



## Reply to de Steenhuijsen Piters and Bogaert

*From the Authors:*

We appreciate the thoughtful letter from de Steenhuijsen Piters and Bogaert in response to our recent report (1). Our proof-of-concept study is the first demonstration that a microbiome signature can be identified in human fetal lung tissues. Newer next-generation culture-independent methodologies, however promising, present numerous challenges and questions, especially with regard to issues involving methodology. Hence, we appreciate the opportunity to address and expand upon our findings, and we welcome a scientific discussion to provide some clarifications or answers.

We concur with de Steenhuijsen Piters and Bogaert that collecting fetal material from human subjects is extremely challenging, and pregnancy complications such as miscarriage could be accompanied by local inflammation. Nevertheless, in our study, all samples were collected from assumed normal subjects after an elective abortion and after informed consent was obtained. No samples were derived from a miscarriage, and therefore the possibility of inflammation/bacterial translocation due to miscarriage or membrane rupture is extremely low.

Given the complication of host DNA contamination coupled with low microbial biomass, these samples were indeed challenging to work with, as alluded to by de Steenhuijsen Piters and Bogaert and others (2). At the time of sample collection, just as with any routine medical procedure, all instruments were sterilized before each procedure (dilatation and curettage or dilatation and evacuation) in each patient. This was the first study to sample both placenta and deep fetal tissue (intestine and lung), and we agree with the concerns about the likelihood of possible contamination from nonbiological sources in low-biomass samples such as these. The abortion material was extracted through the vaginal canal, which is not sterile, and some of the material could have come in contact with the vaginal flora. However, our data showed statistically distinct signatures in the lungs and placenta from the same subjects, suggesting that the DNA signature was specific and not just the result of detecting a contaminant from the vaginal canal. Samples from twin pairs in the cohort also had different microbiome signatures even though they passed through the same vaginal canal. Moreover, a recent study showed no differences in the microbiome signature in placenta, fetal membranes, or meconium between vaginal and Caesarian-section deliveries (3). In addition, a study from our group demonstrated the presence of abundant immune cell signatures in the fetal lung as early as 11

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In addition, the lack of information about the indications for the D&C or D&E procedures makes us question whether pregnancy complications, such as miscarriage, which is likely accompanied by local inflammation (5), bacterial translocation, and infection, might be at the basis of their findings. All of the above information is essential to assess the potential biological origin of the observed bacterial DNA in fetal samples.

In conclusion, although we do not dispute the possibility of the existence of a fetal lung microbiome signature, the study lacked robust controls during both sample collection and laboratory processes, giving rise to speculation about the validity of the reported findings. Therefore, we believe that the presented data insufficiently support the authors' conclusion that the human fetal lung harbors a microbiome signature. We do agree, however, that the road on which they have embarked—studying fetal tissues at early stages of pregnancy—is an interesting and important one, and therefore warrants more research. ■

**Author disclosures** are available with the text of this letter at [www.atsjournals.org](http://www.atsjournals.org).

Wouter A. A. de Steenhuijsen Piters, M.D., Ph.D.\*  
Wilhelmina Children's Hospital/University Medical Center Utrecht  
Utrecht, the Netherlands

and  
National Institute for Public Health and the Environment  
Bilthoven, the Netherlands

Debby Bogaert, M.D., Ph.D.  
Wilhelmina Children's Hospital/University Medical Center Utrecht  
Utrecht, the Netherlands

and  
National Institute for Public Health and the Environment  
Bilthoven, the Netherlands

and  
University of Edinburgh  
Edinburgh, United Kingdom

ORCID IDs: 0000-0002-1144-4067 (W.A.A.d.S.P.);  
0000-0003-2651-7000 (D.B.).

\*Corresponding author (e-mail: [w.a.a.desteenhuijsenpiters@umcutrecht.nl](mailto:w.a.a.desteenhuijsenpiters@umcutrecht.nl)).

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weeks, including B and T cells (4), thus supporting the presence of bacterial products. Although the environment in the clinic/lab where the samples were collected was not sampled, sterile instruments were used to dissect and isolate the tissue, which was then placed in sterile tubes containing sterile media, placed on ice and brought back to the lab, frozen, and shipped before DNA was extracted.

Having initially failed to detect a microbial DNA signature by whole-genome metagenomic shotgun sequencing, partially owing to a high level of contaminating human DNA, we resorted to the use of a more sensitive targeted 16S ribosomal RNA amplicon sequencing approach. We fully acknowledge the challenge and risk associated with the analysis of such material, and we were fully cognizant of the literature concerning low-biomass samples and the caveat of interference from contamination by the so-called “kitome” (5). As such, we exercised caution in this regard and followed published recommendations, including open reporting of important controls as illustrated in Figure 1B of Reference 2. This figure illustrates the background microbial DNA present in extraction controls and sequencing blanks processed at the lab in Singapore, and thus quantifies potential reagent- and sequencing-associated contaminants (2). Although more extensive controls are desirable in any study, we believe that we mitigated against the kitome effect as much as possible, and we acknowledge that further controls would have provided even greater robustness to this study. However, it is critical to note the strictly sterile conditions under which the sampling was undertaken. Although obtaining fetal human samples, as we did in our study, is far more challenging, we acknowledge that the controls fall short of those used by de Goffau and colleagues, who performed a much larger study of nonrespiratory placental samples (6). Importantly, however, we would suggest that our presented data reflecting background contamination in blank samples and library sequencing blanks address this issue and provide adequate insight into the risk of background contamination during the sequencing process. We observed clear and nonrandom microbial signatures across our samples.

Finally, the literature indicates that even histological methods with low sensitivity frequently identify bacteria in the placenta without overt infection (7), and a dynamic fetal microbiota has recently been identified in mice (8). Hence, it was obvious (but not proven) that the human fetus could harbor a microbiome signature. The major strength of our work is the novelty of the clinical samples assessed. Such fetal samples had not been previously analyzed, and therefore our results provide the first insight into potential fetal microbiome development (9). We therefore believe that the presented data are extremely valuable despite the potential limitations. The limits on words and references that can be cited in a letter preclude us from discussing substantial additional work on this subject. We thank you in advance for sharing the thoughtful comments from de Steenhuisen Piters and Bogaert, and we hope that our response provides evidence-based clarity to support our novel observations and propel this scientific field forward. ■

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Denise Al Alam, Ph.D.  
Soula Danopoulos, Ph.D.  
Brendan Grubbs, M.D.  
*Children's Hospital Los Angeles*  
Los Angeles, California

Nur A'tikah Binte Mohamed Ali, B.Sc.  
Micheál Mac Aogáin, Ph.D.  
Sanjay H. Chotirmall, Ph.D.\*  
*Nanyang Technological University*  
Singapore

David Warburton, M.D.  
*Children's Hospital Los Angeles*  
Los Angeles, California

Amit Gaggar, M.D.  
Namasivayam Ambalavanan, M.D.  
Charitharth Vivek Lal, M.D.†  
*University of Alabama at Birmingham*  
Birmingham, Alabama

ORCID IDs: 0000-0002-1726-7700 (M.M.A.);  
0000-0003-0417-7607 (S.H.C.); 0000-0001-9071-4047 (C.V.L.).

\*S.H.C. is Associate Editor of *AJRCCM*. His participation complies with American Thoracic Society requirements for recusal from review and decisions for authored works.

†Corresponding author (e-mail: [clal@peds.uab.edu](mailto:clal@peds.uab.edu)).

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