OBSTETRICS

Is amniotic fluid of women with uncomplicated term pregnancies free of bacteria?

Eva Maria Rehbinder, MD; Karin C. Lødrup Carlsen, MD, PhD[1]; Anne Cathrine Staff, MD, PhD[1]; Inga Leena Angell, MSc; Linn Landrø, MD, PhD; Katarina Hilde, MD; Peter Gaustad, MD, PhD; Knut Rudi, PhD

BACKGROUND: The "sterile womb" paradigm is debated. Recent evidence suggests that the offspring's first microbial encounter is before birth in term uncomplicated pregnancies. The establishment of a healthy microbiota early in life might be crucial for reducing the burden of diseases later in life.
OBJECTIVE: We aimed to investigate the presence of a microbiota in sterilely collected amniotic fluid in uncomplicated pregnancies at term in the Preventing Atopic Dermatitis and Allergies in children (PreventADALL) study cohort.
STUDY DESIGN: Amniotic fluid was randomly sampled at cesarean deliveries in pregnant women in 1 out of 3 study sites included in the PreventADALL study. From 65 pregnancies at term, where amniotic fluid was successfully sampled, we selected 10 from elective (planned, without ongoing labor) cesarean deliveries with intact amniotic membranes and all 14 with prior rupture of membranes were included as positive controls. Amniotic fluid was analyzed by culture-independent and culture-dependent techniques.
RESULTS: The median (min-max) concentration of prokaryotic DNA (16S rRNA gene copies/mL; digital droplet polymerase chain reaction) was low for the group with intact membranes [664 (544—748)]—corresponding to the negative controls [596 (461—679)], while the rupture of amniotic membranes group had >10-fold higher levels [7700 (1066—251,430)] ($P = .0001$, by Mann-Whitney U test). Furthermore, bacteria were detected in 50% of the rupture of amniotic membranes samples by anaerobic culturing, while none of the intact membranes samples showed bacterial growth. Sanger sequencing of the rupture of amniotic membrane samples identified bacterial strains that are commonly part of the vaginal flora and/or associated with intrauterine infections.
CONCLUSION: We conclude that fetal development in uncomplicated pregnancies occurs in the absence of an amniotic fluid microbiota and that the offspring microbial colonization starts after uterine contractions and rupture of amniotic membrane.

Key words: amniotic fluid, bacteria, fetus, microbiome, microbiota, placenta, sterile

Introduction

The human microbiome discovery has developed quickly over the last decades with culture-independent techniques and unique microbial communities being identified in various body sites.[1,2] A diverse and well-balanced maternal and infant microbiome seems important for normal development of the child's immune system, and a dysbiotic maternal gut microbiome has been associated with offspring allergic disease development, as well as other immune-mediated diseases.[3—5] Identifying the timing of the initial microbial colonization of the offspring could therefore be helpful in further understanding the developmental origin of health and disease.[6]

It has recently been suggested, by the use of 16S rRNA sequencing, that amniotic fluid has a microbiome of its own in term uncomplicated pregnancies.[7] These findings are challenging earlier studies, where cultures from amniotic fluid were negative in term uncomplicated pregnancies with intact membranes.[8—10] The emerging evidence of a unique placental microbiome[11,12] are also questioning the "sterile womb" hypothesis.

Although sensitive molecular techniques are suggesting an intrauterine microbiota, the arguments for a sterile womb, including germ-free mice and contamination bias in molecular studies, are still strong.[13—15] However, the current evidence for a sterile intrauterine environment is inconclusive and to what extent, if, and how maternal microbiome influences the fetal immunological development and the shaping of the infant microbiome is not settled.[4,5]

The aim of our study was to investigate the presence of a microbiota in amniotic fluid in term uncomplicated pregnancies. We therefore combined sampling under strictly sterile and DNA-free conditions with highly sensitive techniques to determine the amniotic fluid bacterial load.

Materials and Methods

Study population

Within 22 months from December 2014, 2701 pregnant women were enrolled in the Preventing Atopic Dermatitis and Allergies in children (PreventADALL) study[16] in Norway and Sweden at the 18-week gestational age (GA) ultrasound screening.[16] Investigations included fetal ultrasound and maternal weight, length, and blood pressure on inclusion, with electronic questionnaires completed at 18- and 34-week GA to assess maternal health, family, sociodemographic, and lifestyle factors. The healthy newborn babies of at least GA 35 weeks were included for the mother-child cohort. All mothers consented to amniotic fluid sampling, in case of delivery by cesarean delivery at the Oslo University Hospital location, by signing the study consent form. From the PreventADALL cohort,[16] 65 women at Oslo University Hospital

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AJOG at a Glance

Why was this study conducted?
It is unclear if the amniotic fluid prior to delivery is sterile or not, the latter possibly influencing offspring health programming through in utero microbiota exposure.

Key findings
We found that prior to uterine contractions and rupture of amniotic membranes, amniotic fluid is sterile in uncomplicated term pregnancies.

What does this add to what is known?
This study resolves the uncertainty about a sterile intrauterine environment in uncomplicated pregnancies at term, due to stringent amniotic fluid sampling procedures, together with accurate and high-sensitivity microbiota analyses.

had amniotic fluid sampled during term cesarean delivery by dedicated health personnel in 3 different operating rooms. Out of these 65 women, 51 had intact amniotic membranes and 14 had prior rupture of amniotic membranes (ROM). For the no prior ROM group, we selected 10 amniotic fluid samples, all from elective term cesarean deliveries, none of these having started labor and all sampled in the same operating room. We included all 14 samples with prior ROM (ROM group) as positive controls for the non-ROM group (see Figure 1 for a detailed description on how the study population was selected). The study was approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway (2014/518) as well as registered at clinicaltrial.gov (NCT02449850).

Sampling
Amniotic fluid was collected in a sterile manner during elective (planned, with no ongoing labor) or acute (labor already started) cesarean delivery, after uterotomy, by aspiration of amniotic fluid through intact amniotic membranes using a sterile 19G needle and 10-mL syringe. The amniotic fluid samples were left at 4°C for maximum 24 hours and subsequently aliquoted into 1-2 sterile Cryotubes 4.5 mL SI 363452 (Millipore Sigma, Damstadt, Germany) and 0.5 mL into 1 sterile tube containing 1 mL Aimes medium (ESwab Copan 490CE; Thermo Fischer Scientific). These vials were stored at −80°C until further analysis. Negative controls were sampled from 2 different operating rooms using sterile containers with NaCl (9 mg/mL, 100 mL intravenous infusion; B. Braun), using the same sampling and aliquoting procedure as the amniotic fluid samples. In addition, 2 negative controls from the polymerase chain reaction (PCR) water used in the laboratory were included.

Initial handling and DNA extraction
Amniotic fluid (1 mL) was pulse centrifuged at 1200 rpm × 3 to remove large particles before it was centrifuged at 13,000 rpm for 10 minutes. We included negative controls in all steps, both sterile NaCl from the operating theater and PCR water from the laboratory. Pellet was washed twice in PBS suspended in 100 μL PBS, 50 μL was used for the DNA extraction, done manually by mag midi kit (LGC Genomics, United Kingdom) following the manufacturer's recommendations.

Quantification by digital droplet PCR
Quantification of prokaryotic 16S rRNA gene copies in the amniotic fluid samples was done using digital droplet PCR (ddPCR) (Bio-Rad, Hercules, CA).[17] Droplet generation, droplet transfer, and plate sealing was done according to the manufacturer's instructions. DNA was amplified by PCR using reaction mixes containing 1x QX 200 ddPCR EvaGreen Supermix (Bio-Rad), 0.2 μmol/L of each primers PRK341F (5'-CCTAC GGGRB GCASC AG-3') and PRK806R (5'-GGACT ACYVG GGTAT CTAAT-3') (Thermo Fisher Scientific),[18] and 2 μL DNA. Thermal cycles involved initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 45 seconds, <1 cycle at 4°C for 5 minutes and finally 1 cycle at 90°C for 5 minutes. All reactions were performed on a 2720 Thermal Cycler (Applied BioSystems, Waltham, MA). The droplets were quantified using software (Quantisoft; Bio-Rad). The baseline was set manually with a fluorescence threshold of 15,000 relative fluorescence units. Both the interassay and intraassay variability of ddPCR was validated by *Escherichia coli* spiking of non-ROM amniotic fluid (30,000 and 3000 colony-forming unit/mL) with 3 interassay replicates for each dilution, and duplicates analyses for each interassay replicate. In all cases the coefficient of variation was <15%, with the DNA recovery being ~100%.

Culturing, DNA extraction, and PCR
In all, 150 μL of amniotic fluid in Aimes medium was suspended in 1350 μL of liquid brain heart infusion (BHI) medium, making a 10^{-1} dilution and further diluted to a 10^{-2} dilution, for both aerobic and anaerobic culturing. Tubes for anaerobic culturing were prepared in a closed jar using Oxoid AnaeroGen 3.5-L sachets (Thermo Fisher Scientific) for 48 hours; the closed jar and new sachets were used for the anaerobic culturing both in liquid BHI medium and on the BHI agars. The samples in liquid BHI medium were incubated at 37°C for 48 hours and 10 μL from each sample was plated out on BHI agar for aerobe (48 hours) and anaerobe (120 hours) incubation at 37°C. DNA was extracted manually by mag midi kit (LGC Genomics, United Kingdom) following the manufacturer's recommendations from all the cultures in liquid BHI 10^{-1} dilutions, as well as from the bacterial colonies found on the BHI plates after incubation. Amplification by PCR was performed on DNA from all the liquid culture samples, using 1xHotFirePol DNA polymerase ready to load (Solis BioDyne, Estonia), 0.2 μmol/L of the same PRK primers used in ddPCR, and 2 μL template DNA. Thermal cycles involved initial denaturation at 95°C for