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Measurement of Short-Chain Fatty Acids in Respiratory Samples: Keep Your Assay above the Water Line

To the Editor:

Short-chain fatty acids (SCFAs) are bacterial products that have important biological functions, including maintenance of immune homeostasis (1). Growing evidence indicates that bacteria residing in the airways of patients with numerous pulmonary diseases as well as in those of healthy individuals (2, 3) are capable of making SCFAs (4). Therefore, there is growing interest in measuring respiratory SCFA concentrations because they could provide insight into biological processes in the lungs.

Sampling the lungs is challenging; the most common biospecimen is BAL fluid, which requires bronchoscopy under sedation. This invasive procedure is labor intensive and costly, and it may not be feasible in unstable critically ill patients. Exhaled breath condensate (EBC) is an easily acquired, abundant biofluid that could be used as an alternative to BAL. To test the utility of EBC for this purpose, we measured SCFAs and 16s ribosomal (r)RNA in paired BAL and EBC samples acquired from healthy control subjects.

Methods

The study (clinicaltrials.gov NCT03034642) and its consent procedures were performed in accordance with the Declaration of Helsinki at the Veterans Affairs Ann Arbor Healthcare System and the University of Michigan; protocols were reviewed and approved by the respective Institutional Review Boards (Federalwide Assurance [FWA] 00,000,348 [Veterans Affairs] and FWA 00,004,969 [University of Michigan]). All participants gave written consent.

Participants, who could be never-smokers, current smokers, or former smokers, had normal chest radiographs and post-bronchodilator spirometry and were free of respiratory and gastrointestinal disease. We excluded those with unstable cardiovascular disease, significant renal or hepatic dysfunction, mental incompetence, active psychiatric illness, or infection. We also excluded pregnant women and those on immunosuppression or antibiotic therapy.

On the day of study, EBC (RTube breath condensate collection device; Respiratory Research) was acquired in accordance with the manufacturer's instructions and American Thoracic Society guidelines, and paired BAL was acquired as previously described (5). Total bacterial DNA from each sample was extracted (6), sequenced (7), and quantified for the bacterial 16S rRNA gene (8) as previously described.

Assay of EBC and BAL fluid for SCFAs. Remaining volumes of samples were assayed for SCFAs by gas chromatography (GC)–mass spectroscopy (MS) without derivatization using a previously published protocol (9). A liquid chromatography (LC)-MS method was also used to confirm results (*see* supplemental material at https://doi.org/10.7302/wk4r-7x52). Methyl tert-butyl ether (MTBE) was used for liquid–liquid extraction of SCFAs from acidified EBC. Negative control samples, including MTBE alone, MTBE used to extract acidified water and internal standards, a water wash of the EBC acquisition equipment, and a representative sample of normal saline acquired from the bronchoscopy suite, were also assayed. A postmortem porcine portal vein plasma sample was assayed as a positive control.

Data analysis. The SCFA concentrations for each subject were summed, and Pearson correlation was used to assess the association between the total SCFA concentration and the microbiome signal within each medium. The summed SCFA concentrations of BAL and EBC were compared with a Mann-Whitney *U* test.

Results

Twenty subjects were enrolled into the study. Of these, 13 subjects had sufficient volumes of both EBC and BAL available for SCFA and microbiome assays. The median (interquartile range [IQR]) age of our sample was 59 (48–64) years, 59% of subjects were female, and 85% were white. Four subjects were current smokers, two were former smokers, and seven had never smoked; the median (IQR) pack-years of smokers was 20 (11–21). The median FEV_1% predicted (IQR) was 91% (83–104%), and the FEV_1:FVC ratio (IQR) was 0.87 (0.82–0.95).

SCFAs are in the water. All water samples (negative control samples) had detectable SCFA concentrations as measured by GC-MS (Figure 1); this finding was corroborated by an LC-MS assay (https://doi.org/10.7302/wk4r-7x52). Acetate was profoundly

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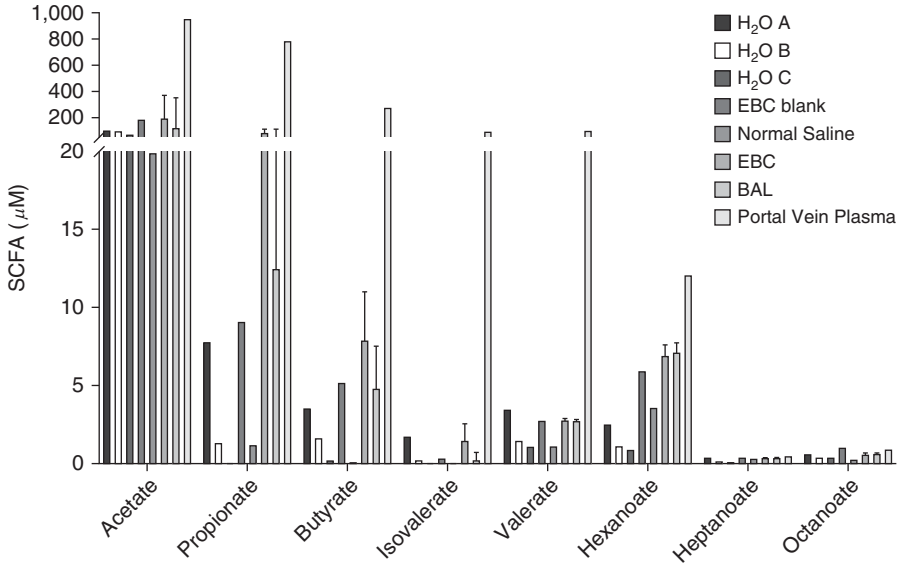

Figure 1. Concentrations of short-chain fatty acids detected by gas chromatography–mass spectroscopy from negative control exhaled breath condensate (EBC) and BAL samples. This includes three water samples from three separate sources. H_2O A: laboratory 1 Millipore Milli-Q academic deionizer, with a nominal resistance reading of 18.2 MΩ at 25°C that uses a Q gard 2 purification pack and a Quantum EX ultrapure cartridge with a 0.22-μm membrane filter; H_2O B: a newly opened bottle of high-performance liquid chromatography (HPLC)-grade water (Fisher W5–4 HPLC grade, submicron 0.5 μm filtered); H_2O C: laboratory 2 Milli-Q Biocel A10 deionizer with a nominal resistance reading of 18.0 MΩ at 25°C that uses a Q gard 2 purification pack and a Quantum EZ ultrapure cartridge with a 0.22-μm membrane filter; EBC blank: a sample of water wash from the exhaled breath acquisition equipment; normal saline: a sample from a newly opened bottle of normal saline from the bronchoscopy suite. The EBC and BAL data are the median (interquartile range) of 13 paired samples. Data are either from single samples (no error bars) or represent the median + interquartile range of three or more technical replicate samples. A portal vein plasma sample acquired from a postmortem swine at the termination of an unrelated experiment was used as a positive control. SCFA = short-chain fatty acid.

abundant in all samples, including the EBC blank and normal saline used for bronchoscopy. In neat MTBE, there were no detectable SCFAs, but this changed when MTBE was used to perform liquid–liquid extraction of any source of water or aqueous solution, resulting in measureable concentrations.

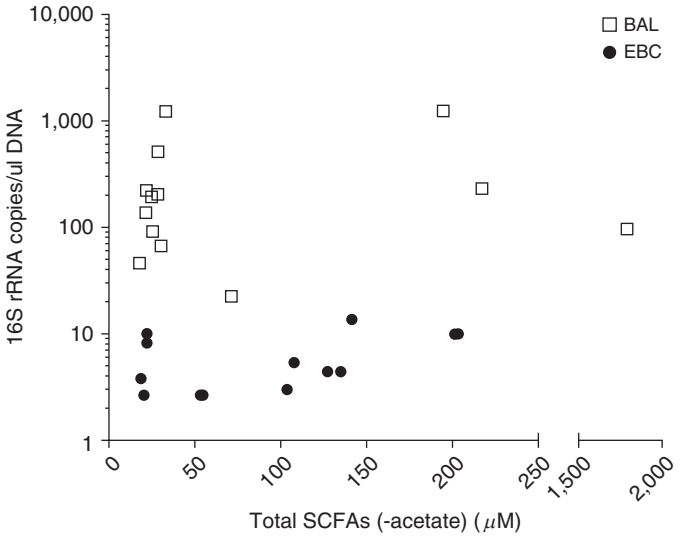

Figure 2. There is no association between total (sum) short-chain fatty acid concentration (exclusive of acetate) detected in BAL fluid ($R^2 = 0.01$; $P = 0.71$) and exhaled breath condensate ($R^2 = 0.19$; $P = 0.13$) and the microbiome (16S ribosomal RNA copies/μl DNA) in healthy control subjects. Note the log scale of the vertical axis. EBC = exhaled breath condensate; rRNA = ribosomal RNA; SCFA = short-chain fatty acid.

The EBC and BAL concentrations of SCFAs are low and are not associated with the amount of bacteria in the sample. The SCFA concentrations in both EBC and BAL were similar to those in water alone (Figure 1). Exclusive of acetate, the median (IQR) total (sum) SCFA concentrations for EBC and BAL were 104 μM (22–138 μM) and 28 μM (23–133 μM), respectively ($P = 0.51$). Furthermore, there was no association for either biofluid between the total (sum) concentration of SCFA (with or without acetate) or each individual SCFA and the amount of bacterial DNA (Figure 2).

Discussion

This work highlights the importance of using negative control samples for the determination of SCFA concentrations, especially in respiratory and other nonfecal samples, including the water that is used in sample and standard curve preparation. SCFA contamination of water sources can occur from microbial sources secondary to sewage treatment plants, septic tank leaching, agricultural livestock, and wildlife (10). Given the low molecular weight, high volatility, and polar nature of SCFAs, reverse osmosis, distillation, or ion exchange may not be sufficient to remove them from high-grade water.

Acetate is the most abundant SCFA. This metabolite is not unique to microbes, as it can be generated in humans by glucose-derived pyruvate (11). It is also ubiquitous in water and in the atmosphere via textiles and other man-made materials composed of cellulose acetate. Accordingly, we recommend that careful consideration be given to interpretation of acetate as a microbial signal in nonfecal samples.

Given the low SCFA signals in BAL and EBC, it is not surprising that we found no association between SCFA concentration and the

microbiome in BAL or EBC samples. However, BAL and EBC with larger bacterial biomasses may have detectable signals of SCFA that correlate with bacterial DNA signatures.

We acknowledge that there are limitations of this small pilot study. First, our tests were done at a single site, on a single instrument, and we did not rigorously test reproducibility. Although the possibility of vial, column, or instrument contamination cannot be completely ruled out, nondetection of SCFA in neat MTBE suggests that these potential sources of contamination did not contribute to our findings. Furthermore, an LC-MS assay confirmed that there are detectable SCFAs in water. We also acknowledge that the limits of detection for SCFA may vary for different assays and that in samples with higher bacterial biomass, correlations between SCFAs and specific microbial taxa may exist.

In conclusion, concentrations of SCFA in BAL and EBC from healthy control subjects are similar to those in water. These results highlight the need to include negative control samples when conducting SCFA assays of respiratory samples and likely other nonfecal samples. Furthermore, because we found detectable SCFA concentrations in samples containing only purified water from numerous different sources, SCFA data generated from such samples need to be interpreted with caution because they may not represent a true biological signal. ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

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A New Configuration for Helmet Continuous Positive Airway Pressure Allowing Tidal Volume Monitoring

To the Editor:

Continuous positive airway pressure (CPAP) is a form of respiratory support that can improve oxygenation (1), limiting the risk of patient–ventilator asynchrony and delivery of high V_T in spontaneous breathing patients with acute hypoxemic respiratory failure. CPAP via helmet compared with face mask could reduce aerosolization of secretion to the environment and operators, increasing safety during viral respiratory infection outbreaks (2).

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