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

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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

Internet address: 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/rcrm.201501-0129OC on August 6, 2015
Internet address: www.atsjournals.org
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-scopen 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)
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 \( \Theta_{YC} \) (ThetaYC) as previously described (27). \( \Theta_{YC} \) 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 \( \Theta_{YC} \) 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**

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

**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.
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](image)

**Table 2. Samples Sequenced Successfully**

<table>
<thead>
<tr>
<th></th>
<th>HIV Negative (n = 86)</th>
<th>HIV/Naive (n = 18)</th>
<th>HIV/HAART (n = 38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral wash</td>
<td>80</td>
<td>18</td>
<td>38</td>
</tr>
<tr>
<td>BAL</td>
<td>67</td>
<td>14</td>
<td>30</td>
</tr>
</tbody>
</table>

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.

Differences in Taxa by Subject Group

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; $P = 0.03$), 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 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 *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...
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...
Veillonella **T. whipplei** in the lungs of negative and is problematic given diets, but **T. whipplei** Campylobacter Prevotella

Beck, Schloss, Venkataraman, and HAART subjects than in the Naive

OTUs in oral washes and BALs, we found

model. In comparing relative abundances of

by approaching potential environmental

subject groups), by analyzing all OTUs, and

for analysis (as there were no signi

by combining smokers and nonsmokers

HIV-infected individuals and also

lung by **T. whipplei** (7). In the current, larger

microbiome in individuals who are

sputa obtained from patients

it is likely that groups of microbes establish

inflammatory reactions. It is important to

subjects. Interestingly, we observed

in **T. whipplei** in Negative and HAART subjects but not in Naive subjects.

Our BAL results agree closely with

Published data obtained from subjects

several of the LHMP sites are conducting serial sampling.

A recent analysis of the Human

microbiota is intimately associated with

formal, or inhibit growth of pathogens or

RNA viruses or fungi. We also did not analyze

gut microbiota or the subjects’ diets, but

significant influence on the upper and

microbiota in the United States and

carrying over from true members of the

In our BAL samples obtained from subjects

with cystic fibrosis in the United States and

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

pathological 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
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 LHM 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, Lisa Caier, 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-A135043): 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, Soo 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/macs/macs.html. Data in this manuscript are available with the text of this article at www.atsjournals.org.
were collected by the Women’s Interagency HIV Study (WIHS) Collaborative Study Group with centers (PI) at The Connie Wofsy Study Consortium (Division of Women’s Health, Roswell Park Cancer Institute, Buffalo, NY), with centers (PI) at the University of North Carolina (Jennifer Kates and Ruth Greenblatt). The WIHS is funded by the National Institute of Allergy and Infectious Diseases (U01-AI-35004, U01-AI-31834, U01-AI-34994, U01-AI-34989, U01-AI-34993, and U01-AI-42590) and by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (U01-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).

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