Lung Microbiota Is Related to Smoking Status and to Development of Acute Respiratory Distress Syndrome in Critically Ill Trauma Patients

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Abstract

Rationale: Cigarette smoking is associated with increased risk of acute respiratory distress syndrome (ARDS) in patients after severe trauma; however, the mechanisms underlying this association are unknown.

Objectives: To determine whether cigarette smoking contributes to ARDS development after trauma by altering community composition of the lung microbiota.

Methods: We studied the lung microbiota of mechanically ventilated patients admitted to the ICU after severe blunt trauma. To do so, we used 16S ribosomal RNA gene amplicon sequencing of endotracheal aspirate samples obtained on ICU admission ($n = 74$) and at 48 hours after admission ($n = 30$). Cigarette smoke exposure (quantified using plasma cotinine), ARDS development, and other clinical parameters were correlated with lung microbiota composition.

Measurements and Main Results: Smoking status was significantly associated with lung bacterial community composition at ICU admission ($P = 0.007$ by permutational

multivariate ANOVA [PERMANOVA]) and at 48 hours ($P = 0.03$ by PERMANOVA), as well as with significant enrichment of potential pathogens, including *Streptococcus*, *Fusobacterium*, *Prevotella*, *Haemophilus*, and *Treponema*. ARDS development was associated with lung community composition at 48 hours ($P = 0.04$ by PERMANOVA) and was characterized by relative enrichment of *Enterobacteriaceae* and of specific taxa enriched at baseline in smokers, including *Prevotella* and *Fusobacterium*.

Conclusions: After severe blunt trauma, a history of smoking is related to lung microbiota composition, both at the time of ICU admission and at 48 hours. ARDS development is also correlated with respiratory microbial community structure at 48 hours and with taxa that are relatively enriched in smokers at ICU admission. The data derived from this pilot study suggest that smoking-related changes in the lung microbiota could be related to ARDS development after severe trauma.

Keywords: microbiota; cigarette smoking; trauma; acute respiratory distress syndrome; acute lung injury

(Received in original form February 28, 2017; accepted in final form October 13, 2017)

Supported by NHLBI grants HL110969 and HL133390 (C.S.C.), and HL115354 and HL087115 (J.D.C.). The analytical chemistry in this study was supported in part by U.S. Public Health Service grant DA12393 from the National Institute on Drug Abuse and in part by the University of California, San Francisco, Helen Diller Family Comprehensive Cancer Center.

Author Contributions: Conception and design: A.R.P., S.V.L., J.D.C., N.L.B., M.J.C., and C.S.C.; acquisition, analysis, or interpretation of data: A.R.P., S.V.L., C.L., J.D.C., K.M., M.N., C.K.C., N.L.B., M.J.C., and C.S.C.; drafting of work and/or revising it for important intellectual content: A.R.P., S.V.L., C.L., J.D.C., K.M., M.N., C.K.C., N.L.B., M.J.C., and C.S.C.; and final approval of version to be published as well as agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: A.R.P., S.V.L., C.L., J.D.C., K.M., M.N., C.K.C., N.L.B., M.J.C., and C.S.C.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Crit Care Med Vol 197, Iss 5, pp 621–631, Mar 1, 2018

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Originally Published in Press as DOI: 10.1164/rccm.201702-0441OC on October 16, 2017

Internet address: www.atsjournals.org

At A Glance Commentary

Scientific Knowledge on the

Subject: Cigarette smoking has been associated with acute respiratory distress syndrome (ARDS) development in multiple settings, but the mechanism of this association is not known. Smoking is associated with changes in the oropharyngeal and nasopharyngeal microbiota, but the impact of smoking on the lung microbiota in critically ill patients has not been studied.

What This Study Adds to the

Field: In this pilot study, we analyzed endotracheal aspirates from 74 critically ill, mechanically ventilated patients after severe blunt trauma and found that smoking status was associated with lung bacterial community composition at intensive care unit admission, including enrichment for potentially pathogenic organisms. ARDS development was associated with lung bacterial community composition at 48 hours, and specific taxa enriched in smokers at baseline were found to persist and be enriched at 48 hours in the lungs of patients who developed ARDS. To our knowledge, this is the largest cohort of critically ill patients to undergo lung microbiota analyses. These findings support further study of how smoking-related changes in lung microbiota taxa could be related to ARDS development after severe trauma.

Although supportive measures such as lung-protective ventilation and conservative fluid therapy have improved outcomes for patients with acute respiratory distress syndrome (ARDS) over the past decade, there are still no known specific pharmacotherapies for ARDS. This lack of treatment options emphasizes the need for improved preventive approaches (1) and highlights the importance of understanding the role of environmental exposures in ARDS development. We previously reported that both active smoking and moderate to heavy passive smoking are associated with an increased risk of ARDS in patients after severe trauma, independent of other ARDS risk factors (2). Similar

associations with smoking have since been reported in nonpulmonary sepsis-related ARDS, transfusion-related ARDS, and primary graft dysfunction (ARDS after lung transplant) (3–5). Although these studies provide strong epidemiologic evidence that cigarette smoke exposure may prime the lung for subsequent ARDS development, the mechanisms underlying this association remain unknown.

Cigarette smoke exposure has significant effects on lung epithelial permeability, endothelial injury, and lung inflammation, mimicking the pathologic abnormalities found in patients with ARDS (6). Smoking may also prime the lung for ARDS by influencing the composition of the resident respiratory microbial communities (microbiota). Recently, a large study of respiratory samples from infants revealed that the existence of compositionally distinct airway microbiota was related to significant differences in susceptibility to lower respiratory tract infection (7). Cross-sectional comparisons of active smokers and nonsmokers have demonstrated that smoking enriches the subgingival, oropharyngeal, and nasopharyngeal microbial communities for potentially pathogenic species (8–10). There have been relatively few studies, however, of the impact of smoking on lower airway microbiota, and the literature to date has been limited by relatively shallow community analyses and inconclusive results (10, 11). In one study, researchers examined the lung microbiota of three healthy never smokers, seven smokers without active respiratory disease, and four smokers with chronic obstructive pulmonary disease (11) and found considerable overlap in bacterial community membership among all groups. In another study of 45 nonsmokers and 19 current smokers, the mouth microbiota was found to differ in smokers versus nonsmokers, but lung communities were not found to differ between these groups (12).

In our present pilot study, we used next-generation sequencing technology to test the hypothesis that exposure to cigarette smoke, as quantified by plasma cotinine (the main proximate metabolite of nicotine and a biomarker of tobacco exposure), is associated with altered respiratory bacterial community composition. Specifically, we hypothesized that cigarette smoke exposure is associated with an increase in the relative

abundance of pathogenic genera in the lung microbiota, which may promote ARDS development. In addition, we evaluated compositional dynamics of the lung microbiota in mechanically ventilated trauma patients over the first 48 hours of ICU hospitalization. Some of the results of this study were previously presented in the form of an abstract (13).

Methods

Patients

We studied intubated and mechanically ventilated patients with severe blunt trauma admitted to the ICU at Zuckerberg San Francisco General Hospital (a level 1 trauma center for the City and County of San Francisco) between November 2012 and October 2013. Adult patients with blunt trauma who met criteria for full trauma team activation and who were subsequently intubated and admitted to the ICU were eligible for inclusion. The institutional review board of the University of California, San Francisco, approved the research protocol for this prospective cohort study and granted a waiver of initial consent for the blood and endotracheal aspirate (ETA) sampling. Informed consent was subsequently obtained from patients or their surrogates for continued study participation, as previously described (2).

Sample Collection

Blood was drawn within 10 minutes of emergency department arrival and again at 2, 4, 6, 12, 24, and 48 hours, as previously described (2, 14). Samples were centrifuged and processed immediately, with plasma stored at -80°C for batch processing. ETA samples were obtained following a standard ICU protocol and using a new, sterile in-line suction catheter, at both the time of ICU admission and again approximately 48 hours later. Specimens were immediately stabilized in RNAlater solution (Life Technologies), stored overnight at 4°C , and then at -80°C until further processing.

Data Collection and Outcome Measures

Clinical data were collected prospectively: patient demographics, time and mechanism of injury, comorbidities, injury severity as measured by the Injury Severity Score (15), and prevalence and severity of organ dysfunction (16). Data on smoking history

and alcohol use were collected from the medical record and from the patient and/or the patient's surrogate, using a standardized questionnaire as in our prior work (2). ARDS was defined using the Berlin criteria (17) and adjudicated by two-physician review of all chest radiographs for each of the first 8 ICU days for all patients with any $\text{PaO}_2/\text{FiO}_2$ ratio less than 300 mm Hg.

Cotinine Measurements and Protein Biomarker Assays

Plasma cotinine was measured in samples drawn in the emergency department using liquid chromatography–tandem mass spectrometry, as previously described (18). The limit of quantitation was 0.08 ng/ml. For the few patients who did not have a plasma sample from 0 hours, plasma from 2 hours, 4 hours, or 6 hours was used. Patients were classified as smokers, passive smokers, or nonsmokers, using previously validated cotinine cutpoints; these cutpoints were selected *a priori* as part of the main study hypothesis (2, 19). ELISAs were used to measure protein biomarkers of lung injury in plasma from 0 hours and 48 hours. Additional details regarding ELISA measurements are provided in the online supplement.

16S Ribosomal RNA Gene-based ETA Microbiota Profiling Using Illumina MiSeq

ETA samples were maintained at -80°C until processing, full details of which are provided in the online supplement. After total DNA extraction, polymerase chain reaction amplification of the 16S ribosomal RNA gene variable region 4 was performed in 25- μl reactions using barcoded 515F/806R primers and either 30 ng/ μl of DNA template or DNA extraction buffer as a negative control. All samples amplified, and amplicons were purified using SPRI beads (Beckman Coulter), analyzed for purity using the Agilent DNA 1000 Kit and Bioanalyzer (Agilent Technologies), quantified using the Qubit double-stranded DNA high-sensitivity assay kit (Life Technologies), and pooled at 50 ng per sample. The pooled library, including negative controls, was quantified using the Qubit double-stranded DNA high-sensitivity assay kit, diluted to 2 nM, and denatured, and then a quantity of 5 pM was loaded onto the MiSeq cartridge (V3; Illumina) in combination with 15% (vol/vol) of denatured 12.5 pM PhiX for

sequencing (50,000 reads per sample). Paired-end sequence reads were combined using FLASH (Fast Length Adjustment of Short reads) version 1.2.7 software. Additional details regarding operational taxonomic unit (OTU; taxa) picking and data analyses are provided in the online supplement.

α -Diversity (within sample) indices were calculated using QIIME (Quantitative Insights into Microbial Ecology) (20). β -Diversity (between sample composition dissimilarity) was assessed using Canberra, unweighted UniFrac, and weighted UniFrac distance matrices for 0-hour and 48-hour samples (21, 22). Distance matrices were visualized using the Emperor tool (23). Cigarette smoke exposure, development of ARDS, and other clinical data were analyzed using the vegan version 2.0.10

package (24) in R version 3.1.1 (R Foundation for Statistical Computing) and permutational multivariate ANOVA (PERMANOVA) for relationships with lower respiratory bacterial β -diversity (composition) (25). To test for significant differences in taxon abundance between smokers and nonsmokers and patients with and without ARDS (ARDS+ and ARDS−, respectively), we simultaneously applied Poisson, negative binomial, and zero-inflated negative binomial regressions (three-model approach) to taxon count data and tested for best fit on a taxon-by-taxon basis using a multiply rarefied OTU table. After correction for false discovery (Benjamini-Hochberg), taxa that exhibited a q value less than 0.1 and a P value less than 0.05 were considered significant, and these data were illustrated on phylogenetic

Table 1. Patient Demographics by Smoking Status as Determined by Plasma Cotinine ($n = 74$)

	Nonsmokers (Cotinine < LOQ; $n = 21$)	Passive Smokers (Cotinine > LOQ and ≤ 3.08 ng/ml; $n = 15$)	Smokers (Cotinine > 3.08 ng/ml; $n = 38$)	P Value*
Age, yr, mean $\pm$ SD	59 $\pm$ 23	43 $\pm$ 23	39 $\pm$ 13	0.01
Male sex, n (%)	11 (52)	10 (67)	30 (79)	0.11
Race, n (%)				0.08
White	12 (57)	13 (87)	28 (74)	
African American	0 (0)	0 (0)	5 (13)	
Asian	6 (29)	2 (13)	1 (3)	
Unknown	3 (14)	0 (0)	4 (11)	
Hispanic ethnicity, n (%)	1 (5)	5 (33)	6 (16)	0.09
ARDS, n (%)	4 (19)	5 (33)	7 (18)	0.32
Injury Severity Score, mean $\pm$ SD	29 $\pm$ 13	24 $\pm$ 21	20 $\pm$ 15	0.12
Medical history, n (%)				
Asthma	0 (0)	2 (13)	5 (13)	0.24
Cirrhosis	1 (5)	2 (13)	0 (0)	0.09
Congestive heart failure	0 (0)	1 (7)	0 (0)	0.14
COPD	0 (0)	0 (0)	2 (5)	0.40
Coronary artery disease	0 (0)	1 (7)	1 (3)	0.50
Diabetes	6 (29)	3 (20)	3 (8)	0.09
HIV	0 (0)	0 (0)	3 (8)	0.2
Malignancy	2 (10)	2 (13)	1 (3)	0.32
Alcohol abuse composite [†] , n (%)				0.001
Yes	1 (5)	5 (33)	23 (61)	
No	14 (67)	7 (47)	13 (34)	
N/A	6 (29)	3 (20)	2 (5)	
ETA sample season, n (%)				0.93
Autumn	4 (19)	2 (13)	10 (26)	
Winter	8 (38)	3 (20)	10 (26)	
Spring	6 (29)	1 (7)	10 (26)	
Summer	3 (14)	9 (60)	8 (21)	

Definition of abbreviations: ARDS = acute respiratory distress syndrome; COPD = chronic obstructive pulmonary disease; ETA = endotracheal aspirate; LOQ = limit of quantitation; N/A = not available.

*By ANOVA.

[†]Defined as a chart history of alcohol abuse or an Alcohol Use Disorders Identification Test score greater than or equal to 8 for men and greater than or equal to 5 for women (39, 40).

trees using the iTOL version 3.0 tool (26). Procrustes and Mantel test analyses combining the 0- and 48-hour coordinate matrices were performed in QIIME to examine relative relationships in community composition over time.

Results

Patient Demographics

We studied 74 mechanically ventilated patients admitted to the ICU after severe blunt trauma using ETA samples obtained at the time of ICU admission ($n = 74$) and at 48 hours postadmission ($n = 30$, including 25 patients who were studied at both time points). Plasma cotinine levels of the 74 patients with 0-hour samples indicated that 21 subjects were nonsmokers, 15 were passive smokers, and 38 were smokers. Smokers were younger than non-smokers and more commonly had a history of alcohol abuse, compared to non-smokers and passive smokers. Further demographic details about these patients are provided in Table 1.

Lung Microbial Composition at ICU Admission

We first examined 0-hour samples to characterize the baseline lung microbiota of trauma patients on ICU admission. α -Diversity measures, including bacterial community richness (number of taxa detected), evenness (distribution of taxa within a community), and diversity (Shannon's, Simpsons, or Faith's phylogenetic index), did not significantly differ between nonsmokers, passive smokers, and smokers ($P > 0.05$ by Kruskal-Wallis test).

We next analyzed whether smoking covaried with lower airway microbial community composition (β -diversity) (Table 2). At baseline, smoking status based on cotinine level categorized into three groups (i.e., smokers, passive smokers, nonsmokers) was significantly associated with microbial community composition in the lower airways (univariate PERMANOVA; Canberra distance; $r^2 = 0.03$; $P = 0.007$) (Figure 1A). Cotinine categorized into two groups (i.e., smokers and passive smokers vs. nonsmokers) was also significantly associated with microbial community composition in the lower airways (univariate PERMANOVA; Canberra distance; $r^2 = 0.02$; $P = 0.003$) (Table 2). In

addition, the presence or absence of congestive heart failure, alcohol abuse based on chart history, and age were significantly associated with bacterial community β -diversity, and season of ETA sample collection and cirrhosis diagnosis trended toward a statistically significant relationship (Table 2). These findings indicate that the presence or absence of lower abundance taxa represents a primary discriminatory feature of the microbial communities associated with these clinical factors. ARDS development during the first 8 days in the ICU was not significantly associated with overall lung bacterial community composition at 0 hours (Table 2).

Because smoking, age, and alcohol abuse may be coassociated, we performed analyses of the association between smoking and microbiota composition adjusted for age and for alcohol abuse, using the varpart function in the vegan package in R. Owing

to missing alcohol abuse data for a number of patients, the sample size for these analyses was reduced to 54. With this smaller sample size and both smoking and alcohol abuse in the model, neither smoking nor alcohol abuse was significantly associated with microbiota composition (see Table E1 in the online supplement). Because alcohol abuse data were frequently missing, thereby reducing sample size and statistical power, and were likely not randomly missing, we also performed a sensitivity analysis in which missing data were treated either as all "yes" responses or all "no" responses. In both the "yes" response model and the "no" response model, the adjusted r^2 value for smoking by cotinine status was larger than the adjusted r^2 value for alcohol abuse (Table E1). The smoking by cotinine r^2 value adjusted for age ($r^2 = 0.00312$; $P = 0.026$) was also higher than the age r^2 value adjusted for smoking by cotinine

Table 2. Significance of Relationship between Clinical Parameters and Time 0 Lung Microbiota ($n = 74$)

Parameter	r^2 Value	P Value
Significant metadata		
Smoking status by plasma cotinine level (nonsmoker or passive smoker/smoker)	0.02	0.003
Smoking status by plasma cotinine level (nonsmoker, passive smoker, smoker)	0.03	0.007
Congestive heart failure	0.02	0.02
Alcohol abuse by chart history	0.02	0.03
Age	0.02	0.04
Demographics		
Race (White, African American, Asian)	0.03	0.11
Insurance source	0.04	0.51
BMI	0.01	0.66
Sex (M/F)	0.01	0.69
Hispanic ethnicity	0.01	0.99
Comorbidities		
Cirrhosis	0.02	0.06
Diabetes	0.02	0.13
Asthma	0.02	0.20
Chronic obstructive pulmonary disease	0.01	0.55
HIV	0.01	0.62
Patient history		
ETA sample season	0.05	0.051
ETA sample month	0.16	0.07
Alcohol abuse composite (Y/N)*	0.02	0.07
Alcohol use by chart history (Y/N)	0.02	0.08
Antibiotics prior to ETA sample (Y/N)	0.01	0.21
Injury Severity Score	0.01	0.47
Pack-years	0.08	0.66
Outcomes		
Acute respiratory distress syndrome during first 8 d in ICU (Y/N)	0.01	0.68

Definition of abbreviations: BMI = body mass index; ETA = endotracheal aspirate.

All analyses were done by univariate permutational multivariate ANOVA.

*Defined as a chart history of alcohol abuse or an Alcohol Use Disorders Identification Test score greater than or equal to 8 for men and greater than or equal to 5 for women (39, 40).

To identify specific bacterial groups enriched in the lower airways of smokers, we compared taxon relative abundance of 0-hour samples obtained from smokers and nonsmokers (Figure 1B; Table E2). Smokers exhibited significant

We also identified taxa enriched at 0 hours in patients with a “yes” response to alcohol abuse by chart history. Several taxa enriched in alcohol abusers were shared with those enriched in smokers at this time point, including *Haemophilus* (*Proteobacteria*), *Prevotella* (*Bacteroidetes*), *Streptococcus* (*Firmicutes*), *Fusobacterium* (*Fusobacteria*), and *Porphyromonas* (*Bacteroidetes*) (Table E2; highlighted in orange); however, the majority of taxa enriched in smokers were not enriched in alcohol abusers. Taxa enriched at 0 hours in both patients under 40 years of age (median age in this cohort) and smokers included distinct *Haemophilus* (*Proteobacteria*), *Streptococcus* (*Firmicutes*), *Prevotella* (*Bacteroidetes*), and *Fusobacterium* (*Fusobacteria*) (Table E2; highlighted in light blue). Taxa enriched in all three categories in patients at 0 hours similarly included specific *Streptococcus* (*Firmicutes*), *Fusobacterium* (*Fusobacteria*), and *Prevotella* (*Bacteroidetes*) taxa (Table E2; highlighted in pink).

To examine lung microbiota dynamics over the first 48 hours post-ICU admission, we next studied community β -diversity in 25 patients from whom paired ETA samples were obtained at 0 and 48 hours. At 48 hours, all patients, regardless of smoking status, exhibited a substantial compositional divergence relative to their baseline bacterial community composition (Figure 2A). This observation was supported by both Procrustes and Mantel statistical analyses, in which we tested whether a significant relationship existed

Figure 2. (A) Procrustes analysis comparing 0-hour and 48-hour sample weighted UniFrac coordinate matrices ($n = 25$). (B) A comparison of mean community distance between all 0-hour samples on the left and between all 48-hour samples on the right, indicating that 48-hour microbiota exhibit significantly greater mean distance between samples (i.e., greater microbiota compositional variability at this time point). The box-and-whisker plot represents median, minimum, and maximum values. The dashed lines represent mean and SD. **** $P < 0.0001$. PC = principal component.

between bacterial community composition of paired 0- and 48-hour samples. Both of these analyses indicated that overall bacterial community composition at 0 and 48 hours was not significantly related ($P = 0.332$ by Monte Carlo method; $P = 0.251$ by Mantel test) (Figure 2A). Calculated mean community distances between 0- and 48-hour samples further confirmed this finding; 48-hour samples exhibited significantly greater community composition variation (as indicated by greater mean distance) than those samples collected at the time of hospital admission ($P < 0.0001$) (Figure 2B).

Lung Microbial Composition at 48 Hours after ICU Admission

We next examined all patients from whom 48-hour samples were collected ($n = 30$) to identify factors that explained the observed variation in bacterial community composition at this time point. At 48 hours, the cohort consisted of 8 nonsmokers, 6 passive smokers, and 16 smokers. The demographics of these patients are described in Table E3. At 48 hours, smoking by cotinine level categorized as three groups or two groups (as described above) was again

significantly associated with the variance in lung bacterial community composition ($r^2 = 0.093$; $P = 0.03$ by univariate PERMANOVA; $r^2 = 0.052$; $P = 0.03$) (Figure E1), explaining larger proportions of variance (9.3 and 5.2%, respectively). This finding indicates that phylogeny and the presence or absence of specific taxa are the primary discriminatory features of 48-hour lung bacterial communities associated with smoking by cotinine level. Several taxa that were significantly enriched in smokers at 0 hours remained significantly enriched in smokers (vs. nonsmokers) at 48 hours post-ICU admission; these included specific *Haemophilus*, *Streptococcus*, *Prevotella*, *Porphyromonas*, and *Campylobacter* taxa (Table 3). These data suggest that smoking exerts a persistent effect on the lower airway microbiota, characterized by the enrichment of specific taxa on ICU admission that remain in high relative abundance at 48 hours postadmission.

Univariate PERMANOVA based on a weighted UniFrac distance matrix (which considers both phylogenetic relationships and relative abundance) showed that ARDS development during the first 8 days in the ICU (Figure 3A) ($r^2 = 0.08$; $P = 0.04$), Injury Severity Score ($r^2 = 0.09$; $P = 0.03$), and presence or absence of asthma ($r^2 = 0.08$; $P = 0.04$) were significantly related to lung bacterial community composition at 48 hours. This finding indicates that lung injury, severity of traumatic injury, and chronic airway disease are related to both bacterial phylogeny and taxon relative abundance in the lower airways after 48 hours of hospitalization. It should be noted that by 48 hours, 80% of patients had received parenteral or enteral antimicrobial therapy. However, antibiotic administration (categorized by antibiotic class or by spectrum of activity) was not significantly associated with lung community composition ($r^2 = 0.04$ for antibiotic class, $P = 0.08$; $r^2 = 0.04$ for antibiotic spectrum; $P = 0.29$; analyses by unweighted UniFrac; similar findings for weighted UniFrac and Canberra), and patients who developed ARDS did not differ in the antibiotic class ($P = 0.47$) or spectrum of antibiotic activity ($P = 0.1$) administered over the initial 48 hours, compared with those who did not develop ARDS.

Table 3. Taxa Significantly Enriched in Smokers Compared with Nonsmokers at Both 0 h and 48 h

OTU	Phylum	Family	Genus	Average Relative Abundance* (%) in Smokers at 0 h	Average Relative Abundance* (%) in Nonsmokers at 0 h	Mean Read Count in Smokers at 0 h	Mean Read Count in Nonsmokers at 0 h	P Value†	Average Relative Abundance* (%) in Smokers at 48 h	Average Relative Abundance* (%) in Nonsmokers at 48 h	Mean Read Count in Smokers at 48 h	Mean Read Count in Nonsmokers at 48 h	P Value†
956702	Proteobacteria	Pasteurellaceae	Haemophilus	1.22	0.0024	732.89	1.43	1.48E-15	0.762	0.0008	456.59	0.5	1.60E-04
4471251	Proteobacteria	Pasteurellaceae	Haemophilus	6.85	0.1727	4,108.19	103.57	4.39E-14	6.219	0.1514	3,728.91	90.75	5.63E-05
4469359	Proteobacteria	Pasteurellaceae	Haemophilus	0.58	0.0188	348.98	11.29	1.53E-12	0.588	0.0140	352.27	8.38	1.11E-04
4416265	Proteobacteria	Pasteurellaceae	Haemophilus	0.05	0.0017	29.4	1	1.88E-11	0.044	0.0019	26.32	1.13	2.98E-03
4443574	Proteobacteria	Pasteurellaceae	Haemophilus	0.10	0.0060	62.85	3.62	6.24E-08	0.109	0.0019	65.23	1.13	1.11E-03
4407433	Proteobacteria	Pasteurellaceae	Haemophilus	0.01	0.0005	7.87	0.29	1.57E-05	0.013	0.0004	8.09	0.25	4.30E-03
1059655	Firmicutes	Streptococcaceae	Streptococcus	5.93	0.7744	3,557.62	464.33	2.83E-05	0.365	0.0527	218.59	31.63	0.014
3678349	Firmicutes	Streptococcaceae	Streptococcus	0.33	0.0458	199.13	27.48	3.61E-05	0.568	0.0492	340.73	29.5	1.28E-03
4428060	Bacteroidetes	Prevotellaceae	Prevotella	0.06	0.0054	38.47	3.24	0.0005	1.609	0.0225	964.95	13.5	1.39E-04
747980	Bacteroidetes	Prevotellaceae	Prevotella	0.21	0.0325	124.68	19.48	0.002	0.355	0.0015	212.82	0.88	6.65E-09
4423790	Bacteroidetes	Porphyromonadaceae	Porphyromonas	0.18	0.0357	109.53	21.43	0.003	0.238	0.0240	142.55	14.38	0.005
32546	Proteobacteria	Campylobacteraceae	Campylobacter	0.02	0.0027	11.53	1.62	0.012	0.009	0.0002	5.55	0.13	0.01

Definition of abbreviation: OTU = operational taxonomic unit.
*Relative abundance was calculated as follows: (read count of taxa from sample/read depth samples normalized to) × 100. The average of these values is presented in the table.
†P value was calculated via three-model approach that considers mean read counts of OTUs for smokers compared with nonsmokers.

Figure 3. (A) Principal coordinates analysis plot (weighted UniFrac distance) of 48-hour samples colored by acute respiratory distress syndrome (ARDS) status. The r^2 value represents the percent variance explained, and the P value denotes the significance of this percentage. (B) Discriminatory taxa (48-h three-model analysis comparing ARDS-positive [ARDS+] with ARDS-negative [ARDS-] patients) labeled by genus. OTU = operational taxonomic unit; PC = principal component.

Lung Microbial Community Composition Associated with ARDS Development

We next determined taxonomic differentials that characterized the 48-hour lung microbiota of patients who developed ARDS during the first 8 days in the ICU (ARDS+; $n = 13$) and those who did not develop the syndrome during that time period (ARDS-; $n = 17$) (Figure 3B; Table E4). We hypothesized that some of these ARDS-associated taxa would be enriched in smokers versus nonsmokers at baseline. ARDS+ patients were most significantly

enriched for a specific taxon belonging to the *Enterobacteriaceae* (OTU2119418) (Figure 3B); others exhibiting relative expansion in ARDS+ patients included specific *Preotella* (OTU4447248) and *Fusobacterium* (OTU4319899) (Figure 3B). These latter two taxa were also among those significantly enriched in smokers at baseline (Figure 1B; Table E2).

Plasma Biomarkers and Lung Microbiota Composition

As an intermediate phenotype of lung injury, selected plasma biomarkers of

inflammation, lung epithelial injury, and endothelial injury were measured at 0 and 48 hours. These biomarkers (complete list provided in the online supplement) have previously been associated with lung injury pathogenesis and/or outcomes. Using paired microbiota and biomarker data at 0 hours, soluble intercellular adhesion molecule-1, IL-8, and vascular endothelial growth factor (VEGF) categorized into quartiles were all found to be significantly associated with lung bacterial community variation (Table 4). Using paired microbiota and biomarker data generated from 48-hour

samples, we found that IL-6 and IL-8 categorized into quartiles were also significantly associated with community variation at this time point (Table 4). Interestingly, 0-hour microbiota composition was significantly related to 48-hour levels of VEGF, RAGE (receptor for advanced glycation end-products), ANG-2 (angiopoietin-2), PENT3 (pentraxin 3), and IL-8, and these markers explained the largest proportion of variation in the baseline microbiota (Table 4). These data indicate that microbiota composition is related to contemporary markers of endothelial and epithelial injury and inflammation, as well as that baseline microbiota composition on ICU admission is related to markers of lung injury and inflammation observed after 48 hours of hospitalization.

Discussion

In this pilot study, we found that cigarette smoking is associated with differences in lung microbial community composition with enrichment of specific taxa, both at the time of ICU admission and at 48 hours postadmission. In addition, lung microbiota composition at 48 hours was associated with ARDS development and with injury severity. We also found that for all patients, bacterial community composition in the lung significantly diverged at 48 hours post-ICU admission from that observed on admission to the ICU. To our knowledge,

this study represents the largest cohort of critically ill patients to undergo sequence-based analyses of the lung microbiota, as well as the first study of the impact of smoking on the lung microbiota in the setting of critical illness.

Lung bacterial community composition at 0 hours and 48 hours was significantly associated with smoking status based on cotinine level. These relationships were observed using distinct distance matrices (Canberra at 0 h and unweighted UniFrac at 48 h) and were robust after adjustment for confounding factors. This finding indicates that whereas at baseline smoking is related to lung bacterial communities that differ primarily on the basis of relative abundance of specific taxa, at 48 hours this relationship is driven by the presence or absence of specific phylogenetic groups. Specific taxonomic enrichments associated with smokers at baseline included *Haemophilus*, *Streptococcus*, *Prevotella*, and *Campylobacter*. A small number of these taxa were also enriched in patients who abused alcohol and were younger, indicating that these factors may also shape airway community composition. At 48 hours, these same taxa were significantly enriched in smokers. These data suggest smoke exposure leads to increased relative abundance of specific bacterial taxa in the lung microbiota, which remain persistently enriched in smokers over time. In a recent study of infants, *Haemophilus*-dominated airway microbiota were associated with an

odds ratio of 11 for acute respiratory infection (ARI), and *Streptococcus*-dominated communities were associated with an odds ratio of 2.0 for ARI (7). Thus, it is plausible that smoke exposure shapes the lung microbiota—or alternatively that it shapes the oropharyngeal microbiota which subsequently migrates lower in the respiratory tract in the setting of intubation and critical illness—and enriches for specific potentially pathogenic organisms that increase susceptibility to ARDS.

Patients who developed ARDS over the first 8 days in the hospital also exhibited taxonomic differences in their lung microbial communities at 48 hours compared with those who did not develop ARDS during that period. ARDS+ patients were most significantly enriched for *Enterobacteriaceae* (OTU2119418), which are found as both commensals and pathogens in the human gut. Although controversial (27), some studies have suggested that trauma can lead to bloodstream translocation of gut microbiota, which offers a potential explanation for these findings (28, 29). Alternatively, microaspiration (either prior to or despite endotracheal intubation) could potentially lead to increased presence of gut microbiota members in the lungs of patients with ARDS at 48 hours. Notably, these findings are also consistent with a recently published report that gut-specific bacteria were the dominant microbiota members in BAL fluid from humans with ARDS (30). Other taxa associated with ARDS development at 48 hours include specific *Fusobacterium* and *Prevotella* taxa also found to be significantly enriched in smokers at 0 hours. Members of both of these genera common in oral microbiota have been found in anaerobic respiratory infections, in some cases associated with the development of severe ARDS (31, 32). Furthermore, both *Fusobacterium* and *Prevotella* have been shown to induce increased production of the proinflammatory cytokine IL-6 (33, 34). Lung microbial community composition at 0 hours, based on a distance metric that weights relative abundance, was related to endothelial injury (plasma intercellular adhesion molecule-1), epithelial injury (VEGF), and inflammation (IL-8), whereas at 48 hours microbiota variation was associated with inflammation (IL-6 and IL-8). At 48 hours, the observed relationship between microbiota and inflammation was

Table 4. Plasma Biomarkers Significantly Associated with Lung Microbiota Composition

Lung Microbiota Time Point	Plasma Biomarker, Time Point	r^2 Value	P Value
Baseline (0 h)	ICAM-1, 0 h*	0.02	0.02
	IL-8, 0 h*	0.02	0.04
	VEGF, 0 h*	0.02	0.04
	VEGF, 48 h [†]	0.07*; 0.09 [†]	0.03*; 0.02 [†]
	RAGE, 48 h [†]	0.08	0.04
	Ang-2, 48 h [†]	0.17	0.02
	Pentraxin-3, 48 h [†]	0.15	0.02
48 h	IL-8, 48 h [†]	0.13	0.04
	IL-6, 48 h [†]	0.08	0.01
	IL-8, 48 h [†]	0.07	0.02

Definition of abbreviations: Ang = angiopoietin; ICAM = intercellular adhesion molecule; RAGE = receptor for advanced glycation end-products; VEGF = vascular endothelial growth factor.

All biomarkers were analyzed in quartiles using univariate permutational multivariate ANOVA.

*Canberra (distance matrix that weights rare taxa).

[†]Unweighted UniFrac (distance matrix weighted by phylogenetic relationships).

[‡]Weighted UniFrac (distance matrix weighted by both phylogenetic relationships and relative abundance).

driven by the presence or absence of specific taxa, suggesting that loss or outgrowth of specific bacteria after the first 48 hours in the ICU may contribute to inflammation observed at this time point. In addition, bacterial β -diversity on ICU admission was related to severity of both lung injury and inflammation, as reflected by plasma biomarkers, at 48 hours. Taken together, these data suggest that smoke exposure is associated with a distinct bacterial community enriched for potential pathogens that could increase susceptibility to ARDS and contribute to pulmonary inflammation and injury during hospitalization.

Compared with the relatively modest variation in lung community composition observed at admission, lung microbiota in patients after 48 hours of hospitalization significantly diverged. Although we did not detect a significant association between antibiotic administration and composition of the lung microbiota at 48 hours, this analysis was likely underpowered owing to the wide variety of antimicrobials administered and the relatively small number of patients at the 48-hour time point. Studies in which researchers have observed a relationship between antibiotic administration and microbial community composition have had far larger patient numbers, as much as six times the number of patients as in our present study (35).

In this study, we also identified taxon-level variation (i.e., different OTUs) but genus-level conservation (e.g., *Neisseria* or *Streptococcus*) in smokers versus nonsmokers. This finding suggests that smoking may select for specific species or strains within airway-adapted genera, potentially resulting in replacement of commensal strains with phylogenetically related but genomically distinct members of the same genera. Further exploration of this

possibility will require metagenomic or targeted isolate sequencing efforts, because the 16S ribosomal RNA gene variable region 4 sequenced in this study cannot confidently assign identity at the species or strain level.

This pilot study has several strengths, including the use of next-generation sequencing to identify members of the lung microbiota, early sampling of patients at the time of ICU admission and subsequent sampling 48 hours later, meticulous clinical phenotyping for ARDS, and quantitative measurement of tobacco exposure using a well-validated biomarker. This pilot study also has some limitations. First, the sample size, though to our knowledge the largest published on this topic, remains relatively modest, both at time 0 and at 48 hours, limiting the insights gained from analyses adjusted for potential confounders. Future studies with larger sample sizes and more complete data on alcohol abuse must be performed to further discern the relationship between these variables, because it remains possible that alcohol abuse and/or age may be contributing to some of the observed differences between smokers and nonsmokers. Similarly, the analyses of association between lung microbiota and both clinical variables and plasma biomarkers were not controlled for multiple comparisons, owing to the sample size, and, as such, merit replication in larger cohorts. Second, we were unable to obtain 48-hour specimens from all patients enrolled at baseline, because some patients died or were extubated between the two time points. Third, we used ETA rather than BAL specimens, which some investigators have argued may be superior for capturing the distal lung microbiota, although this is still controversial. For logistical reasons, it was not possible to obtain immediate BAL specimens from patients with severe injuries

at the time of ICU admission. Although there may be regional differences in the microbiota of the upper and lower airways and the alveolar compartments in the lung (36), these microbial communities likely represent a continuum (37, 38); thus, we believe that ETA is a practical and useful approach for microbial community analysis in critically ill patients in whom more invasive procedures may be prohibitively risky or temporally impossible. Similarly, although ETA samples may be affected by colonization of the endotracheal tube (more so at 48 h than at baseline), these samples have the advantage of bypassing the oropharynx, and there are no feasible alternative approaches to studying in ventilated patients the lung microbiota that do not pass through the endotracheal tube. Finally, although ARDS developed after 48 hours in most patients, because 5 patients developed ARDS between 0 and 48 hours, it is possible that the link between lung microbiota and ARDS could be driven in part by ARDS development, either via antibiotic administration spurred by the development of radiographic opacities or by dysfunction of the alveolar-capillary barrier.

In conclusion, we identified significant differences in the lung microbiota composition in critically ill patients related to both smoking status and subsequent development of ARDS, as well as a marked divergence in bacterial community composition between 0 and 48 hours of ICU admission in critically ill patients with blunt trauma injuries. Further investigations with larger sample sizes, and ideally with concurrent experimental models, are needed to confirm the findings of this pilot and to further evaluate the role of the lung microbiota in ARDS pathogenesis. ■

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

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