

Multicenter Comparison of Lung and Oral Microbiomes of HIV-infected and HIV-uninfected Individuals

James M. Beck^{1,2}, Patrick D. Schloss³, Arvind Venkataraman³, Homer Twigg III⁴, Kathleen A. Jablonski⁵, Frederic D. Bushman⁶, Thomas B. Campbell¹, Emily S. Charlson⁷, Ronald G. Collman⁷, Kristina Crothers⁸, Jeffrey L. Curtis^{3,9}, Kimberly L. Drews⁵, Sonia C. Flores¹, Andrew P. Fontenot¹, Mary A. Foulkes⁵, Ian Frank⁷, Elodie Ghedin¹⁰, Laurence Huang¹¹, Susan V. Lynch¹¹, Alison Morris¹², Brent E. Palmer¹, Thomas M. Schmidt³, Erica Sodergren¹³, George M. Weinstock¹³, and Vincent B. Young³; for the Lung HIV Microbiome Project

¹Department of Medicine, University of Colorado Denver, Aurora, Colorado; ²Veterans Affairs Eastern Colorado Health Care System, Denver, Colorado; ³Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan; ⁴Department of Medicine, Indiana University, Indianapolis, Indiana; ⁵Department of Epidemiology and Biostatistics, George Washington University, Washington, District of Columbia; ⁶Department of Microbiology and ⁷Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania; ⁸Department of Medicine, University of Washington, Seattle, Washington; ⁹Veterans Affairs Ann Arbor Healthcare System, Ann Arbor, Michigan; ¹⁰Department of Computational and Systems Biology and ¹²Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania; ¹¹Department of Medicine, University of California San Francisco, San Francisco, California; and ¹³The Jackson Laboratory for Genomic Medicine, Farmington, Connecticut

Abstract

Rationale: Improved understanding of the lung microbiome in HIV-infected individuals could lead to better strategies for diagnosis, therapy, and prophylaxis of HIV-associated pneumonias. Differences in the oral and lung microbiomes in HIV-infected and HIV-uninfected individuals are not well defined. Whether highly active antiretroviral therapy influences these microbiomes is unclear.

Objectives: We determined whether oral and lung microbiomes differed in clinically healthy groups of HIV-infected and HIV-uninfected subjects.

Methods: Participating sites in the Lung HIV Microbiome Project contributed bacterial 16S rRNA sequencing data from oral washes and bronchoalveolar lavages (BALs) obtained from HIV-uninfected individuals (n = 86), HIV-infected individuals who were treatment naive (n = 18), and HIV-infected individuals receiving antiretroviral therapy (n = 38).

Measurements and Main Results: Microbial populations differed in the oral washes among the subject groups (*Streptococcus*, *Actinomyces*, *Rothia*, and *Atopobium*), but there were no individual

taxa that differed among the BALs. Comparison of oral washes and BALs demonstrated similar patterns from HIV-uninfected individuals and HIV-infected individuals receiving antiretroviral therapy, with multiple taxa differing in abundance. The pattern observed from HIV-infected individuals who were treatment naive differed from the other two groups, with differences limited to *Veillonella*, *Rothia*, and *Granulicatella*. CD4 cell counts did not influence the oral or BAL microbiome in these relatively healthy, HIV-infected subjects.

Conclusions: The overall similarity of the microbiomes in participants with and without HIV infection was unexpected, because HIV-infected individuals with relatively preserved CD4 cell counts are at higher risk for lower respiratory tract infections, indicating impaired local immune function.

Keywords: lung; microbiome; HIV infection; bronchoscopy; bronchoalveolar lavage

(Received in original form January 21, 2015; accepted in final form August 5, 2015)

Supported by U.S. Public Health Service grants U01HL98961 (J.M.B., J.L.C., and V.B.Y.), R01HG005975 (P.D.S.), U01HL098960 (H.T. and G.M.W.), U01HL098958 (K.A.J., M.A.F., P.D.S., and K.L.D.), U01HL098957 (F.D.B. and R.G.C.), R01HL090342 (K.C.), U01HL098996 (T.B.C., S.C.F., and A.P.F.), U01HL098962 (A.M. and E.G.), U01HL098964 (L.H. and S.V.L.), and National Institutes of Health/Human Microbiome Project grants R0100417746 (T.M.S.) and T32 HL007749 (A.V.). Additional National Institutes of Health support was provided by University of Pittsburgh Clinical and Translational Science Institute grants UL1 RR024153 and UL1 TR000005, the University of California San Francisco Clinical and Translational Science Institute grant UL1 TR000004, and the University of Colorado Clinical and Translational Science Institute grant UL1 TR001082.

Author Contributions: Conception and design: J.M.B., P.D.S., A.V., H.T., K.A.J., F.D.B., T.B.C., R.G.C., J.L.C., S.C.F., A.P.F., M.A.F., E.G., L.H., S.V.L., A.M., E.S., G.M.W., and V.B.Y. Acquisition of data: J.M.B., H.T., F.D.B., T.B.C., E.S.C., R.G.C., K.C., J.L.C., S.C.F., A.P.F., I.F., E.G., L.H., S.V.L., A.M., B.E.P., E.S., G.M.W., and V.B.Y. Analysis and interpretation of data: J.M.B., P.D.S., A.V., K.A.J., K.L.D., M.A.F., T.M.S., and G.M.W. Writing the manuscript: J.M.B., P.D.S., A.V., H.T., K.A.J., F.D.B., T.B.C., R.G.C., S.C.F., A.P.F., M.A.F., E.G., L.H., S.V.L., A.M., and T.M.S. Final approval of the manuscript: All authors.

Correspondence and requests for reprints should be addressed to James M. Beck, M.D., Medicine Service (111), Veterans Affairs Eastern Colorado Health Care System, 1055 Clermont Street, Denver, CO 80220. E-mail: james.beck@ucdenver.edu

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 192, Iss 11, pp 1335–1344, Dec 1, 2015

Copyright © 2015 by the American Thoracic Society

Originally Published in Press as DOI: 10.1164/rccm.201501-0128OC on August 6, 2015

Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the

Subject: Analysis of the lung microbiome is a relatively new field and may suggest novel therapeutic approaches to pulmonary infections and disease processes. Differences in oral and lung microbiomes between HIV-infected and HIV-uninfected subjects require further definition, including possible differences in microbiome composition in HIV-infected subjects who are treatment naive and those receiving antiretroviral therapy.

What This Study Adds to the

Field: This multicenter study enrolled subjects who were HIV-negative, HIV-infected and treatment naive, and HIV-infected and receiving antiretroviral therapy. We compared oral washes and bronchoalveolar lavages (BALs) and used a novel neutral model procedure to exclude environmental contaminants. We demonstrated significant differences among the groups in oral washes, but BALs were similar. Each subject group demonstrated differences between oral washes and BALs. There was no relationship between CD4 counts and microbiome data.

The diversity and frequency of infections and other pathogenic lung processes in HIV-infected individuals suggest significant impairments in host defense in this population. A better understanding of the respiratory tract microbiome in HIV-infected individuals, including the lung and the upper respiratory tract, could lead to improved strategies for diagnosis, therapy, and prophylaxis of HIV-associated pneumonias in this population.

The lung was long considered to be a sterile compartment, but recent molecular investigations from multiple groups demonstrate that the lung contains bacterial DNA sequences, although at low levels relative to the upper respiratory tract (1). However, clarifying the relationship between the microbiome of the lung and the microbiome of other compartments, including the oropharynx, presents a challenge for lung microbiome analysis

(2, 3). Because sampling of the lower airways by bronchoscopy depends on insertion of a bronchoscope through the nose or mouth, careful methodologic attention to possible contamination from the upper airway is required (4). Environmental sources that may confound the lung data must be evaluated, particularly in low bioburden samples. Although bacterial taxa in the lung closely resemble those of the upper airway from the oropharynx (5), there seem to be differences between the mouth and the lungs in both health and disease (6, 7). A growing body of literature documents changes in the microbiome that occur during pulmonary disease processes, including chronic obstructive pulmonary disease (8–11), bronchiectasis (12), asthma (13–15), lung transplantation (16–19), bronchopulmonary dysplasia (20, 21), and cystic fibrosis (22, 23).

The Lung HIV Microbiome Project (LHMP) is a multicenter consortium established by the NHLBI to understand the respiratory tract microbiome during HIV infection. Previous work from the LHMP compared smokers and nonsmokers without HIV infection and demonstrated that the oropharyngeal microbiome in smokers differs from that in nonsmokers (6). However, significant differences were not detected in the lung microbiome between smokers and nonsmokers. The LHMP then demonstrated that in HIV-infected individuals there is widespread colonization of the lung by the bacterium *Tropheryma whippelii*, and the institution of antiretroviral therapy decreases the relative abundance of this organism (7). However, the broader bacterial community characteristics of the upper and lower respiratory tract were not addressed in HIV-infected individuals in this study.

In the current study, we performed a comprehensive analysis of upper and lower respiratory tract bacterial microbiomes from individuals with and without HIV infection. We examined volunteers who were HIV-uninfected, who were HIV-infected but untreated with antiretroviral therapy, and who were HIV-infected and effectively treated with antiretroviral therapy. We chose to study clinically healthy individuals, rather than those with active pulmonary infections or other disease processes, to understand baseline changes in microbial populations that occur during HIV infection and treatment. These studies will help to inform further investigations aimed at understanding HIV-associated lung diseases.

Methods

Study Design

The LHMP performed a prospective, multisite cohort study that was approved by the institutional review boards at each participating site. A second level of institutional review was provided by the Data Coordinating Center and the NHLBI, including an Observational Safety Monitoring Board. All subjects provided written informed consent as approved by the review board at each site.

Participants

Participants were healthy individuals with and without HIV infection, recruited from eight cities participating in the LHMP. Men and women aged 18 to 80 years were eligible. We enrolled HIV-negative subjects (referred to as “Negative”), HIV-positive subjects who had not been treated with any antiretroviral medications (referred to as “Naive”), and HIV-positive subjects currently receiving highly active antiretroviral medications (referred to as “HAART”).

Subjects completed questionnaires including medical history and use of medications including nonprescription drugs, illicit drugs, tobacco, and/or marijuana. At the time of microbiome sampling, individuals were asymptomatic for upper respiratory or lung infections and had no reported fever, cough, or other acute respiratory symptoms during the previous 4 weeks. Subjects had not used any antibacterial medications in the previous 3 months and had not used corticosteroids (including inhaled medications) in the previous 6 months. Individuals with known respiratory diagnoses, including chronic obstructive pulmonary disease and asthma, were ineligible for enrollment.

Sample Collection

Oropharyngeal and lung samples were collected as previously described (6). Smokers were asked to stop smoking at least 12 hours before collection, and all subjects were instructed to avoid food and water from midnight before the procedure. Subjects gargled with 10 ml sterile 0.9% saline at the start of the procedure, and this oral wash sample was collected and iced immediately. To potentially reduce the presence of bacteria in the upper airway, all subjects then gargled with antiseptic

mouthwash immediately before bronchoscopy. Bronchoscopy was performed as previously described (6). Using minimal sedation, the bronchoscope was inserted through the mouth and was advanced quickly to the wedge position in the right middle lobe or lingula. Bronchoalveolar lavage (BAL) was collected by instillation and aspiration of 0.9% sterile saline to a maximum instillation volume of 300 ml. Subjects from the University of Pennsylvania cohort underwent bronchoscopy with a two-scope method as previously described (5). Samples obtained by this method did not differ systematically from those obtained at the other sites (data not shown); thus, data were combined for the current analysis.

Controls obtained at the time of sampling included a neat sample of the sterile saline immediately after the container was opened and 10 to 50 ml of the same saline aspirated through the suction channel of the designated bronchoscope before the procedure. Control samples were processed and sequenced in parallel with the subjects' samples.

Sample Processing, Sequencing, and Curation

DNA was extracted at each site, using consensus protocols. To assure consistency in sequencing, all samples were sequenced at Washington University. Sequencing was performed on a Roche 454 FLX Titanium platform using primers for variable regions 1 through 3 (V1–3) and regions 3 through 5 (V3–5). Successful sequencing was substantially more robust for V1–3 than V3–5. Additionally, V3–5 primers resulted in fewer specific amplifications that included more host sequences (data not shown). Therefore, we focused on the V1–3 sequences for this study. The 16S rRNA sequences were assigned to organizational taxonomic units (OTUs) using the mothur software package according to previously described methods (6, 24, 25).

Neutral Model Analysis for Potential Contaminant Sequences

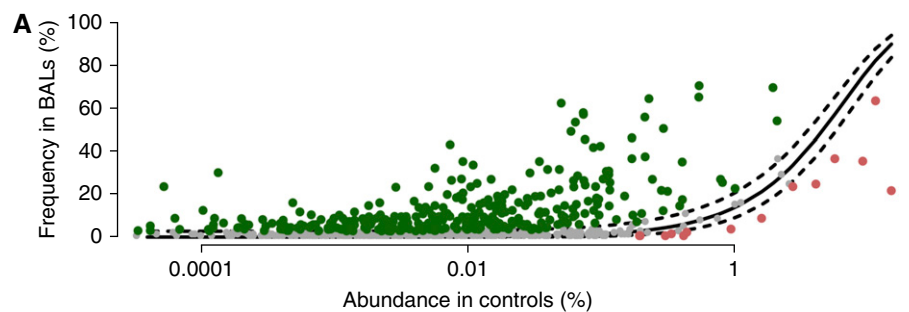
We previously used the neutral model to examine enrichment of oral versus lung samples (6, 26). In this analysis, frequency describes the percentage of samples in which an OTU was detected, and abundance describes the percentage of sequences assigned to an individual OTU. For the present study, we used the neutral model to identify sequences in BAL

samples that may have arisen from contamination introduced by the bronchoscope or from reagents used in sample collection or processing. In the implementation of the model, the control samples (bronchoscope and reagents) were considered as sources contributing DNA sequences to the BAL samples (Figure 1).

Statistics

Demographic characteristics were compared using Chi-square tests or Fisher

exact test for categorical variables, analyses of variance for normally distributed continuous variables, and the Wilcoxon rank test for skewed data (for example, in comparing CD4 counts between groups). Alpha diversity was evaluated by comparing the number of observed OTUs and the Shannon Diversity Index by rarefying each sample to 500 sequences with 1,000 randomizations. The Shannon Diversity Index combines measurements of evenness (distribution of OTUs across a population)



B Sequence classification	Abundance (%)		Frequency (%)	
	BAL	Controls	BAL	Controls
Unclassified Actinomycetales	6.8	14.9	18	20
Unclassified Bacillales	1.7	2.1	36	21
<i>Ralstonia</i>	1.4	11.3	60	70
Unclassified Rhizobiales	0.9	5.6	36	42
<i>Ralstonia</i>	0.8	9.1	32	29
<i>Rhizobium</i>	0.8	4.0	25	39
<i>Pelomonas</i>	0.6	2.2	29	29
<i>Streptococcus</i>	0.7	0.4	5	5
<i>Streptococcus</i>	0.5	0.2	7	6
<i>Bosea</i>	0.3	2.6	25	26

C Sequence classification	Abundance (%)		Frequency (%)	
	BAL	Controls	BAL	Controls
<i>Streptococcus</i>	10.7	1.9	70	25
<i>Veillonella</i>	3.2	0.3	51	11
<i>Veillonella</i>	2.7	0.5	70	14
<i>Streptococcus</i>	2.6	0.5	65	15
<i>Streptococcus</i>	1.6	0.2	55	9
<i>Prevotella</i>	1.5	0.4	35	8
<i>Tropheryma</i>	1.6	0.0005	10	0.4
<i>Veillonella</i>	1.5	0.06	23	4
<i>Prevotella</i>	1.5	0.4	35	8
<i>Prevotella</i>	1.2	0.2	27	7

Figure 1. Application of the neutral model to assess possible contaminant sequences identified in control samples. The bronchoscope, saline solution for sample collection, and extraction reagents were considered as potential sources of contaminating DNA. Sequences consistent with the model prediction (gray dots) and sequences enriched in the control samples (red dots) were considered as potential contaminants and removed from subsequent analyses. Sequences overrepresented in bronchoalveolar lavage (BAL) samples (green dots) were retained (A). The 10 most abundant sequences excluded from subsequent analysis (B) and retained for analysis (C) are shown. Some genus names are repeated in B and C as they reflect different sequences with the same taxonomic classification.

and richness (number of different OTUs in a population) to express diversity numerically. After randomly selecting 500 sequences per sample with 1,000 randomizations, the distances between populations were measured using Θ_{YC} (Θ_{YC}) as previously described (27). Θ_{YC} measures dissimilarity between the structures of communities. We tested for differences in the average distance between each group of samples by analysis of molecular variance, a statistical tool analogous to analysis of variance (28). OTUs were compared by the Wilcoxon signed rank test for all OTUs with average relative abundances of greater than 1% across all samples. These tools produce similar results to the weighted and unweighted UniFrac distances (data not shown); we chose the Θ_{YC} distance metric over the UniFrac metrics because the former is based on the relative abundance of individual OTUs, whereas the latter is based on a phylogenetic tree without the use of OTU designations.

Results

Demographics

We enrolled 142 subjects from the clinical LHMP sites (Table 1). Eighty-six subjects

were HIV negative (“Negative”), 18 subjects were HIV positive but had not received antiretroviral medications (“Naive”), and 38 subjects were HIV positive and treated with highly active antiretroviral medications (“HAART”). The groups were not matched for demographic characteristics. The Negative group contained a higher proportion of women than both HIV-positive groups, and the HAART group was older than the other groups. The HIV-positive groups contained larger proportions of African Americans, and the Naive group contained more Hispanics.

As expected, plasma HIV RNA was significantly lower in the HAART group than in the Naive group. However, the CD4 counts in the Naive and HAART groups did not differ significantly and reflected relatively preserved immune function in this clinically healthy cohort. There were no significant differences in smoking status among the groups, with each cohort containing nonsmokers, current smokers, and former smokers. Analysis of molecular variance revealed no differences in microbiome data by smoking status when the data were corrected for multiple comparisons. Therefore, data were combined for analysis. Although we

excluded subjects who reported antibacterial use within 3 months of sampling, we also examined more distant antibacterial use. The Naive group contained a higher proportion of subjects who had used antibacterial medications in the 3 to 6 months before enrollment, but the difference was not statistically significant.

Neutral Model Application, Sequencing Yield, Observed OTUs, and Diversity Indices

We applied the neutral model to the BAL samples to identify and to remove sequences that might have resulted from contamination sources (Figure 1). After curation and removal of sequences potentially derived from control sources, samples containing greater than or equal to 500 sequences were subject to analysis. The increased microbial biomass present in oral wash samples, compared with BAL samples, resulted in improved 16S rRNA sequence amplification and successful sequencing of 136 oral wash samples (Table 2). Despite the relatively low biomass present in BAL, DNA recovery was adequate and sequencing was successful for 111 BAL samples. Sequences were binned at the 97% identity levels and were assigned OTUs as described in the METHODS.

Table 1. Characteristics of Participants

	HIV Negative (n = 86)	HIV/Naive (n = 18)	HIV/HAART (n = 38)	P Value
Sex, n (%)				
Male	50 (58)	16 (89)	31 (82)	<0.01*
Female	36 (42)	2 (11)	7 (18)	
Age, mean ± SD, yr	44.9 ± 13.0	41.3 ± 7.8	51.7 ± 8.5	<0.01†
Race, n (%)				
White	56 (65)	6 (33)	18 (47)	<0.01‡
African American	26 (30)	9 (50)	20 (53)	
Other	4 (5)	3 (17)	0	
Ethnicity, n (%)				
Hispanic	5 (6)	4 (22)	3 (8)	0.02‡
Not Hispanic	81 (94)	13 (72)	35 (92)	
Decline to state		1 (6)		
CD4 count/μl, median (min–max)	ND	668 (290–1,192)	618 (208–1,265)	0.62†
Plasma HIV RNA copies/ml, median (min–max)	ND	6,432 (38–88,246)	36 (10–604)	<0.01§
Smoking status, n (%)				
Nonsmoker	46 (53)	5 (28)	15 (40)	0.25*
Current	30 (35)	9 (50)	18 (47)	
Former	10 (12)	4 (22)	5 (13)	
Antibiotics < 6 mo, n (%)	7 (8)	4 (22)	5 (13)	0.14*

Definition of abbreviations: HIV/HAART = HIV-positive subjects treated with highly active antiretroviral therapy; HIV/Naive = HIV-positive but treatment-naive subjects; HIV Negative = HIV-negative subjects; ND = not done.

*Chi-square test.

†Analysis of variance.

‡Fisher exact test.

§Wilcoxon rank test.

Table 2. Samples Sequenced Successfully

	HIV Negative (n = 86)	HIV/Naive (n = 18)	HIV/HAART (n = 38)
Oral wash	80	18	38
BAL	67	14	30

Definition of abbreviations: BAL = bronchoalveolar lavage; HIV/HAART = HIV-positive subjects treated with highly active antiretroviral therapy; HIV/Naive = HIV-positive but treatment-naive subjects; HIV Negative = HIV-negative subjects.

Samples considered successfully sequenced contained at least 500 sequences after curation and contaminant removal.

Numbers of observed OTUs in the oral washes and BALs did not differ among groups (*see online supplement*). Similarly, the Shannon Diversity Index did not differ among groups (*see online supplement*).

Community Structure Comparison by Subject Group

We compared nonmetric multidimensional scaling plots using all V1–3 sequences from oral washes obtained from subjects in the three groups (Figure 2A). Nonmetric multidimensional scaling plots provide two-dimensional visualization of differences between members of a population, with similar members grouping together, and this analysis allows measurement of overall differences in microbial populations among the subject groups. Oral washes from the Negative subjects differed significantly

from those obtained from Naive subjects ($P < 0.01$) and also differed significantly from those obtained from HAART subjects ($P = 0.01$). In comparison of the two HIV-infected groups, there were also significant differences between the oral washes obtained from Naive and HAART subjects ($P = 0.01$). Thus, oral microbial populations differ significantly between HIV-uninfected and HIV-infected subjects and also differ significantly according to HIV treatment status.

We then compared all sequences from BALs from the subjects in the three groups (Figure 2B). Unlike the oral washes, we did not detect statistically significant differences among the groups. Therefore, our findings do not support differences in the microbial populations sampled in BAL according to HIV infection status or by HIV treatment status.

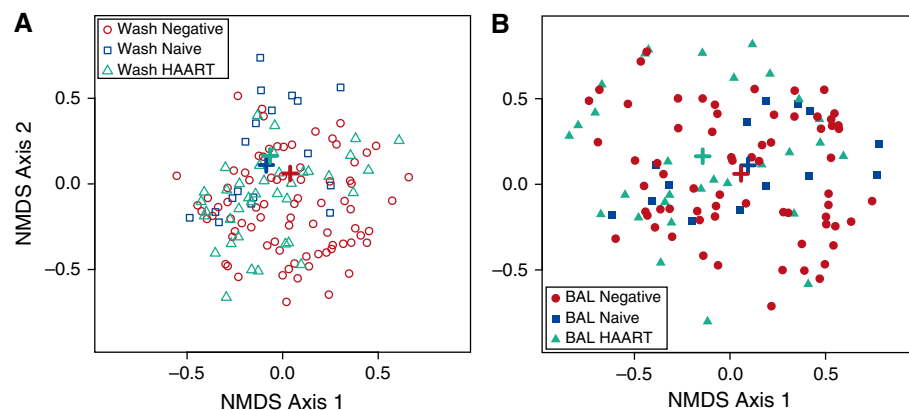


Figure 2. Nonmetric multidimensional scaling (NMDS) plots for all subjects showing oral wash (A) and bronchoalveolar lavage (BAL) (B). Samples were obtained as detailed from HIV-negative subjects (“Negative,” red circles), HIV-positive but treatment-naive subjects (“Naive,” blue squares), and HIV-positive subjects treated with highly active antiretroviral therapy (“HAART,” green triangles). Analysis is based on organizational taxonomic units (OTUs) from sequences of the V1–3 regions of the 16S rRNA. Centroids are indicated by crosses. For oral washes, NMDS analysis demonstrated significant differences in OTUs between Negative and Naive subjects ($P < 0.01$), and between Negative and HAART subjects ($P = 0.01$), and between Naive and HAART subjects ($P = 0.01$). There were no significant differences in the BALs.

Community Structure Comparison by Body Site

We next examined the relationships in microbial communities between oral washes and BALs in each subject group. There were significant differences in communities between oral wash and BAL in the Negative group (Figure 3A; $P < 0.01$), in the Naive group (Figure 3B; $P = .03$), and in the HAART group (Figure 3C; $P < 0.01$). These results support our previous investigations (6) and provide further evidence that the lung compartment, as sampled by BAL, does not completely mirror the oropharyngeal compartment.

Differences in Taxa by Subject Group

We examined the taxa corresponding to the most abundant sequences to determine which specific taxa accounted for the differences in community structure described above. Comparison of sequences in all oral washes demonstrated that a *Streptococcus* OTU and two *Actinomyces* OTUs were significantly more abundant in Naive subjects than in the other groups, and an *Atopobium* OTU was significantly more abundant in Naive and HAART groups than in Negative subjects (Figure 4). Additionally, a *Rothia* OTU was significantly more abundant in oral washes from the HAART group than in the other groups. In contrast to the oral washes, there were no significant differences in the most common taxa identified in BALs from the three subject groups (Figure 5).

Differences in Taxa by Body Site

Comparison of oral washes and BALs from the Negative subjects demonstrated significant differences in abundances of 11 OTUs, including increased abundances of *Streptococcus*, *Veillonella*, *Prevotella*, *Fusobacterium*, *Rothia*, *Porphyromonas*, *Granulicatella*, and *Gemella* OTUs in oral washes than in BAL (Figure 6A). In contrast, a *Veillonella*, a *Tropheryma*, and a *Prevotella* OTU were found in greater abundance in BAL than in oral wash. For the Naive subjects, only *Veillonella*, *Rothia*, and *Granulicatella* OTUs were more abundant in oral wash than in BAL (Figure 6B). The pattern of differences in the HAART group resembled the Negative group much more closely, including the increased abundance of *Tropheryma* in BAL than in oral wash samples (Figure 6C). Thus, each group of subjects demonstrated

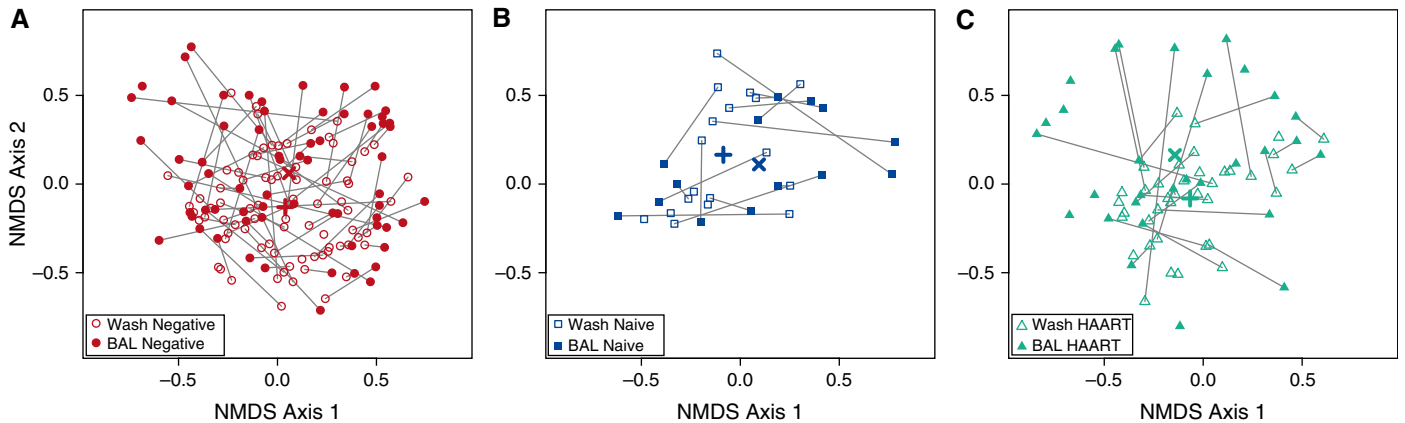


Figure 3. Nonmetric multidimensional scaling (NMDS) plots comparing oral washes (*open symbols*) and bronchoalveolar lavages (BALs) (*solid symbols*) within subject groups. Individual subjects are linked by lines. Samples were obtained as detailed in METHODS, and V1–3 regions were sequenced. There were significant differences in oral wash and BAL samples within HIV-negative subjects (Negative subjects) (A) ($P < 0.01$), HIV-positive but treatment-naive subjects (Naive subjects) (B) ($P = 0.03$), and HIV-positive subjects treated with highly active antiretroviral therapy (HAART subjects) (C) ($P < 0.01$).

a unique pattern in the taxa differing in oral washes and BALs.

Effect of CD4 Count on Microbiome

Finally, we examined whether CD4 count influenced oral wash or BAL microbiome composition in the two groups of HIV-positive subjects. When CD4 count was evaluated as a dichotomous value, there were no significant differences regardless of the nominal value of CD4 used as a cutoff (data not shown). We also determined whether CD4 count, evaluated

as a continuous variable, might modulate the microbiome, and again determined that there were no overall differences, including examination of specific OTUs (*see* Figures E1 and E2 in the online supplement).

Discussion

This large, multicenter study conducted at sites across the United States contributes unique information about the oral and lung microbiomes in healthy HIV-uninfected and HIV-infected individuals and

represents the first data directly comparing these subject groups. We did not detect overall differences in numbers of OTUs or in the Shannon diversity index. Microbial populations in oral washes differed between HIV-uninfected and HIV-infected subjects, but BAL populations did not differ significantly. The most surprising finding in our study was the overall similarity of the microbiota in subjects with and without HIV infection. This result was unexpected, since HIV-infected individuals (even with relatively preserved CD4 counts) are at higher risk for lower respiratory tract bacterial infections, including bacterial pneumonia and tuberculosis, indicating significantly impaired local immune function. We did identify greater differences in oral washes than in BALs. It is possible that these differences reflect the greater complexity of the oropharyngeal compartment and/or increased exposure to the environment, but we did observe significant differences in our subject groups related to HIV infection and treatment status. Alterations in the oral microbiome have important implications for disease processes (29), such as periodontal diseases (30), but may precede changes in the lung. Previous work by one of the LHMP collaborators identified increased abundances for a large number of taxa in oral samples from HIV-infected patients with pneumonia, in comparison to HIV-uninfected control subjects (31). A culture-based study identified abundant *Streptococcus* spp. and *Staphylococcus* spp. in oral samples from HIV-infected subjects, but uninfected subjects were not examined

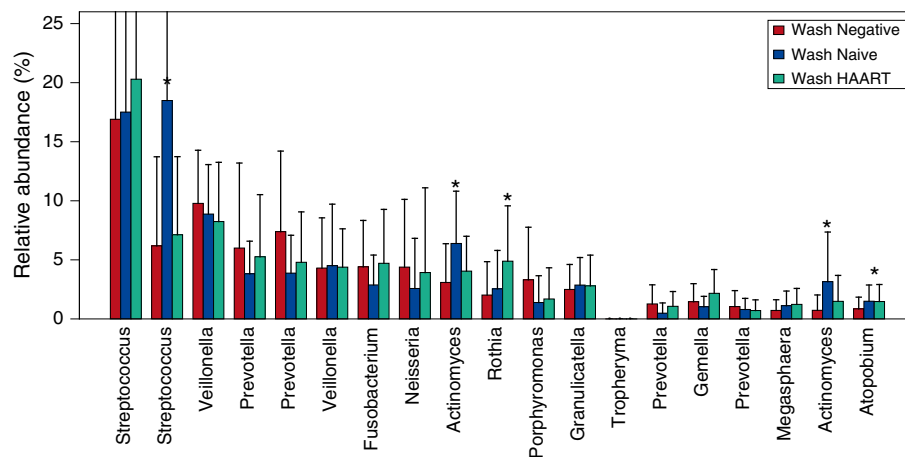


Figure 4. Relative abundances of the most common organizational taxonomic units (OTUs) identified in oral washes from all subject groups. Samples were obtained as detailed in METHODS and V1–3 regions were sequenced. OTUs were compared by the Wilcoxon signed-rank test for all OTUs with average relative abundances of greater than 1% across all samples, and significant differences are demonstrated by asterisks. *Streptococcus* and *Actinomyces* were more abundant in oral washes from HIV-positive but treatment-naive subjects (Naive) than in the other groups. *Rothia* was more abundant in oral washes from HIV-positive subjects treated with highly active antiretroviral therapy (HAART) than in the other groups. Some genus designations appear more than once because multiple OTUs have the same consensus taxonomy. Negative = HIV-negative subjects.

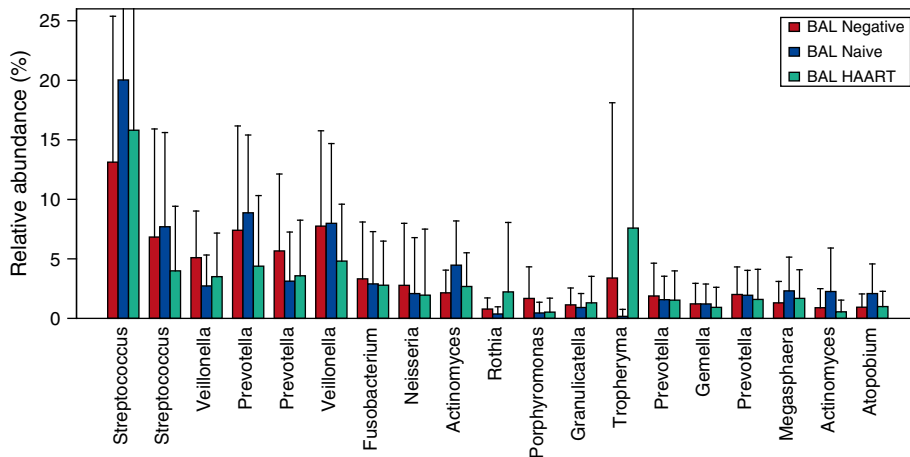


Figure 5. Relative abundances of the most common organizational taxonomic units (OTUs) identified in bronchoalveolar lavage (BAL) from all subject groups. Samples were obtained as detailed, and V1–3 regions were sequenced. OTUs were compared by the Wilcoxon signed-rank test for all OTUs with average relative abundances of greater than 1% across all samples. Overall, there were no significant differences. Some genus designations appear more than once because multiple OTUs have the same consensus taxonomy. HAART = HIV-positive subjects treated with highly active antiretroviral therapy; Naive = HIV-positive but treatment-naive subjects; Negative = HIV-negative subjects.

in this study (32). A study of the lingual microbiome associated increased proportions of *Veillonella*, *Prevotella*, *Megasphaera*, and *Campylobacter* with HIV viremia (33).

The current study extends earlier observations from the LHMP cohorts. We previously compared HIV-uninfected smokers and nonsmokers and found that the lung microbiome in HIV-uninfected individuals was largely derived from the oral microbiome, except for some unique lung species such as *Tropheryma whipplei* (6). The mouth microbiome differed in smokers and nonsmokers, whereas smoking did not alter the lung microbiome significantly. The LHMP then conducted a targeted analysis of colonization of the lung by *T. whipplei* in the lungs of HIV-infected individuals and also demonstrated that the use of antiretroviral therapy decreased the relative abundance of this organism (7). In the current, larger cohort, we adopted a different approach by combining smokers and nonsmokers for analysis (as there were no significant differences in smoking status between subject groups), by analyzing all OTUs, and by approaching potential environmental controls conservatively using the neutral model. In comparing relative abundances of OTUs in oral washes and BALs, we found more OTUs that differed in the Negative and HAART subjects than in the Naive

subjects. Interestingly, we observed differences in *T. whipplei* in Negative and HAART subjects but not in Naive subjects.

Our BAL results agree closely with published data obtained from subjects without respiratory disease and further support the current consensus regarding communities of bacteria identified in the lungs (6, 10, 13, 15, 34). The possible pathogenic, or protective, effects of these taxa require further investigation. However, it is likely that groups of microbes establish lung environments that may encourage or inhibit growth of pathogens or inflammatory reactions. It is important to note that all subject groups in this study were clinically well at the time of enrollment and sampling. Very different results are observed during episodes of clinical pneumonia and with decreased CD4 counts (35).

There are several unique aspects of the current study that advance the literature in this field. First, we collected samples from individuals at eight different clinical sites, whereas most of the existing literature examines subjects enrolled from a single center. Previous, small studies have suggested geographic variation, for example in sputa obtained from patients with cystic fibrosis in the United States and the United Kingdom (36). We did not detect systematic variation among our sites. The current results support our previous

multicenter investigation in which patterns of diversity were not driven by cohort location (7). Next, none of the patients received oral or inhaled corticosteroids. The role of inhaled corticosteroids in modulation of the upper and/or lower airway microbiota remains unclear (14).

Several limitations of the present study require comment. Our groups were not matched demographically, and it is possible that differences influenced the results. However, the statistical power to analyze these factors *post hoc* is problematic given the small numbers in the subgroups. We focused on bacteria and did not analyze viruses or fungi. We also did not analyze gut microbiota or the subjects' diets, but it is likely that the gut and diet have significant influence on the upper and lower airway microbiota (11). In mice, the gut microbiota is intimately associated with pulmonary phenotypes, including allergic and asthma-like conditions (37, 38). Next, the samples were obtained at a single point in time, although several of the LHMP sites are conducting serial sampling. A recent analysis of the Human Microbiome Project data demonstrated that samples from oral communities were the least stable over time, in comparison to the relative stability of gut samples (39). We chose oral washes as integrative samples of the mouth, realizing that it was impractical to control accurately for differences in oral hygiene. Thus, small differences in composition of bacterial communities in different regions of the oropharynx may impact the most appropriate comparator against which to assess BAL specimens (40). Bronchoscopy, although providing the only practical method to sample the lung in large numbers of subjects, runs an inherent risk of contamination from the oropharynx and upper airway during the procedure. The lower biomass of BAL samples from clinically well individuals necessitated the sequencing depth we reported, and future investigations using higher biomass samples should consider deeper sequencing. It also appears that the lung microbiome in individuals who are clinically well and without lung disease is largely derived from the oral microbiome, making it challenging to distinguish carry-over from true members of the community (6). Although sampling during surgery can approach a gold standard (9, 10), this methodology is

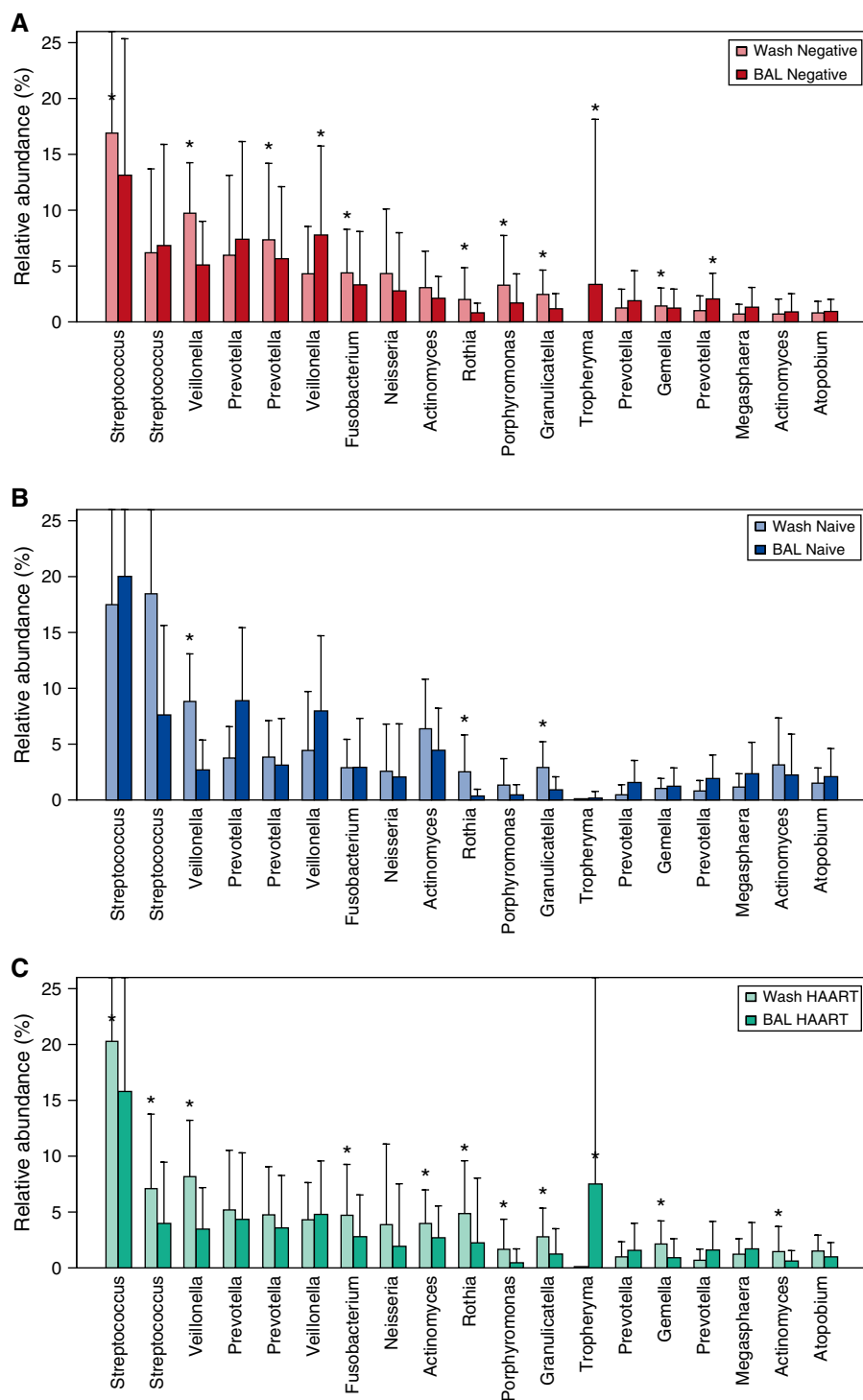


Figure 6. Relative abundances of the most common organizational taxonomic units (OTUs) identified in oral wash versus bronchoalveolar lavage (BAL) for each subject group. Samples were obtained as detailed in METHODS, and V1–3 regions were sequenced. OTUs were compared by the Wilcoxon signed-rank test for all OTUs with average relative abundances of greater than 1% across all samples. The OTUs indicated by asterisks demonstrated significant differences between BAL and oral wash in HIV-negative subjects (Negative) (A), HIV-positive but treatment-naive subjects (naive) (B), and HIV-positive subjects treated with highly active antiretroviral therapy (HAART) (C). Some genus designations appear more than once because multiple OTUs have the same consensus taxonomy.

less practical for studies examining large numbers of subjects or for performing serial sampling.

In conclusion, this study compares the oropharyngeal and lung microbiomes in a healthy population of HIV-uninfected and HIV-infected individuals in a large, multicenter cohort. Oral bacterial communities differed significantly depending on HIV status, whereas lung communities sampled by BAL were similar. We identified specific taxa in oral samples that differed among the subject groups. Within each subject group, we identified a unique signature of taxa differing between the mouth and the lung; the patterns in the Negative and HAART subjects were similar to one another but differed from the pattern in the Naive subjects. These LHMP data serve as a basis for further investigations of the role of the lung microbiome during health and disease in HIV-infected individuals (3). ■

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

Acknowledgment: The authors thank Sandra Colombini-Hatch, Hannah Peavey, Lis Caler, and James Kiley for NHLBI's leadership in lung microbiome research. The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health. Data in this manuscript were collected by the Multicenter AIDS Cohort Study (MACS) with centers at Los Angeles (U01-AI35040): University of California Los Angeles, Schools of Public Health and Medicine; Roger Detels (Principal Investigator [PI]), Otoniel Martinez-Maza (Co-PI), Aaron Aronow, Robert Bolan, Elizabeth Breen, Anthony Butch, Beth Jamieson, Eric N. Miller, John Oishi, Harry Vinters, Dorothy Wiley, Mallory Witt, Otto Yang, Stephen Young, Zuo Feng Zhang; Pittsburgh (U01-AI35041): University of Pittsburgh, Graduate School of Public Health; Charles R. Rinaldo (PI), Lawrence A. Kingsley (Co-PI), James T. Becker, Ross D. Cranston, Jeremy J. Martinson, John W. Mellors, Anthony J. Silvestre, Ronald D. Stall; and the Data Coordinating Center (UM1-AI35043): The Johns Hopkins University Bloomberg School of Public Health; Lisa P. Jacobson (PI), Alvaro Munoz (Co-PI), Alison Abraham, Keri Althoff, Christopher Cox, Jennifer Deal, Gypsyamber D'Souza, Priya Duggal, Janet Schollenberger, Eric C. Seaberg, Sol Su, Pamela Surkan. The MACS is funded primarily by the National Institute of Allergy and Infectious Diseases, with additional cofunding from the National Cancer Institute. MACS data collection is also supported by UL1-TR000424 (JHU CTSA). Website located at <http://www.statepi.jhsph.edu/mac/mac.html>. Data in this manuscript

were collected by the Women's Interagency HIV Study (WIHS) Collaborative Study Group with centers (PI) at The Connie Wofsy Study Consortium of Northern California (Ruth Greenblatt). The WIHS is funded by the National Institute of Allergy and Infectious Diseases

(UO1-AI-35004, UO1-AI-31834, UO1-AI-34994, UO1-AI-34989, UO1-AI-34993, and UO1-AI-42590) and by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (UO1-HD-32632). The study is cofunded by the National Cancer

Institute, the National Institute on Drug Abuse, and the National Institute on Deafness and Other Communication Disorders. Funding is also provided by the National Center for Research Resources (UCSF-CTSI grant number UL1 RR024131).

References

- Beck JM, Young VB, Huffnagle GB. The microbiome of the lung. *Transl Res* 2012;160:258–266.
- Twigg HL III, Morris A, Ghedin E, Curtis JL, Huffnagle GB, Crothers K, Campbell TB, Flores SC, Fontenot AP, Beck JM, et al.; Lung HIV Microbiome Project. Use of bronchoalveolar lavage to assess the respiratory microbiome: signal in the noise. *Lancet Respir Med* 2013; 1:354–356.
- Huang YJ, Charlson ES, Collman RG, Colombini-Hatch S, Martinez FD, Senior RM. The role of the lung microbiome in health and disease: a National Heart, Lung, and Blood Institute workshop report. *Am J Respir Crit Care Med* 2013;187:1382–1387.
- Beck JM. ABCs of the lung microbiome. *Ann Am Thorac Soc* 2014;11: S3–S6.
- Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, Bushman FD, Collman RG. Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am J Respir Crit Care Med* 2011;184:957–963.
- Morris A, Beck JM, Schloss PD, Campbell TB, Crothers K, Curtis JL, Flores SC, Fontenot AP, Ghedin E, Huang L, et al.; Lung HIV Microbiome Project. Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med* 2013;187:1067–1075.
- Lozupone C, Cota-Gomez A, Palmer BE, Linderman DJ, Charlson ES, Sodergren E, Mitreva M, Abubucker S, Martin J, Yao G, et al.; Lung HIV Microbiome Project. Widespread colonization of the lung by *Tropheryma whipplei* in HIV infection. *Am J Respir Crit Care Med* 2013;187:1110–1117.
- Huang YJ, Kim E, Cox MJ, Brodie EL, Brown R, Wiener-Kronish JP, Lynch SV. A persistent and diverse airway microbiota present during chronic obstructive pulmonary disease exacerbations. *OMICS* 2010;14:9–59.
- Sze MA, Dimitriu PA, Hayashi S, Elliott WM, McDonough JE, Gosselink JV, Cooper J, Sin DD, Mohn WW, Hogg JC. The lung tissue microbiome in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2012;185:1073–1080.
- Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, Young VB, Toews GB, Curtis JL, Sundaram B, et al. Analysis of the lung microbiome in the “healthy” smoker and in COPD. *Plos One* 2011;6:e16384.
- Sze MA, Hogg JC, Sin DD. Bacterial microbiome of lungs in COPD. *Int J Chron Obstruct Pulmon Dis* 2014;9:229–238.
- Tunney MM, Einarsson GG, Wei L, Drain M, Klem ER, Cardwell C, Ennis M, Boucher RC, Wolfgang MC, Elborn JS. Lung microbiota and bacterial abundance in patients with bronchiectasis when clinically stable and during exacerbation. *Am J Respir Crit Care Med* 2013;187:1118–1126.
- Huang YJ, Nelson CE, Brodie EL, Desantis TZ, Baek MS, Liu J, Woyke T, Allgaier M, Bristow J, Wiener-Kronish JP, et al.; National Heart, Lung, and Blood Institute's Asthma Clinical Research Network. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J Allergy Clin Immunol* 2011;127: 372–381.e1, 3.
- Huang YJ. Asthma microbiome studies and the potential for new therapeutic strategies. *Curr Allergy Asthma Rep* 2013;13: 453–461.
- Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, Davies J, Ervine A, Poulter L, Pachter L, et al. Disordered microbial communities in asthmatic airways. *Plos One* 2010;5:e8578.
- Dickson RP, Erb-Downward JR, Freeman CM, Walker N, Scales BS, Beck JM, Martinez FJ, Curtis JL, Lama VN, Huffnagle GB. Changes in the lung microbiome following lung transplantation include the emergence of two distinct *Pseudomonas* species with distinct clinical associations. *Plos One* 2014; 9:e97214.
- Willner DL, Hugenholtz P, Yerkovich ST, Tan ME, Daly JN, Lachner N, Hopkins PM, Chambers DC. Reestablishment of recipient-associated microbiota in the lung allograft is linked to reduced risk of bronchiolitis obliterans syndrome. *Am J Respir Crit Care Med* 2013;187:640–647.
- Borewicz K, Pragman AA, Kim HB, Hertz M, Wendt C, Isaacson RE. Longitudinal analysis of the lung microbiome in lung transplantation. *FEMS Microbiol Lett* 2013;339:57–65.
- Charlson ES, Diamond JM, Bittinger K, Fitzgerald AS, Yadav A, Haas AR, Bushman FD, Collman RG. Lung-enriched organisms and aberrant bacterial and fungal respiratory microbiota after lung transplant. *Am J Respir Crit Care Med* 2012;186:536–545.
- Stressmann FA, Connett GJ, Goss K, Kollamparambil TG, Patel N, Payne MS, Puddy V, Legg J, Bruce KD, Rogers GB. The use of culture-independent tools to characterize bacteria in endo-tracheal aspirates from pre-term infants at risk of bronchopulmonary dysplasia. *J Perinat Med* 2010;38:333–337.
- Beeton ML, Maxwell NC, Davies PL, Nuttall D, McGreal E, Chakraborty M, Spiller OB, Kotecha S. Role of pulmonary infection in the development of chronic lung disease of prematurity. *Eur Respir J* 2011;37:1424–1430.
- Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoli JD, VanDevanter DR, Murray S, Li JZ, et al. Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc Natl Acad Sci USA* 2012;109:5809–5814.
- Carmody LA, Zhao J, Schloss PD, Petrosino JF, Murray S, Young VB, Li JZ, LiPuma JJ. Changes in cystic fibrosis airway microbiota at pulmonary exacerbation. *Ann Am Thorac Soc* 2013;10:179–187.
- Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;75: 7537–7541.
- Schloss PD, Gevers D, Westcott SL. Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies. *Plos One* 2011;6:e27310.
- Hubbell SP. Neutral theory in community ecology and the hypothesis of functional equivalence. *Funct Ecol* 2005;19:166–172.
- Yue JC, Clayton MK. A similarity measure based on species proportions. *Commun Stat Theory Methods* 2005;34:2123–2131.
- Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol* 2001;26:32–46.
- Wade WG. The oral microbiome in health and disease. *Pharmacol Res* 2013;69:137–143.
- Razzouk S, Termechi O. Host genome, epigenome, and oral microbiome interactions: toward personalized periodontal therapy. *J Periodontol* 2013;84:1266–1271.
- Iwai S, Fei M, Huang D, Fong S, Subramanian A, Grieco K, Lynch SV, Huang L. Oral and airway microbiota in HIV-infected pneumonia patients. *J Clin Microbiol* 2012;50:2995–3002.
- Sharifzadeh A, Khosravi AR, Shokri H, Asadi Jamnani F, Hajiabdolbaghi M, Ashrafi Tamami I. Oral microflora and their relation to risk factors in HIV+ patients with oropharyngeal candidiasis. *J Mycol Med* 2013;23:105–112.

33. Dang AT, Cotton S, Sankaran-Walters S, Li CS, Lee CY, Dandekar S, Paster BJ, George MD. Evidence of an increased pathogenic footprint in the lingual microbiome of untreated HIV infected patients. *BMC Microbiol* 2012;12:153.
34. Pragman AA, Kim HB, Reilly CS, Wendt C, Isaacson RE. The lung microbiome in moderate and severe chronic obstructive pulmonary disease. *Plos One* 2012;7:e47305.
35. Iwai S, Huang D, Fong S, Jarlsberg LG, Worodria W, Yoo S, Cattamanchi A, Davis JL, Kaswabuli S, Segal M, *et al*. The lung microbiome of Ugandan HIV-infected pneumonia patients is compositionally and functionally distinct from that of San Franciscan patients. *Plos One* 2014;9:e95726.
36. Stressmann FA, Rogers GB, Klem ER, Lilley AK, Donaldson SH, Daniels TW, Carroll MP, Patel N, Forbes B, Boucher RC, *et al*. Analysis of the bacterial communities present in lungs of patients with cystic fibrosis from American and British centers. *J Clin Microbiol* 2011;49:281–291.
37. Huffnagle GB. The microbiota and allergies/asthma. *Plos Pathog* 2010;6:e1000549.
38. Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL, *et al*. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nat Med* 2014;20:159–166.
39. Ding T, Schloss PD. Dynamics and associations of microbial community types across the human body. *Nature* 2014;509:357–360.
40. Charlson ES, Bittinger K, Chen J, Diamond JM, Li H, Collman RG, Bushman FD. Assessing bacterial populations in the lung by replicate analysis of samples from the upper and lower respiratory tracts. *Plos One* 2012;7:e42786.