SUPPLEMENTARY MATERIAL S1

Diagnosis of neonatal morbidity

Respiratory distress syndrome (RDS) was diagnosed as the presence of respiratory distress, an increased oxygen requirement (FiO_2 >0.4), and diagnostic radiological and laboratory findings in the absence of evidence of any other causes of respiratory distress.[1] Bronchopulmonary dysplasia (BPD) was diagnosed using the criteria of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Workshop definition, i.e., the treatment with oxygen >21% for at least 28 days, and also diagnosed in the presence of typical findings at autopsy.[2] Congenital neonatal sepsis was diagnosed in the presence of a positive blood culture result within 72 hours of delivery.[3] Necrotizing enterocolitis (NEC) was diagnosed in the presence of abdominal distension and feeding intolerance (vomiting or increased gastric residual) for at least 24 hours with clear evidence of intramural air, perforation, and meconium plug syndrome by radiological examination, or definite surgical or autopsy findings of NEC.[4] Intraventricular hemorrhage (IVH) was diagnosed by ultrasonographic examination or magnetic resonance imaging (MRI) of the neonatal head (≥Grade II).[5] Periventricular leukomalacia (PVL) was diagnosed as the presence of cystic lesions within the periventricular white matter by ultrasonographic examination or MRI. Significant neonatal morbidity was defined when one or more neonatal outcomes including RDS, BPD, congenital neonatal sepsis, NEC, and IVH were diagnosed.

SUPPLEMENTARY MATERIAL S2

Detailed description of each patient who received antimicrobial agents

The reasons for the administration of antibiotics include: 1) the MMP-8 rapid test was positive (n=9); 2) the MMP-8 rapid test was positive and amniotic fluid white blood cell count was elevated (n=5); 3) the amniotic fluid white blood cell count was elevated, and the MMP-8 rapid test was not available (n=2); 4) rupture of membranes was suspected (positive result of a nitrazine test but without gross leakage or pooling of amniotic fluid by speculum examination) (n=2) (cases 11 and 14 in Table 3); rupture of the membranes was confirmed afterwards in these patients (case 11; 7 weeks and case 14; 8 weeks after the initial amniocentesis, respectively); and 5) MMP-8 concentration was elevated (80.1 ng/mL) (n=1, case 16 in Table 3).

Outcome of patients with intra-amniotic infection

Two patients (2/19) had a positive culture for microorganisms; details are listed in Table 3 (case 14 had a positive culture for *Ureaplasma* spp. and case 18 had a mixed infection with *Ureaplasma spp.* and *Mycoplasma hominis*). One responded favorably to antimicrobial treatment and had a negative amniotic fluid culture one week after the initial amniocentesis (case 14 in Table 3). The other patient (case 18 in Table 3) had persistent intra-amniotic infection with the same microorganisms at serial amniocenteses and delivered 6 weeks later at 32.6 weeks. The placenta revealed acute histologic chorioamnionitis and funisitis; the newborn weighed 2,060 gm and did not develop complications.

This case demonstrates that although antibiotics did not eradicate the infection, the magnitude of the inflammatory response decreased as the amniotic fluid interleukin-6

concentration dropped from 18 ng/mL to 12 ng/mL, and the amniotic fluid white blood cell count dropped from 50 to 10 cells/mm^3 (from 26 to 31 weeks of gestation).

Two patients had a positive amniotic fluid PCR for *Ureaplasma spp*. and a negative culture. In one patient (case 1 in Table 3), amniotic fluid PCR for *Ureaplasma spp*. became negative in the follow-up amniocentesis (26.4 weeks) performed nine days after the initial amniocentesis (25.1 weeks). Resolution of intra-amniotic inflammation was confirmed retrospectively by a decrease in amniotic fluid interleukin-6 concentration at the time of the third amniocentesis (29.1 weeks, interleukin-6 concentration: from 5.01 ng/mL in initial amniocentesis to 0.56 ng/mL in the third amniocentesis). The patient delivered spontaneously at 35.6 weeks. The newborn did not develop any major complications. In the other patient with a positive amniotic fluid PCR for *Ureaplasma spp*. (case 10 in Table 3), eradication of intra-amniotic infection and resolution of intra-amniotic inflammation was confirmed in the follow-up amniocentesis performed 13 days after the initial amniocentesis (interleukin-6 concentration from 42.6 ng/mL to 2.2 ng/mL).

Patients with intra-amniotic inflammation without demonstrable microorganisms

There were 15 patients who had intra-amniotic inflammation without intra-amniotic infection (positive culture or positive PCR for *Ureaplasma* spp.) at the time of initial amniocentesis, and were treated with antimicrobial agents. Antibiotics were continued despite culture and PCR results, because it was not possible to exclude the presence of microorganisms that had escaped detection with conventional culture techniques used in the clinical setting.[6-8] These patients continued to receive antibiotics, and were subsequently offered amniocentesis to monitor fetal lung maturity (depending on gestational age) and the intra-amniotic inflammatory response.

Resolution of intra-amniotic infection/inflammation and delivery after 34 weeks (Group A in Tables 3 and 4)

A description of the patients who had objective evidence of the resolution of intra-amniotic inflammation at follow-up amniocentesis, classified according to whether delivery occurred after (Group A) or before (Group B) 34 weeks, is listed in Table 3.

Nine patients (cases 1-9 in Table 3, Group A in Tables 3 and 4) delivered after 34 weeks, and resolution of intra-amniotic inflammation was confirmed by interleukin-6 concentrations measured retrospectively in amniotic fluid obtained during follow-up amniocenteses. Neonates born to these 9 mothers did not have major complications. The median gestational age at initial amniocentesis and delivery were 25.6 weeks and 35.4 weeks, respectively. The median interval from the first amniocentesis to diagnosis of the resolution of intra-amniotic inflammation was 14 days (range 7-67), and to delivery was 73 days (range 32-109). There were no significant differences in the median gestational age at amniocentesis, interleukin-6 concentration, and the rate of a positive amniotic fluid culture between the 4 groups of patients (A, B, C, and D described in Table 4; $p>0.1$ for each).

Resolution of intra-amniotic infection/inflammation and delivery before 34 weeks (Group B in Tables 3 and 4)

Resolution of intra-amniotic inflammation was confirmed retrospectively by a decreased concentration of interleukin-6 in amniotic fluid determined at follow-up amniocentesis; however, 6 patients delivered before 34 weeks (cases 10-15 in Table 3, Group B in Tables 3 and 4). The median gestational ages at initial amniocentesis and delivery were 22.5 weeks and 31.1 weeks,

respectively. The median interval from the first amniocentesis to the diagnosis of resolution of amniotic fluid inflammation was 18 days (range 10-30), and to delivery was 48 days (range 23-86).

Case 10 had a positive PCR for *Ureaplasma spp*. in amniotic fluid from the initial amniocentesis. Amniotic fluid PCR for *Ureaplasma spp*. became negative in the second amniocentesis (30.3 weeks) performed 13 days after the initial procedure (28.4 weeks). The amniotic fluid white blood cell count was persistently high (720, 342, and 486 cells/mm^3) in this patient, despite the use of antibiotics for 3.5 weeks. The patient underwent a Cesarean delivery because of documented fetal lung maturity at 31.7 weeks and breech presentation. Amniotic fluid interleukin-6 concentration measured retrospectively revealed resolution of intra-amniotic inflammation (from 42.6 ng/mL to 1.22 ng/mL). The rapid MMP-8 test was not available at that time (October 2011). The newborn weighed 1,840 gm and had no complications.

Resolution of intra-amniotic inflammation occurred in four patients (Cases 11-14) in whom rupture of membranes occurred several weeks after the procedure (67% [4/6]) (7 weeks, 3 weeks, 10 weeks, and 8 weeks, respectively). If patients had ruptured membranes before term, they were offered admission, amniocentesis, and antibiotic administration, and management occurred as previously described.[9] The newborns of cases 11 and 12 delivered at 32.9 weeks and 30.6 weeks, respectively, and had no significant complications. The newborn of case 13 delivered at 33.3 weeks, weighed 1800 gm, and developed atypical BPD.[2] However, placental pathology showed no evidence of acute histologic chorioamnionitis or funisitis.

Case 14 had a positive amniotic fluid culture for *Ureaplasma spp.* at 21 weeks of gestation, and was given antibiotics. The amniotic fluid culture was negative for microorganisms at follow-up amniocentesis (performed at 21.9 weeks), and the interleukin-6 concentration

measured in stored amniotic fluid obtained from the third procedure (23.9 weeks) decreased (from 4.84 ng/mL at initial to 1.93 ng/mL). However, at the time of a subsequent amniocentesis, performed because of the suspicion of rupture of membranes at 29 weeks, there was a positive amniotic fluid culture for *Morganella morganii*. The patient delivered shortly thereafter (29.6 weeks), and the placenta showed acute histologic chorioamnionitis and funisitis; however, the newborn had no significant complications. This case demonstrates that intra-amniotic infection can recur after successful treatment.

In case 15, the first amniocentesis at 20.1 weeks of gestation showed a positive MMP-8 rapid test, and antibiotics were administered. Repeat amniocentesis revealed another positive MMP-8 rapid test, and the patient went into spontaneous labor and delivered a neonate weighing 700 gm at 25.4 weeks of gestation. Retrospective amniotic fluid analysis showed that there was an improvement of intra-amniotic inflammatory processes, as amniotic fluid interleukin-6 concentrations at the follow-up amniocenteses performed at 21.6 weeks and at 23.1 weeks had decreased substantially (from 11.1 ng/mL to 0.9 ng/mL, and 0.49 ng/mL). There was no evidence of funisitis; however, the newborn died five hours after delivery. The parents declined an autopsy.

Patients without evidence of intra-amniotic infection/inflammation resolution who delivered after 34 weeks (Group C in Tables 3 and 4)

One patient (case 16 in Table 3, Group C in Tables 3 and 4) delivered at term after antibiotic treatment. The patient had received antibiotics for two weeks because the MMP-8 concentration of amniotic fluid obtained at initial amniocentesis (21.6 weeks of gestation) was elevated (80.1 ng/mL): antibiotics were discontinued after the follow-up amniocentesis at 23.7

weeks, which showed an absence of white blood cells in the amniotic fluid, and preterm labor had stopped. The maternal C-reactive protein concentration decreased over time (from 1.99 mg/mL to 0.12 mg/mL). When this patient was admitted (January 2004), the rapid MMP-8 test was not yet available. However, the interleukin-6 concentration of the amniotic fluid obtained at the follow-up amniocentesis was 6.6 ng/mL (down from 19.4 ng/ml at the first amniocentesis). Resolution of intra-amniotic inflammation could not be confirmed. The patient delivered at 38 weeks, the newborn had no complications, and the placenta was not examined.

Patients without evidence of resolution of intra-amniotic infection/inflammation who delivered before 34 weeks (Group D in Tables 3 and 4)

In three patients, the resolution of intra-amniotic fluid inflammation was not confirmed – these patients delivered before 34 weeks (cases 17, 18 and 19 in Table 3; Group D in Tables 3 and 4).

Case 17 received antibiotics for 10 days and delivered because of placental abruption at 32.9 weeks of gestation. The effect of antibiotics could not be assessed, because there was no remaining amniotic fluid for interleukin-6 determination obtained at the follow-up amniocentesis. However, the amniotic fluid white blood cell count dropped from 100 to 14 cells/mm^3. The newborn had no significant complications. The placenta showed acute histologic chorioamnionitis, but not funisitis.

Cases 18 and 19 did not have a demonstrable response to antibiotic treatment. Both had intra-amniotic inflammation at the time of admission. However, after antibiotic treatment, follow-up amniocenteses showed persistent inflammation. Although the absolute amniotic fluid interleukin-6 concentrations decreased, both patients went into spontaneous labor after about 10

days of the initial amniocentesis. Acute histologic chorioamnionitis was present in both cases, but funisitis was absent. Case 18 delivered at 32.6 weeks and did not have significant neonatal complications. However, the neonate of case 19 was born at 23.6 weeks, weighed 620 gm and had RDS, IVH grade II, PVL, and was discharged from hospital on day 105.

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