL-8 (≥1.3 ng/mL).

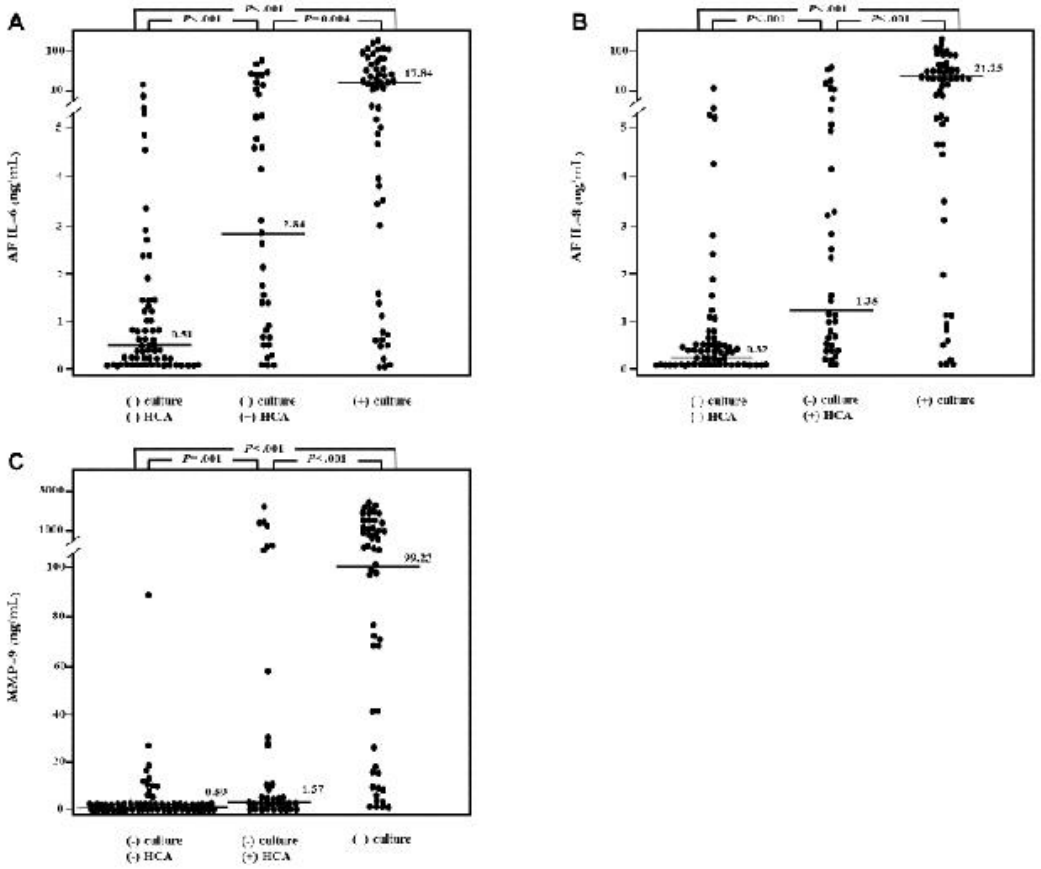

Figure 1. Amniotic fluid (AF) interleukin (IL)-6, IL-8, and metalloproteinase-9 (MMP-9) levels of the study population according to the results of the placental histological examination and AF culture. AF IL-6, IL-8, and MMP-9 levels were lowest in women with a negative AF culture without histologic chorioamnionitis (HCA) (group 1), followed by those with HCA but a negative AF culture (group 2), and highest in those with a positive AF culture (group 3) (AF IL-6: group 1, median, 0.509 ng/mL [range, 0.004–11.825 ng/mL] vs. group 2, median, 2.842 ng/mL [range, 0.047–59.934 ng/mL] vs. group 3, median, 17.839 ng/mL [range, 0.009–104.121 ng/mL]; AF IL-8: group 1, median, 0.318 ng/mL [range, 0.012–11.492 ng/mL] vs. group 2, median, 1.348 ng/mL [range, 0–52.335 ng/mL] vs. group 3, median, 21.253 ng/mL [range, 0.046–113.969 ng/mL]; AF MMP-9: group 1, median, 0.593 ng/mL [range, 0–87.920 ng/mL] vs. group 2, median, 1.572 ng/mL [range, 0–2267.941 ng/mL] vs. group 3, median, 99.232 ng/mL [range, 0–2303.970 ng/mL]; each *P*-value is shown on the graph).

Table 3**.** Neonatal outcome of the study population according to the results of the placental histological examination and amniotic fluid (AF) culture[a]

	Negative AF culture						Positive AF culture		
		P value[b]			*P* value[c]			*P* value[d]	
Neonatal outcome	HCA negative (group 1; n=64)	Un-adjusted	Adjusted[e]	HCA positive (group 2; n=36)	Un-adjusted	Adjusted[e]	(Group 3; n=53)	Un-adjusted	Adjusted[e]
Birthweight (kg)[f]	1.97 ± 0.68	.028	−	1.66 ± 0.67	.353	−	1.51 ± 0.70	**.001**	−
Apgar <7 at 1min[g]	43% (26/60)	.042	.171	66% (21/32)	.616	.279	60% (27/45)	.091	.922
Apgar <7 at 5 min[g]	7% (4/60)	**.004**	.067	31% (10/32)	.694	.995	36% (16/45)	**< .001**	.053
Composite neonatal morbidity[g]	20% (12/60)	**.003**	.217	50% (16/32)	.630	.725	56% (25/45)	**< .001**	.088
EONS[g]	3% (2/60)	.047	.377	16% (5/32)	> .999	.988	16% (7/45)	.036	.457
RDS[g]	13% (8/60)	.018	.710	34% (11/32)	.924	.411	33% (15/45)	**.014**	.925
BPD[h]	8% (5/60)	**.014**	.370	29% (9/31)	.456	.141	21% (9/42)	.059	.363

NEC[h]	5% (3/60)	> .999	.318	7% (2/31)	> .999	.933	7% (3/42)	.688	.505
IVH (grade 2 or more)[h]	2% (1/60)	> .999	.553	3% (1/31)	.387	.262	10% (4/42)	.156	.308
PVL[h]	5% (3/60)	> .999	.584	3% (1/31)	.127	.069	17% (7/42)	.087	.134
Neonatal mortality	0% (0/60)	.355	> .999	3% (1/33)	.634	.676	7% (3/45)	.076	.999

AF, amniotic fluid; HCA, histologic chorioamnionitis; EONS, early onset neonatal sepsis; RDS, respiratory distress syndrome; BPD, bronchopulmonary dysplasia; NEC, necrotizing enterocolitis; IVH, intraventricular hemorrhage; PVL, periventricular leukomalacia.

Data are mean ± standard deviation, analyzed by Mann-Whitey *U* test among the 3 groups; and % (n/N), analyzed by χ^2 test or Fisher's exact test among the 3 groups.

[a]Significant findings ($p < 0.0167$) after Bonferroni correction are presented in bold letters.

[b]Comparison between group 1 and 2.

[c]Comparison between group 2 and 3.

[d]Comparison between group 3 and 1.

[e]Adjusted for gestational age at delivery and the state of fetal membranes (logistic regression analysis).

[f]$P < .01$, by Kruskal-Wallis analysis of variance test.

[g]Sixteen infants were excluded from the analysis because they died in utero after amniocentesis (n=1) or were not actively resuscitated at birth because of extreme prematurity (n=15) and thus could not be evaluated with respect to the presence or absence of complications.

[h]Based on 133 subjects who survived for at least 30 days after birth.

Table 4. Demographic and clinical characteristics of women with preterm labor involved in the discovery work using membrane-based microarray assay

	Histologic chorioamnionitis		*p*-value
	Absent (n =14)	Present (n = 14)	
Age (years)	32.2 ± 3.4	32.5 ± 4.1	.764
Nulliparity	35.7% (5/14)	35.7% (5/14)	1.000
Gestational age at sampling (weeks)	31.3 ± 2.4	30.8 ± 2.5	.475
Gestational age at delivery (weeks)	31.4 ± 2.4	30.9 ± 2.5	.476
Positive amniotic fluid culture	7.7% (1/13)	28.6% (4/14)	.326
Use of tocolytics	78.6% (11/14)	92.9% (13/14)	.596
Use of corticosteroids	85.7% (12/14)	78.6% (11/14)	1.000
Use of antibiotics	21.4% (3/14)	28.6% (4/14)	1.000

Data are given as the mean ± SD or % (n).

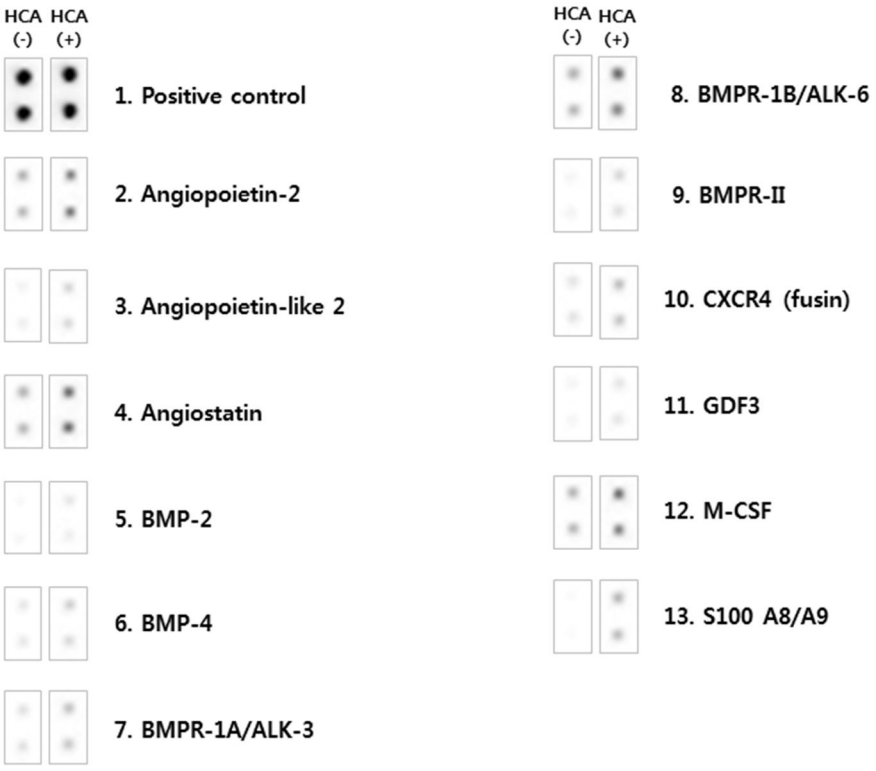

Figure 2. A list of the molecules with significantly different levels of expression in the maternal plasma of women with preterm labor and histologic chorioamnionitis versus control subjects (without histologic chorioamnionitis). Twelve proteins exhibited increased density in women with histologic chorioamnionitis

Table 5. Demographic and clinical characteristics of women with preterm premature rupture of membrane involved in the discovery work using membrane-based microarray assay

	Histologic chorioamnionitis		*p*-value
	Absent (n =14)	Present (n = 14)	
Age (years)	32.4 ± 3.3	32.4 ± 3.8	.926
Nulliparity	42.9% (6/14)	50.0% (7/14)	.705
Gestational age at sampling (weeks)	32.4 ± 1.4	31.8 ± 1.5	.221
Gestational age at delivery (weeks)	32.5 ± 1.3	31.9 ± 1.6	.357
Positive amniotic fluid culture	35.7% (5/14)	71.4% (10/14)	.058
Use of tocolytics	50.0% (7/14)	57.1% (8/14)	.705
Use of corticosteroids	85.7% (12/14)	85.7% (12/14)	1.000
Use of antibiotics	92.9% (13/14)	100% (14/14)	1.000

Data are given as the mean ± SD or % (n).

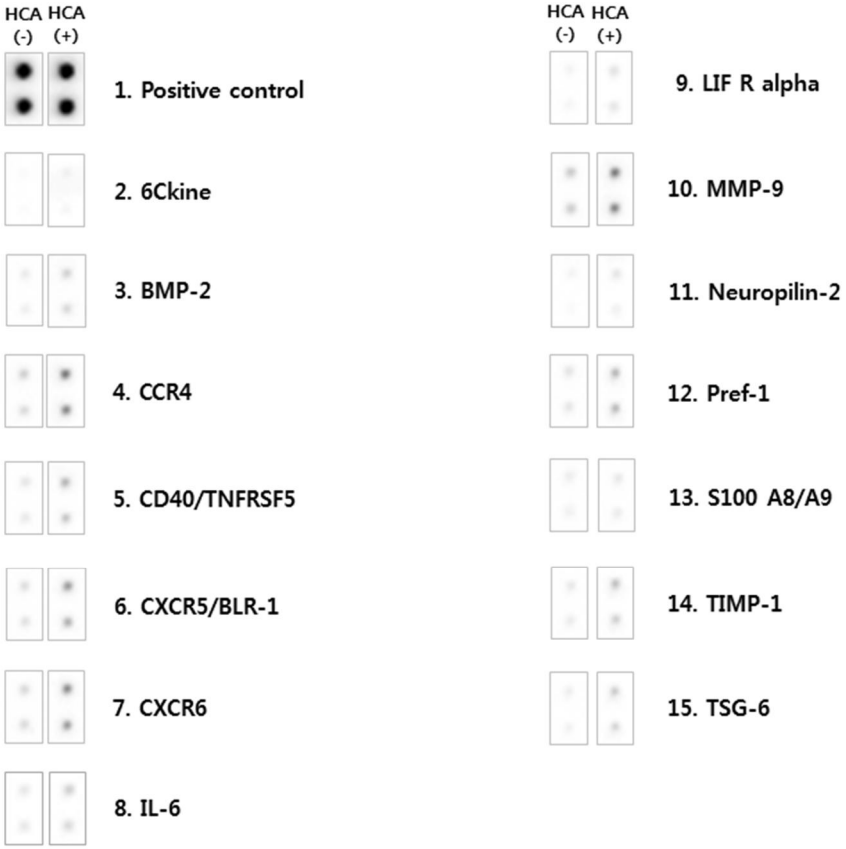

Figure 3. A list of the molecules with significantly different levels of expression in the maternal plasma of women with preterm premature rupture of membranes and histologic chorioamnionitis versus control subjects (without histologic chorioamnionitis). Fourteen proteins exhibited higher density in women with histologic chorioamnionitis

Table 6. Demographic and clinical characteristics of women presenting with preterm labor according to the presence or absence of histological chorioamnionitis

	Histologic chorioamnionitis		*p*-value
	Absent (n =30)	Present (n = 44)	
Age (years)	32.27 ± 3.7	33.02 ± 4.2	.429
Nulliparity	50.0% (15)	31.8% (14)	.116
Gestational age at sampling (weeks)	32.0 ± 2.3	29.9 ± 3.4	.011
Gestational age at delivery (weeks)	32.1 ± 2.4	30.1 ± 3.4	.015
Amniotic fluid IL-6 (ng/mL)	1.97 ± 2.80	22.51 ± 28.92	< .001
Positive amniotic fluid culture	3.3% (1)	45.5% (20)	< .001
Serum CRP (mg/dL)	0.91 ± 1.61 (28)	1.97 ± 1.80	.002
Plasma S100 A8/A9 (ng/mL)	295.09 ± 252.71	425.62 ± 306.91	.023
Plasma angiopoietin-2 (ng/mL)	3.99 ± 2.86	5.40 ± 5.54	.226
Use of tocolytics	78.6% (22)	81.8% (36)	.734
Use of corticosteroids	73.3% (22)	81.8% (36)	.384
Use of antibiotics	43.3% (13)	50.0% (22)	.573
Clinical chorioamnionitis	0% (0)	6.8% (3)	.267
Funisitis	0%(0)	38.6% (17)	< .001

CRP, C-reactive protein; IL, interleukin; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase-1; IL-6, interluekin-6.

Data are given as the mean ± SD or % (n).

Table 7. Demographic and clinical characteristics of women presenting with preterm premature rupture of membrane according to the presence or absence of histological chorioamnionitis

	Histologic chorioamnionitis		*p*-value
	Absent (n =47)	Present (n = 35)	
Age (years)	30.9 ± 3.6	31.6 ± 4.1	.436
Nulliparity	46.8% (22)	48.6% (17)	.874
Gestational age at sampling (weeks)	33.2 ± 1.2	30.7 ± 2.7	< .001
Gestational age at delivery (weeks)	33.5 ± 1.2	30.9 ± 2.7	< .001
Amniotic fluid IL-6 (ng/mL)	0.96 ± 1.31	16.85 ± 24.52	< .001
Positive amniotic fluid culture	23.4% (11)	51.4% (18)	.009
Serum CRP (mg/dL)	0.42 ± 0.31 (45)	2.09 ± 2.06	< .001
Plasma S100 A8/A9 (ng/mL)	256.05 ± 245.66	488.39 ± 364.40	< .001
Plasma MMP-9 (ng/mL)	146.44 ± 87.40	301.10 ± 197.88	< .001
Plasma TIMP-1 (ng/mL)	94.20 ± 23.70	100.30 ± 27.30	.341
Plasma IL-6 (pg/mL)	3.44 ± 2.39	11.62 ± 12.65	.001
Use of tocolytics	29.8% (14)	74.3% (26)	< .001
Use of corticosteroids	60.9% (28)	85.7% (30)	.014
Use of antibiotics	95.7% (44)	91.4% (32)	.647
Clinical chorioamnionitis	0% (0)	11.4% (4)	.030
Funisitis	0% (0)	40.0% (14)	< .001

IL, interleukin; CRP, C-reactive protein; MMP-9, matrix metalloproteinase-9; TIMP-1, tissue inhibitor of metalloproteinase-1.

Data are given as the mean ± SD or % (n).

Table 8. Multivariable logistic regression model showing the unadjusted and adjusted odds ratios of association between various proteins in maternal plasma and histologic chorioamnionitis in preterm labor

Variables	Odds ratio (95% confidence interval)		*p*-value[b]
	Unadjusted	Adjusted[a]	
Plasma S100 A8/A9 (ng/mL)	1.002 (1.000 - 1.004)	1.001 (0.999 - 1.003)	.200
Plasma angiopoietin-2 (ng/mL)	1.087 (0.951 - 1.241)	1.047 (0.928 - 1.181)	.456

[a] Adjustment for gestational age at sampling.

[b] For the adjusted odds ratio.

Table 9. Multivariable logistic regression model showing the unadjusted and adjusted odds ratios of association between various proteins in maternal plasma and histologic chorioamnionitis in preterm premature rupture of membranes

Variables	Odds ratio (95% confidence interval)		*p*-value[b]
	Unadjusted	Adjusted[a]	
Plasma S100 A8/A9 (ng/mL)	1.003 (1.001 - 1.004)	1.002 (1.000 - 1.004)	.124
Plasma MMP-9 (ng/mL)	1.010 (1.005 - 1.015)	1.009 (1.003 - 1.016)	.004
Plasma IL-6 (pg/mL)	1.225 (1.069 - 1.404)	1.148 (0.987 - 1.334)	.073

MMP-9, matrix metalloproteinase-9; IL-6, interluekin-6.

[a] Adjustment for gestational age at sampling, and use of tocolytics and corticosteroids.

[b] For the adjusted odds ratio.

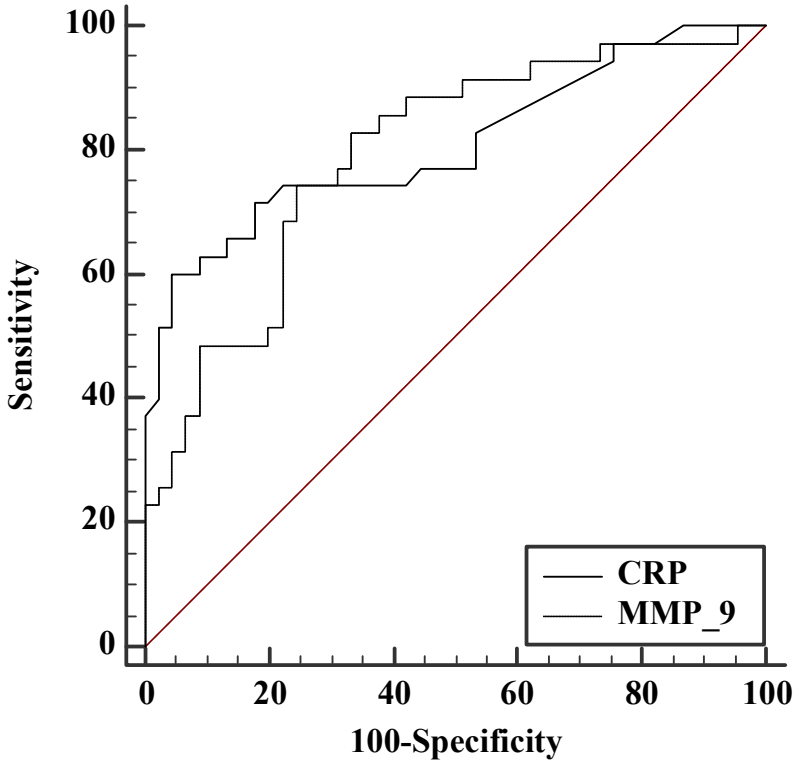

Figure 4. Receiver operating characteristics curve analysis of serum C-reactive protein and plasma matrix metalloproteinase-9 for predicting histologic chorioamnionitis in preterm premature rupture of membranes.

Table 10. Diagnostic indices of serum C-reactive protein levels and plasma matrix metalloproteinase-9 to predict histologic chorioamnionitis in preterm premature rupture of membranes

Variables	Area (±SE) under the ROC curve	95% CI	Cut-off value[a]	Sensitivity[b] (95% CI)	Specificity[b] (95% CI)	PPV[b]	NPV[b]
Serum CRP (mg/dL)	0.808 ± 0.052	0.706 – 0.910	0.55	74.3 (56.7 – 87.5)	77.8 (62.9 – 88.8)	72.2	79.6
Plasma MMP-9 (ng/mL)	0.788 ± 0.052	0.686 – 0.889	175.91	74.3 (56.7 – 87.5)	74.5 (59.7 – 86.1)	68.4	79.6

ROC, receiver operating characteristics; SE, standard error; CI, confidence interval; CRP, C-reactive protein; MMP-9, matrix metalloproteinase-9; PPV, positive predictive value; NPV, negative predictive value.

[a]Cut-off values corresponding to the highest sum of sensitivity and specificity.

[b]Values are given as % (95% CI).

The areas under the ROC curves, sensitivities, and specificities **were not significantly different between** plasma MMP-9 and serum CRP testing for detecting HCA in women with PPROM (McNemar's test, $P > 0.05$).

Discussion

In the first phase of this study, we show that (1) among women who delivered preterm neonates owing to PTL or PPROM, the prevalence of HCA with a negative AF culture was 23% and that (2) similar to pregnant women with positive AF cultures, women with HCA with a negative AF culture are at increased risk of clinical presentation of symptoms and delivery at earlier gestational ages, intra-amniotic inflammation, and prematurity-associated composite neonatal morbidity. These findings underscore the role of HCA, regardless of AF culture results, as a major risk factor of preterm birth and adverse perinatal outcome, and the need to develop novel rapid biomarkers with greater sensitivity, especially noninvasive ones, for detecting early HCA in the preterm setting. Therefore, we designed the second phase of the study to identify the best novel biomarkers in maternal plasma for predicting HCA non-invasively.

In regard to HCA and MIAC in women who delivered preterm neonates, the prevalence, inverse correlation with gestational age at presentation and birth, and the relationship with adverse outcome are in line with the observations previously reported by our group and other groups.[6, 7, 10, 24, 34] Moreover, in the present study, the frequency of HCA with negative AF culture was 23%, also in keeping with the findings previously reported by Yoon et al. and our group.[7, 10] The most plausible causes of HCA in women with negative AF culture are as follows: (1) early stage of ascending intrauterine infection in which microorganisms reside in the decidua; (2) an extra-amniotic infection; (3) intra-amniotic viral infection; (4) non-infectious cause of placental inflammation; and (5) MIAC that do not grow in standard culture conditions because of a small inoculum size but can be detected by molecular methods such as polymerase chain reaction (PCR). In support of our speculation regarding inoculum size, a previous study by Kacerovsky et al. demonstrated that the presence of HCA in women with PPROM was associated with a higher bacterial load of genital mycoplasma DNA in AF.[38] On the contrary, we found that 25% (13/53) of women with MIAC had no evidence of

HCA, of whom 92% (12/13) had PPROM and 8% (1/13) had PTL. This is likely to be due to the possibility that microorganisms directly traverse intact membranes or that microbial invasion may have developed in the interval between amniocentesis and delivery, especially by an ascending route through ruptured membranes in cases with PPROM.[39]

As mentioned above, acute HCA is not equated with MIAC. Acute inflammatory lesions of the placenta have been considered to reflect MIAC. However, MIAC was found in approximately one third in patients with HCA.[10, 40] Cultivation-based microbiologic methods require several conditions (i.e., species that grow rapidly on generic media, under aerobic conditions, and at moderate temperatures) to detect microbial invasion. Therefore, it has been possible that species recovered most frequently in culture methods might not be numerically dominant nor of foremost clinical significance among the microorganisms present in a sampled environment.[41] In regard to investigation of the potential association between negative AF culture in women with HCA and a small inoculum size into the AF that cannot be detected by conventional culture technique or non-viable microorganisms that may release chemotactic factors that lead to placental inflammation, molecular microbiologic approaches such as PCR enable the detection of microorganisms more sensitively. Yoon et al, reported that the prevalence of *Ureaplasma urealyticum* in women with PPROM was 28% (43/154) based on taxon-specific PCR and 16% (25/154) based on culture.[42] Moreover, a study using a panel of 16 taxon-specific PCR assays found the prevalence of MIAC to be 46% by means of PCR and 12% by means of culture in women with PTL and intact membranes.[43] These studies based on taxon-specific PCR demonstrated that the true rate of MIAC caused by *Ureaplasma urealyticum* was higher than that indicated by culture methods. Based on broad-range PCR using multiple taxa, it was reported to be higher rates of MIAC by 1.5-3.5 times the number recovered by culture methods only.[41] Although our study used culture methods only, it is thought to introduce broad-range PCR to further determine the clinical significance of HCA with negative AF culture in the future study.

One question unanswered in regard to an inclusion criterion of our study that restricted the study subjects to those with a time interval of ≤48 hours from amniocentesis to delivery was whether a time interval from symptom onset at the time of amniocentesis to delivery at the time of placental examinations was very short or not. It was supposed to require sufficient time duration to detect microorganisms after onset of placental inflammation. According to a recent review of acute HCA and MIAC, however, most intra-amniotic infections are due to ascending microbial invasion from the lower genital tract and bacteria are primarily found in the amnion in cases of intra-amniotic infection, which indicates that MIAC is a prerequisite for substantial invasion of the amnion and chorion.[3] One evidence by the authors using FISH with a bacterial 16S ribosomal RNA probe was that bacteria are detected more frequently in the amniotic cavity than in the chorioamniotic membranes of women with positive AF culture (100% vs 33%; P <.0001),[44] although some investigators believe that there is a stage in which the bacteria are found diffusely in the choriodecidual layer.

Several investigators have reported that the incidence of intra-amniotic inflammation (as assessed by a mass-restricted score or glucose level) was significantly higher in patients with HCA than in those without HCA, all of whom delivered within 48 hours of amniocentesis.[9, 45] In accordance with previous reports,[9, 45] our results demonstrated that women with HCA who had negative AF culture results showed stronger intra-amniotic inflammatory response (elevated AF IL-6, IL-8, and MMP-9 levels) and a higher rate of intra-amniotic inflammation than those without HCA with negative AF culture. However, with respect to its design, our study is different from previous studies in that women with positive AF culture were excluded from the HCA group for evaluation of the direct effect of HCA per se (not in combination with MIAC) on the inflammatory status of the AF. Potential explanations for our aforementioned observations can be largely similar to the explanations invoked for the presence of HCA in cases with negative AF culture in the previous paragraph. Furthermore, we found that the magnitude of intra-amniotic inflammatory response was greater in the women with positive AF culture than in those with HCA with negative AF culture. This finding is

natural, given the fact that the presence of a large number of microbes to the extent of proliferation into the AF by using the standard culture technique may induce more intense cell-mediated immune system activation and cytokine release than the presence of microbes in the chorioamnion in the absence of AF infection or a small number of microbes in the AF.[46, 47]

In the literature, although inconsistent, data suggest that HCA may be a risk factor of preterm birth, and neonatal morbidity and mortality, including early onset neonatal sepsis, BPD, IVH, PVL, and cerebral palsy.[2, 4, 6] However, whether such morbidity and mortality are independent of gestational age remains unclear. In a recent report with a relatively large sample size, Lee et al. showed that the higher incidence of neonatal morbidity according to increased stage of HCA or funisitis was due to an earlier gestational age at delivery.[48] Similarly, in terms of the association of MIAC and elevated AF IL-6 levels with neonatal morbidity, Rodriguez-Trujillo et al. and Comb et al. demonstrated that these significant associations disappeared after adjusting for gestational age at delivery.[49, 50] In concurrence to the results of previous studies,[48-50] our results showed that HCA with negative AF culture was associated with increased risk of composite neonatal morbidities, but this risk is dependent on gestational age at birth. Collectively, our findings and those of other groups[48-50] suggest that gestational age at delivery may have a more important role in the development of composite neonatal morbidities than exposure to prenatal infection/inflammation.

The first phase of the present study has several limitations. First, the study was of retrospective nature, potentially leading to selection bias, although most data were collected prospectively. Second, the study population consisted of a heterogeneous status of fetal membranes, including intact or rupture membranes. However, our results remain valid because we derived our results from a multivariable analysis, adjusting for this confounder. Third, the present study used neither placental culture, including the space between chorioamniotic membranes, nor molecular technique (i.e., PCR or next generation sequencing*)* to detect microbes or their DNA in AF. Therefore, we could not specifically explain the causes of HCA with negative AF culture. The strength of our

study is the relatively large sample size, yielding clear results, given the low frequency of delivery within 48 hours of amniocentesis. Furthermore, it is the first study to examine the effects of HCA with negative AF culture on maternal and neonatal outcomes, and inflammatory status of the AF.

The principal findings of the second phase of the study are as follows: (1) in women with PPROM, MMP-9 in the maternal plasma may be useful as non-invasive predictor of HCA; (2) However, we failed to find a biomarker for prediction of HCA in PTL group. These findings underscore the importance of biomarker discovery using maternal blood samples, especially in women with PPROM.

The second phase of the present study using membrane-based antibody microarray assay has several limitations. This study was of a retrospective nature and was performed at a single center; these may limit the generalization of the study findings. Second, we could not validate the levels of all the candidate biomarkers showing density differences between the groups via membrane-based microarray assay because of no availability for validation of certain proteins in our laboratory. The main strength of the study was that we evaluated maternal plasma expression of inflammatory and immune-related proteins in women with PTL and PPROM, separately, which made it possible to reflect the different biologic characteristics of HCA in PPROM and in PTL. From a clinical perspective, this may help clinicians determine whether the plasma proteins studied could be a clinically useful biomarker for HCA.

In women with PPROM, serum CRP and gestational age at sampling have been consistently reported to be associated with the presence of HCA,[7, 10, 19, 24, 51, 52] which is in line with the present study. However, few studies screened the extensive proteins via membrane-based microarray method, especially including those using maternal blood, for non-invasive detection of HCA. From a clinical perspective, to identify a biomarker for predicting HCA non-invasively and prenatally is useful for

classifying women with PPROM into low- and high-risk groups, and women with high-risk for HCA may then be targeted for additional treatment and prevention strategies for adverse neonatal outcomes, such as reinitiation of antimicrobial agents or expedition of delivery.

We have previously reported that an elevated level of AF MMP-9 is associated with the presence of HCA in a mixed population of PTL and PPROM women.[7, 11] In fact, MMP-9 has been shown to be involved in modulation of cytokines and chemokines, inflammation, and extracellular matrix remodeling.[53] We found that MMP-9 activity was also increased in plasma compartment in the presence of HCA in women with PPROM, which is consistent with the previous study by Caloone et al.[52] Taken together, these findings suggest that MMP-9 may play an important role in biological pathway resulting in PPROM complicated by HCA at a systemic as well as a local level, as its role is well characterized for basement membrane degradation. Contrary to PPROM, a change in plasma MMP-9 levels was not associated with HCA in women with PTL, which is consistent with the findings of a previous study conducted in an asymptomatic cohort.[54] While we cannot explicitly explain this discrepancy, altogether, these findings suggest that there are differences in the biological characteristic of HCA at the systemic level in PTL and PPROM.

Based on our data, diagnostic indices were not significantly different between serum CRP and plasma MMP-9 in the PPROM group (Table 10). Although plasma MMP-9 was not superior to serum CRP which has been in clinical use for predicting HCA, the diagnostic accuracy can be increased if this novel biomarker and conventional factors were used in combination. The clinical usefulness of their combined use of various biomarkers and thus improved diagnostic accuracy is to decrease the numbers of unnecessary invasive diagnostic procedures (eg, transabdominal amniocentesis) by excluding HCA.

Several studies have demonstrated a rise in matermal serum IL-6 in the presence of HCA as well as funisitis in women with PPROM.[55-59] In accordance with the previous reports, our results also

showed that an elevated plasma level of IL-6 was associated with the presence of HCA in women with PPROM although it did not remained significant after adjusting for potential confounding factors. However, few studies have investigated the changes in IL-6 levels in maternal plasma in relation to HCA in women with PTL. We observed that HCA was not associated with IL-6 level in plasma of women with PTL by evaluating via antibody microarray assay.

Studies in the literature have reported TIMP-1 levels in AF to be elevated in the presence of intra-amniotic infection.[60, 61] However, to the best of our knowledge, no studies have investigated the changes in TIMP-1 levels in maternal plasma in relation to HCA. We observed that HCA was not associated with a change in plasma TIMP-1 levels.

Although statistical significance was not remained after adjusting for gestational age at sampling, S100 A8/A9 had higher level in women with HCA in PTL and PPROM. S100 A8/A9, known to be one of principal mediator of innate immune response, has been traditionally used to predict or diagnose inflammatory process of intestines, especially preterm infants. The protein was reported to be likely to be an early diagnostic marker for necrotizing enterocolitis in neonates.[62] In our study population, especially in fetuses of women with HCA, elevated S100 A8/A9 in maternal plasma suggests an involvement of fetal intestines and thus inflammatory process of placentas, although confirmation in other larger cohorts is necessary.

Conclusion

Our studies show that (1) among the women who delivered preterm neonates, HCA with a negative AF culture was associated with increased risks of preterm birth, intense intra-amniotic inflammatory response, and prematurity-associated composite neonatal morbidity. These risks are similar to the risk

posed by positive AF culture and (2) plasma MMP-9 is an important non-invasive predictors of HCA of women with PPROM.

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국문 초록

조기진통 및 조기양막파열 산모에서 양수배양 음성인 조직학적 융모양막염의 임상적 의미 및 조직학적 융모양막염의 비침습적 예측지표 개발

목적: 본 연구의 목적은 (1) 조기진통 및 조기양막파열 산모에서 양수배양 음성인 조직학적 융모양막염이 임신 및 신생아 예후와 양수 내 염증 상태에 미치는 영향을 평가하고자 하였고, (2) 막기반시토카인마이크로어레이 기법을 이용하여 조직학적 융모양막염과 연관된 모체 혈장 내 새로운 생물표지자를 확인하고자 하였다.

방법: 본 연구는 2 단계로 구성되었다. (1) 첫 번째 단계에서, 임신 20-34 주에 조기진통 및 조기양막파열로 진단받고 양수검사 시행 후 48 시간 이내에 분만한 153 명의 연속적인 단태임신 여성을 연구대상으로 하였다. 양수검사를 통해 균 배양 검사 및 양수 내 IL-6, IL-8 및 MMP-9 의 농도를 측정하였으며 태반 조직검사를 시행하였다. (2) 두 번째 단계에서는, 새로운 혈장 생물표지자를 확인하기 위해 코호트 내 환자-대조군 연구를 수행하였다. 두 번째 단계는 총 2 기로 구성되었는데, 제 1 기는 분만 96 시간 이내에 채혈된 모체 혈장 검체를 환자군(조직학적 융모양막염) 14 명과

태반염증이 없는 대조군 14 명을 조기진통 산모에서 채취하였다. 막기반단백마이크로어레이 분석법을 이용하여 모체혈장의 면역조절 단백질 계수를 비교하여 후보 생물표지자를 발견하는 작업을 수행하였다. 제 2 기는 선별된 후보 생물표지자의 혈장 농도를 ELISA 기법을 이용하여 측정하였고 46 명의 혈장 검체를 추가하여 총 74 명의 조기진통 환자에서 비교하였다. 조기양막파열 환자에서도 조기진통 환자에서와 동일한 과정으로 제 1 기 작업 (n=28)과 54 명의 혈장 검체를 추가하여 총 82 명에서 선정된 후보 생물표지자의 모체 혈장 농도를 비교 분석하였다.

결과: (1) 연구의 첫 번째 단계에서, 조직학적 융모양막염의 유병률은 23.5% (36/153)이었다. 양수배양 음성인 조직학적 융모양막염(집단 2) 여성과 양수배양 양성인 여성(집단 3)에서 양수배양 음성이면서 조직학적 융모양막염이 없는 여성(집단 1)에 비해 평균 양수검사 시행 주수 및 분만 주수가 유의하게 낮았다. 양수 내 IL-6 및 IL-8, MMP-9 농도는 집단 3 에서 가장 높았으며, 집단 2, 집단 1 순서대로 농도의 차이를 보였다. 복합 신생아 이환율은 집단 1 에 비해 집단 2 및 집단 3 에서 유의하게 높았으나 재태주수를 보정하였을 때 더 이상 유의하지 않았다. (2) 연구의 두 번째 단계에서, 막기반단백마이크로어레이 분석에서 상이하게 발현된 단백질은 조기진통에서 12 개, 조기양막파열에서 14 개가 확인되었다. 조기진통 여성(74 명)에서 ELISA 기법으로 모체 혈장 내 농도를 측정하였을 때 S100 A8/A9 은 대조군에 비해 조직학적 융모양막염군에서 유의하게 높았으나 검체 채취 재태 주수를 보정하였을 때에는 더 이상 통계학적 유의성은 없었다. 조기양막파열 여성(82 명)에서 ELISA 기법을 통한 모체 혈장 농도를 측정하였을 때 S100 A8/A9, MMP-9 및 IL-6 에서 조직학적 융모양막염이 존재할 때 유의하게 더 높은 농도를 보였으며, 임신 주수, 자궁수축억제제 및 코르티코스테로이드 사용을 보정하였을 때, MMP-9 의 모체 혈장 농도 증가가 조직학적 융모양막염과 독립적인 관련성이 있었다.

결론: (1) 조산아를 분만한 여성에서 양수배양 음성인 조직학적 융모양막염은 조기 분만, 강한 양막 내 염증반응 및 조산 관련 신생아 이환의 증가와 관련이 있으며 이는 양수배양 양성인 군과 비슷한 위험도를 보인다. (2) 모체 혈장 내 단백 발현 분포는

조직학적 융모양막염 존재 유무에 따라 유의한 차이가 있다. 막기반단백마이크로어레이 및 ELISA 기법을 통해 조직학적 융모양막염을 예측할 수 있는 모체 혈장 내 특정 단백질과의 관련성 조기진통에서 발견되지 않았다. 반면 조기양막파열 환자에서는 모체 혈장 내 증가한 MMP-9 이 조직학적 융모양막염을 산전에 비침습적으로 예측할 수 있는 새로운 후보 생물표지자로서의 가능성을 보여 준다.

주요어: 양수 배양, 조직학적 융모양막염, 양수 내 염증, 신생아 이환, 조산, 비침습적 예측인자, 막기반단백마이크로어레이

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