Analysis of microbial differences in amniotic fluid between advanced and normal age pregnant women

Ya Wang†, Chunyu Luo†, Yiwei Cheng2, Li Li1, Dong Liang1, Ping Hu1* and Zhengfeng Xu1*

Keywords: Amniotic fluid, Microorganisms, 16S rRNA gene sequencing, Proteomics

To the Editor
Amniotic fluid (AF) has been considered sterile for nearly a century because no microorganisms were identified by traditional culture methods [1]. However, this opinion paradigm has been challenged by recent studies based on culture-independent sequencing techniques [2–4] and AF proteomics [5]. Recently, a conclusion that no microorganisms were present in the mid-trimester AF of healthy pregnancies was reached using culture-independent sequencing techniques [6] and seemed to settle the argument [7]. However, it could not explain why non-human proteins were identified in normal human AF supernatants [8] and why microbial exposure primes fetal immune cells in fetal tissues during fetal development [9].

Intra-amniotic infection caused by microbial invasion of the amniotic cavity (MIAC) was associated with adverse pregnancy outcomes, when the bacteria were at high concentrations [10]. This may be a reason why people think that AF is sterile and that bacteria in AF are abnormal because of their negative effects.

Meanwhile, we could identify bacterial proteins in the AF proteomics database that were consistent with the results of 16S ribosomal RNA (rRNA) gene sequencing. Nine amniotic fluid samples were collected from 9 pregnant women and the pregnancy outcomes of the participants were followed. No bacteria were found by cultivation, but a sparse microbial presence was found by proteomics analysis and 16S rRNA gene sequencing approach. The 148 microbes found in the human AF proteomics database were consistent with the microbes found in the 16S rRNA gene sequencing database. The species composition and the structure of communities in the normal age (< 35 years old) and advanced maternal age (AMA) (> 35 years old) pregnancies differed significantly. However, all of the newborns were healthy and had no allergic reactions.

Proteomics analysis has identified nearly 2000 proteins in AF during the past 20 years [11], including many non-human proteins [8]. In a recent study, 7 normal AF samples were used to generate human AF proteomes, which were divided into 4 groups: original proteins, bound proteins, flow-through proteins, and iTRAQ-labeled individual/mixed digested peptides [11]. We reanalyzed the human AF protein database and found that a part of the non-human proteins were derived from microorganisms. A total of 148 microbial-associated proteins in the normal human AF proteome, which could potentially play important roles in cellular/metabolic processes and binding/catalytic activity (Additional file 1). Notably, all of the microbial-associated proteins were present at a low level in human AF. These data suggest that there may be low concentrations of microorganisms in normal human AF samples.

To test our hypothesis, we conducted a study to investigate the presence of microorganisms in the mid-trimester AF of 9 women [4 normal age [normal] and 5 advanced
maternal age [AMA]; advanced maternal age is generally defined as age above 35 years at the time of delivery [12, 13]) by cultivation and 16S rRNA gene sequencing, and to follow their pregnancy outcomes. If the AMA group differed from the normal group in the microbial-associated 16S rRNA genes, it could reflect an endogenous microbial difference between the two groups and support the hypothesis that the AF is not sterile; otherwise, the microorganisms could be generated by exogenous contamination, which could not rule out the possibility that AF is sterile.

The main findings were: (1) B-ultrasonography and karyotype findings of the fetuses were normal and no bacteria were found by cultivation; (2) all 148 microbial-associated proteins in the normal human AF proteome were found in the 16S rRNA gene sequencing database, in which *Bacillus*, *Mycobacterium*, and *Pseudomonas* accounted for ~20%; (3) the bacterial richness of the AF samples showed no significant difference between the AMA and normal groups (Chao1 index, Welch’s *t* test, *P* = 0.540; Additional file 2); (4) a significant difference in the species composition and structure of communities in the AF samples was found between the normal and AMA groups (Fig. 1A, B, Weighted_unifrac, OUT, Welch’s *t* test, *P* = 0.017); (5) the newborns were healthy and had no allergic reactions up to 90 days (Table 1). Collectively, these data suggest that the normal AF is not sterile and that the species composition and structure of communities change in the AMA group, although the bacterial richness may be similar and have no effect on the babies’ health.

The differences between the AF samples from the normal and AMA groups with regard to bacterial species composition and structure of communities were as follows. Five bacterial operational taxonomic units (OTUs) were increased in the AMA AF samples: *Lactobacillus helveticus*, *Pediococcus acidilacticii*, *Pasteurella multocida*, *Bacillus indicus*, and *Bacteroides vulgatus*. We used the LEfSe (Linear discriminant analysis Effect Size) method to identify bacterial OTUs that were likely to explain most of the differences between the normal and AMA AF samples. The bacterial orders of OTUs differed between the two groups. *Sphingomonadales* were more abundant in the normal group, while *Lactobacillales* were more abundant in the AMA group (Fig. 1C, LDA scores > 4, Additional file 3). The differences in OTUs mainly spanned two Orders, with the Families Lactobacillaceae (primarily *Lactobacillus helveticus*) and *Sphingomonadaceae* accounting for the majority of the differences. Collectively, there may be a low concentration of microorganisms in the normal human AF samples and significant differences between the normal and AMA AF samples.

In the present study, we found that microbial-associated proteins and 16S rRNA genes could be identified in human AF at a low concentration. Furthermore, the species composition and structure of communities differed significantly between the normal and AMA AF samples. Thus, we conclude that the microbial-associated 16S rRNA gene in human AF is real, rather than occurring through microbial contamination, and that the bacteria in AF differ between normal age and AMA pregnancies. We already know that AF neutrophils can phagocytize bacteria during intra-amniotic infection [14], but we do not know the function of bacteria in normal AF. Two possible hypotheses were proposed based on our results. First, we suggest that the AF is not sterile, but the level of microbiota may be very low and under the mother’s immune system control. Therefore, the microbial communities may be related to the establishment of fetal immune function. Immunoglobulins from the mother may help to control the number or activity of the bacteria, to precisely control and activate the fetal immune system, given that recent studies demonstrated the presence of microbes or microbial DNA in the placenta, amniotic fluid [15], and meconium. Furthermore, Florent et al. [9] found live microbes in human fetuses such as *Lactobacillus*, and suggested that the selective presence of live microbes in fetal organs may have broader implications toward the establishment of immune competency and priming before birth, with the microbial exposure priming fetal immune cells during early human development. Second, we suggest that the bacterial 16S rRNA gene may come from the mother, because Rodriguez and colleagues proved that bacteria are transferred to the fetus from the mother by testing the meconium of healthy babies [16]. In addition, circulating cell-free DNA fragments are able to transfer between the fetus and the mother [17], and the bacterial 16S rRNA gene can be successfully detected in cell-free plasma DNA [18]. In this way, the bacterial 16S rRNA gene could possibly enter the AF through the umbilical cord, and this would explain why we were able to identify the 16S rRNA gene and peptides, but could not cultivate the bacteria.

**Materials and methods**

**AF sample collection and preparation**

Human amniotic fluid samples (~10 ml) were obtained by amniocentesis from women at 18–22 weeks of gestation who were undergoing prenatal diagnosis due to AMA or noninvasive prenatal testing for high-risk pregnancy after receiving written informed consent. The samples were collected in an operating room with the help of
ultrasonic guidance; the operating room was sterile and the surgical instruments underwent aseptic processing and packaging. Nine samples from chromosomally normal pregnancies were chosen randomly.

16S rRNA gene sequencing
Microbial DNA was extracted from AF samples using an E.Z.N.A. Stool DNA Kit (Omega Biotek, Norcross, GA, USA) according to the manufacturer’s protocol.
16S rDNA V3–V4 region of the eukaryotic rRNA gene was amplified by PCR using the following thermal profile: 95 °C for 2 min, followed by 27 cycles of 98 °C for 10 s, 62 °C for 30 s, and 68 °C for 30 s, and a final extension at 68 °C for 10 min. The primers used were 341-F: 5′-CCT ACG GGNGGC WGC AG-3′ and 806-R: 5′-GGA CTATCGTGATCTTAAT-3′, where the barcode was an 8 bp sequence unique to each sample. The PCR amplifications were performed in triplicate using 50-μl mixtures containing 5 μl of 10× KOD buffer, 5 μl of 2.5 mM dNTPs, 1.5 μl of each primer (5 μM), 1 μl of KOD polymerase, and 100 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer’s instructions, and quantified using a QuantiFluor-ST System (Promega, Madison, WI, USA). Purified amplicons were pooled in equimolar quantities and subjected to paired-end sequencing (2 × 250) on an Illumina HiSeq 2500 Platform (Illumina Inc., San Diego, CA, USA) according to standard protocols.

Statistical analysis
Bioinformatic analysis was performed using Omicsmart (http://www.omicsmart.com).

Abbreviation
AF: Amniotic fluid.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12967-021-02996-y.

Table 1 Clinical information of the newborns

| No. | Age | Apgar | Delivery modes | Gestational weeks | W/L (birth) (kg/cm) | W/L (90 days) (kg/cm) | Feeding | Allergy |
|-----|-----|-------|----------------|-------------------|---------------------|----------------------|---------|---------|
| YS01 | 29 | 10/10 | Eutocia | 38±6 | 3.0/50 | 7.0/61 | PB | No |
| YS02 | 33 | 10/10 | Cesarean | 37±5 | 3.4/49 | 6.3/64 | B | No |
| YS03 | 30 | 10/10 | Eutocia | 38±3 | 2.9/50 | 6.8/62 | PB | No |
| YS04 | 28 | 10/10 | Cesarean | 38±5 | 3.4/50 | 6/61.5 | B | No |
| YS06 | 41 | 10/10 | Cesarean | 39±2 | 3.4/50 | 10/63 | B | No |
| YS07 | 40 | 10/10 | Eutocia | 39±0 | 3.5/50 | 6.5/61 | PB | No |
| YS08 | 40 | 10/10 | Cesarean | 37±5 | 3.3/48 | 7/62 | B | No |
| YS09 | 39 | 10/10 | Eutocia | 39±5 | 4.1/52 | 5/65 | B | No |
| YS10 | 40 | 10/10 | Eutocia | 40±3 | 4.0/51 | 9/65 | B | No |

Advanced maternal age was defined as age > 35 years
W weight; L length; PB partial breastfeeding; B breastfeeding

Acknowledgements
We are grateful to the affected individuals and the family for permitting us to publish this information, Wei Sun (Peking Union Medical College, China) for offering human AF proteomics database, and Guangzhou Genedenovo Biotechnology Co., Ltd., for providing technical support.

Authors’ contributions
PH, YW designed and performed the experiments; CYL and LL were responsible for the clinical samples and data collection; ZFX, PH, YWC analyzed the data; YW and DL drafted the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by Grants from the National Key R&D Program of China (No. 2018YFC011029), the National Natural Science Foundation of China (No. 81971398).

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Additional file 1. Human AF protein groups.
Additional file 2. Bacterial diversity indexes of the AF samples.
Additional file 3. LEfSe analysis of bacterial communities of the AF samples.
Declarations

Ethics approval and consent to participate
Samples of amniotic fluid were taken from each patient on the same day. All procedures performed in studies involving human participants were in accordance with the Declaration of Helsinki and the patients gave their written consent. The Research Ethics Committee of Nanjing Maternal and Child Health Hospital approved the study ([2019] KY-081).

Consent for publication
Not applicable.

Competing interests
The authors have declared that no competing interests exists.

Author details
1 Department of Prenatal Diagnosis, Obstetrics and Gynecology Hospital Affiliated to Nanjing Medical University, Nanjing Maternity and Child Health Care Hospital, Nanjing 210004, China. 2 Nanjing Jiangbei New Area Biopharmaceutical Public Service, Platform No.3-1 Xinjinhua Road, Zhongdan Life Science and Ecology Industrial Park, Nanjing 210000, China.

Received: 13 April 2021   Accepted: 11 July 2021
Published online: 27 July 2021

References
1. Willyard C. Could baby’s first bacteria take root before birth? Nature. 2018;553:264–6.
2. Lim ES, Rodriguez C, Holtz LR. Amniotic fluid from healthy term pregnancies does not harbor a detectable microbial community. Microbiome. 2018;6,87.
3. Payne MS, Keehan JA, Stinson LF. Re: “Amniotic fluid from healthy term pregnancies does not harbor a detectable microbial community.” Microbiome. 2019;7:20.
4. Lim ES, Rodriguez C, Holtz LR. Reply Re: “Amniotic fluid from healthy term pregnancies does not harbor a detectable microbial community.” Microbiome. 2019;7:21.
5. Cho C-KJ, Shan SJ, Winsor EJ, Diamandis EP. Proteomics analysis of human amniotic fluid. Mol Cell Proteom. 2007;6:1406–15.
6. Liu Y, Li X, Zhu B, Zhao H, Ai Q, Tong Y, et al. Midtrimester amniotic fluid from healthy pregnancies has no microorganisms using multiple methods of microbiologic inquiry. Am J Obstet Gynecol. 2020;223:248.
7. Blaser MJ, Devkota S, McCoy KD, Relman DA, Yassour M, Young VB. Lessons learned from the prenatal microbiome controversy. Microbiome. 2021;9:1–7.
8. Tsangaris GT, Kolialexi A, Karamessinis PM, Anagnostopoulos AK, Antsaklis A, Fountoulakis M, et al. The normal human amniotic fluid supernatant proteome. Vivo. 2006;20:479–90.
9. Mishra A, Lai GC, Yao L, Ang T, Shental N, Rotter-Maskowitz A, et al. Microbial exposure during early human development primes fetal immune cells. Cell. 2021;184:1–16.
10. Romero R, Dey SK, Fisher SJ. Preterm labor: one syndrome, many causes. Science. 2014;345:760–5.
11. Liu X, Song Y, Guo Z, Sun W, Liu J. A comprehensive profile and inter-individual variations analysis of the human normal amniotic fluid proteome. J Proteome. 2019;192:1–9.
12. Jeffner LJ. Advanced maternal age—how old is too old? N Engl J Med. 2004;351:1927–9.
13. Zhang X, Chen L, Wang X, Wang X, Jia M, Ni S, et al. Changes in maternal age and prevalence of congenital anomalies during the enactment of China’s universal two-child policy (2013–2017) in Zhejiang Province, China: an observational study. PLoS Med. 2020;17:1–19.
14. Gomez-Lopez N, Romero R, Garcia-Flores V, Xu Y, Leng Y, Alhousseini A, et al. Amniotic fluid neutrophils can phagocytize bacteria: a mechanism for microbial killing in the amniotic cavity. Am J Reprod Immunol. 2017;78:139–48.
15. Collado MC, Rautava S, Aakkio J, Isolauri E, Salminen S. Human gut colonization may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep. 2016;6:23129.
16. Jiménez E, Marín ML, Martin R, Odrozola JM, Olivares M, Xaus J, et al. Is meconium from healthy newborns actually sterile? Res Microbiol. 2008;159:187–93.
17. Dennis Lo YM, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CWG, et al. Presence of fetal DNA in maternal plasma and serum. Lancet. 1997;350:485–7.
18. Vernon SD, Shukla SK, Conradt J, Unger ER, Reeves WC. Analysis of 16S rRNA gene sequences and circulating cell-free DNA from plasma of chronic fatigue syndrome and non-fatigued subjects. BMC Microbiol. 2002;2:1–6.
19. Magrane M, UniProt Consortium. UniProt Knowledgebase: a hub of integrated protein data. Database. 2011;2011:bai009.
20. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 2008;26:1367–72.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.