

Overview Protocol of the Lung HIV Microbiome Project (LHMP)

Prepared by the

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Preface

This overview of the Lung HIV Microbiome Project (LHMP) describes the design and organization of the overall effort. This overview will be maintained by the LHMP Data Analysis and Coordinating Center (DACC) at The George Washington University throughout the course of the project through new releases of the entire overview, or issuance of updates, or in the form of supplemental memoranda.

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Abbreviations Used

BAL	Bronchoalveolar Lavage
BioLINCC	Biologic Specimen and Data Repository Information Coordinating Center
CRF	Clinical Research Form
DACC	Data Analysis and Coordinating Center
HIV	Human immunodeficiency virus
HMP	NIH Roadmap Human Microbiome Project
IRB	Institutional Review Board
LHMP	Lung HIV Microbiome Project
NHLBI	National Heart, Lung, and Blood Institute
NIH	National Institutes of Health
OSMB	Observational Study Monitoring Board

1. INTRODUCTION

1.1. Abstract

Humans interact with countless microorganisms on skin, in the gut, and in multiple other environments. While the NIH Roadmap Human Microbiome Project (HMP) will characterize nasal, oral, skin, gastro-intestinal, and urogenital microbial communities in a large number of healthy and non-healthy individuals, the Lung HIV Microbiome Project (LHMP) will characterize the microbiome of the lung and respiratory tract. This effort will also provide initial data to develop further hypotheses addressing differences between HIV-infected and HIV-uninfected individuals. The project will involve multiple studies, either single center or multiple-center studies. The research design of each study will vary with the objective of that study.

1.2. Purpose of the Overview Protocol

The Lung HIV Microbiome Project (LHMP) will work collaboratively to ensure the clinical and scientific integrity and success of this project. The LHMP will characterize the microbiome of the lung alone or in combination with the nasal and/or oropharyngeal cavities in HIV-infected individuals and matched HIV-uninfected controls using molecular techniques to identify bacteria and, if possible, other organisms. The Overview Protocol of the LHMP includes, as appendices, the protocols for all center-specific studies and the cross-center collaborative studies which contribute to the LHMP. The Overview Protocol also serves to document the infrastructure by which the collaborative development of protocols, data acquisition, standardization and sharing of data (phenotype, sequencing) and specimens will occur including the planned contribution to data and specimen repositories.

1.3. Objectives

Characterizing the lung microbiome will enable us to learn which microbes are present and where they are located. Starting from this basic knowledge, we will study how these microbes grow, interact among themselves, and relate to other microbial species co-existing in the same niche and with the cells in the lung; and how they are altered by other environmental factors such as the physical environment and cigarette smoke. This knowledge will enhance our understanding of the role of the lung microbiome in preserving health or causing disease and in the divergent effects observed in HIV-infected versus uninfected individuals. Knowledge of the lung microbiome and that of other components of the respiratory tract in health and diseased states may lead to the identification of predictors of disease progression and therapeutic targets for translation into better preventive and treatment strategies

2. OVERVIEW, SPECIFIC AIMS, AND HARMONIZATION

2.1. Overview

The LHMP brings the distinct efforts at each of the clinical/sequencing centers together under a single infrastructure, creating a network to combine the expertise accumulated across the range of involved disciplines in a focused and concentrated collaboration. This network assembles a group of investigators and centers ready to cooperate in collaborative research, allows deliberate and strategic staging of resources and methodical implementation of high quality research, and provides a mechanism for systematically studying disease processes from the broadest possible perspective. In a dynamic research environment, this network is able to take advantage of findings from its own and other reported research efforts to develop and pursue the most meaningful paths of study. The network will prioritize the use of limited resources and take advantage of efficiencies to pursue research. Various subcommittees including the Sequencing Working Group, the Clinical Working Group, and the Sampling Working Group, provided the standardization of the sequencing methods, clinical data collection, and sampling procedures used by the network.

The collaborative LHMP network includes both the clinical and sequencing centers which are listed here and each of the center-specific studies are listed below the center (study numbering system:M00X). The study protocols are located in the appendices.

Overview of the Lung HIV Microbiome Project

2.1.1. Clinical Centers:

Center C001 University of Michigan, Ann Arbor (UM)		
<i>Parent Protocol:</i>	M008	Understanding the Lung Microbiome in HIV-Infected Individuals and HIV-Uninfected Individuals
<i>Sub-study Cohorts:</i>	M008-A	University of Michigan Medical Center
	M008-B	Ann Arbor VAMC
Center C002		
<i>Parent Protocol:</i>	M012	Pennsylvania Lung Microbiome Project
<i>Sub-study Cohorts:</i>	M012-A	Group 1A – HIV+ off HIV Therapy, CD4 ≥ 400, = Number of Smokers and Non-Smokers
	M012-B	Group 1B – HIV+ off HIV Therapy, 200-400 CD4+T, Non-Smokers
	M012-C	Group 2A – HIV+ on HIV Therapy, COPD/Emphysema
	M012-D	Group 2B – HIV+ on HIV Therapy, No Lung Disease
	M012-E	Group 3A – HIV-, COPD/Emphysema, Former Smokers/Current Non-Smokers
	M012-F	Group 3B – HIV-, No COPD, Healthy, Smokers & Non-Smokers
	M012-G	Group 3C – HIV-, Enrolled in the Diet Study, URT Sampling Only/ No Bronchoscopy
	M012-H	Group 3D – HIV-, One scope, Healthy/No Lung Disease, Smokers & Non-Smokers
Center C005		
<i>Overarching Protocol:</i>	The Lung Microbiome in Cohorts of HIV-Infected Persons (Lung MicroCHIP):	
<i>Parent Protocols:</i>	M009	The Options Project (Options)
	M010	Observational Study of the Consequences of the Protease Inhibitor Era (Scope)
	M011	The International HIV-Associated Opportunistic Pneumonias Study (IHOP)
<i>Sub-study Cohorts:</i>	M011-A	San Francisco-San Francisco General Hospital
	M011-B	Kampala, Uganda-Mulago Hospital
Center C006 University of Colorado, Denver (UC Denver)		
<i>Parent Protocol:</i>	M003	Alterations in Lung Microbiome in Acute and Chronic HIV Infection
<i>Sub-study Cohorts:</i>	M003-A	Cohort A1 – Acute or recent HIV-1 infection
	M003-B	Cohort A2 - HIV-1 Seronegative, healthy controls
	M003-C	Cohort B1- HIV-1 infection who are antiretroviral therapy naïve
	M003-D	Cohort B2 – HIV-1 infection who are on stable antiretroviral therapy
<i>Parent Protocol:</i>	M014	Longitudinal Studies of HIV-1 Nef and Pulmonary Arterial Hypertension
Center C007 University of Pittsburgh (Pitt)		
<i>Overarching Protocol:</i>	Pathogens of Obstruction/Emphysema and the Microbiome (POEM):	
<i>Parent Protocol:</i>	M001	Multicenter AIDS Cohort Study (MACS)
<i>Sub-study Cohorts:</i>	M001-A	Pittsburgh
	M001-B	Los Angeles
<i>Parent Protocol:</i>	M002	Women's Interagency HIV Study (WIHS)
<i>Sub-study Cohorts:</i>	M002-B	San Francisco

Center C008 Indiana University (IU)		
<i>Parent Protocol:</i>	M004	Analysis of Immunologic Responses in the Lung
<i>Sub-study Cohorts:</i>	M004-A	New Participants: Smokers vs. Nonsmokers
<i>Parent Protocol:</i>	M006	Examination of HIV Associated Lung Emphysema (EXHALE)
<i>Sub-study Cohorts:</i>	M006-A	Atlanta VAMC
	M006-B	Houston- Michael E. DeBakey VA Medical Center

2.1.2. Sequencing Centers:

Center S001	University of Michigan, Ann Arbor (UM)
Center S002	University of Pennsylvania (Penn)
Center S004	University of Colorado, Boulder (CU Boulder)
Center S005	University of California at San Francisco (UCSF)
Center S007	University of Pittsburgh (Pitt)
Center S003	Washington University in St. Louis (Wash U)

2.2. Specific Aims

The specific aims for each center-specific protocol and each collaborative protocol are included in the appendices.

The aim of this overview protocol is to document the structure and processes of the LHMP including (1) the establishment of a combined database resource of standardized contributed clinical and sequencing core data from center-specific studies and all of the collaborative studies; and (2) the participants' corresponding set of specimens that will ultimately reside in the BioLINCC repository.

Overview of the Lung HIV Microbiome Project

Data Harmonization

2.2.1. Data Acquisition and Management

The LHMP's approach to data acquisition and storage is shown in Figure 1. There are six clinical centers collecting clinical data, and specimens and six sequencing centers producing sequencing data, each using multiple protocols. Each protocol includes data definitions and formats. The methods used for data transfer and acquisition, the curation process, and plans for transfers to the NIH repository are also described. Figure 1 shows the overall data flow for this project.

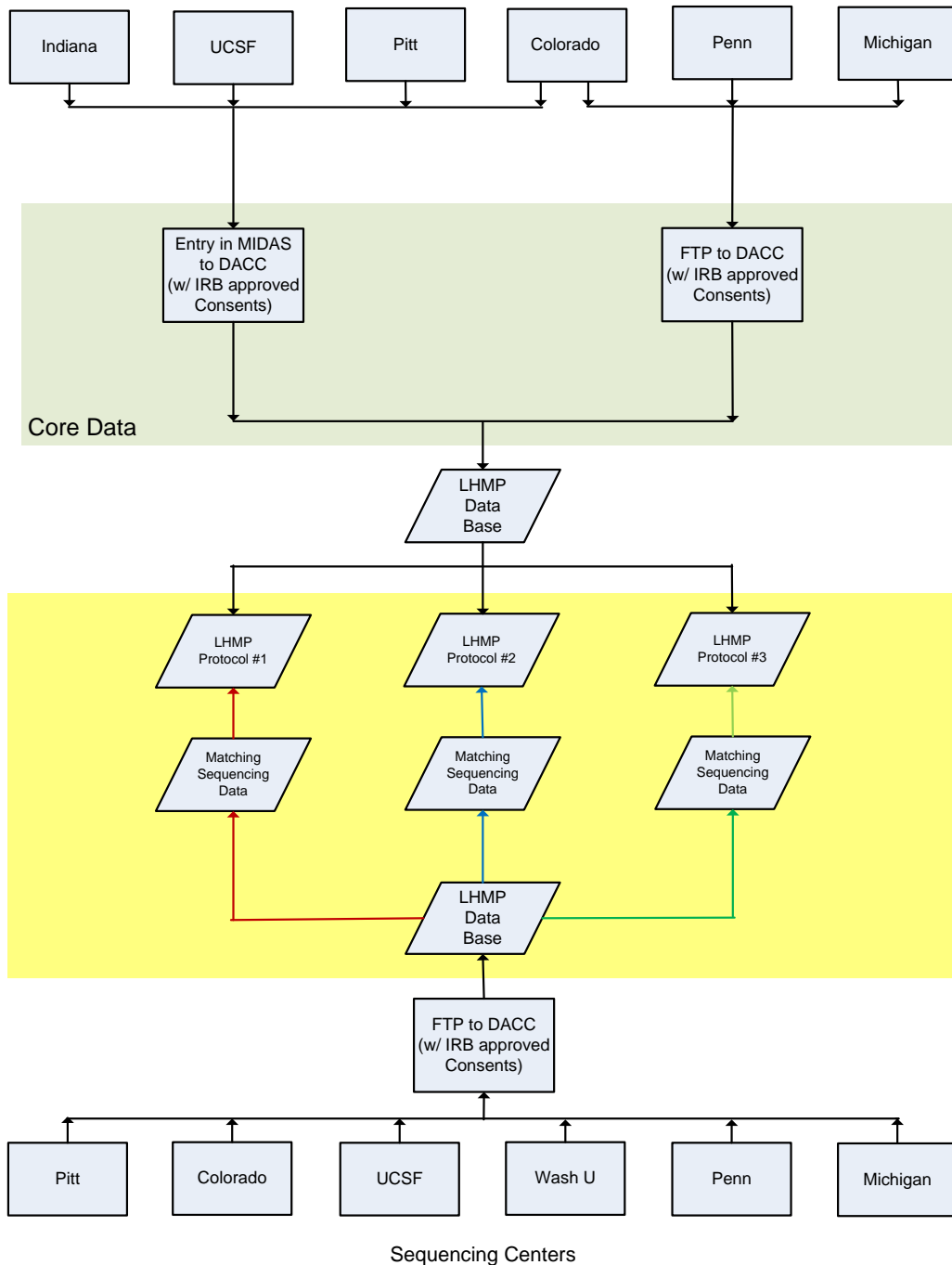


Figure 1. Data Flow Diagram

2.2.2. Collaborative Procedural Design

The LHMP working groups undertook the task to develop a harmonized, collaborative approach to data acquisition and management. The Clinical Working Group developed the data collection forms and clinical procedures for the LHMP. The Sampling Working Group developed procedures for specimen collection including forms to collect the characteristics of the specimens in a standard manner and in accordance with the anticipated requirements of the BioLINCC repository. The Sequencing Working Group developed procedures for processing and analysis of sequencing data.

2.2.2.1. Clinical Data

To standardize the collection of clinical data across centers the LHMP Clinical Working Group has specific data items, definitions and data collection forms which have been agreed upon for this project. These include the Demographic Form, Diagnosis Form, the Pulmonary HIV Questionnaire and Consent Form which have specified core information that all centers agree collect and contribute to the database, where appropriate. ([LHMP Manual of Procedures](#))

Additional data collected during center-specific studies will be the responsibility of the respective center to collect, store, analyze and contribute to a repository as appropriate.

Additionally some of the centers are including lung CT scans in their protocol and they will be following the Longitudinal Lung HIV Study (<http://www.lunghiv.com>) procedure which states that the phantom protocol is scanned onto each sites CT equipment every 6 months to ensure standardization of all CT scans for this. [[Link to MOP]]

2.2.2.2. Sampling Procedures

The sampling procedures were developed by the LHMP Sampling Working Group to standardize the procedures used for bronchoalveolar lavage (BAL), blood and oral wash collections for use by the LHMP Centers in all studies for which they are contributing data. Sampling collection forms were also developed. ([LHMP Manual of Procedures](#)) These standard procedures were developed to meet the requirements of Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC), the National Heart, Lung, and Blood Institute (NHLBI) specimen repository where the remaining specimens will be stored.

2.2.2.1. Sequencing Data and Methods

([LHMP Manual of Procedures](#))

2.2.3. Informed Consent

Information about each study's Informed Consent can be found in the specific protocol (see appropriate appendices). The DACC maintains a template of each Center's Informed Consent Form and documents the individual participant's consent for phenotype and sequencing data and specimens use, which will be linked with the LHMP Specimen Inventory - Characterization Data form.

2.2.3.1. Stored Sample(s)

With the participant's consent, residual sample(s) will be stored indefinitely at the NHLBI BioLINCC.

2.2.4. Quality Assurance

The quality and integrity of the data is of primary importance, particularly in collaborative research projects. The careful development of the protocols, forms, and written procedures has proceeded with that goal in mind. Training of clinical site personnel on the protocol, Clinical Research Form (CRF) completion instructions and data transfer procedures are also essential components of the quality assurance process.

2.2.4.1. Site Visits

At least one visit per site will occur for data quality assurances purposes. Visits for cause may also be conducted if the need arises. Site visits will be conducted to confirm such activities as the study protocol being followed, IRB approval has been documented and accurate, complete and current records are maintained.

2.3. Specimen Inventory – Characterization Data

The LHMP Specimen Inventory data base has a primary goal of maintaining a complete record of the specimens to be submitted to BioLINCC, sponsored by the NHLBI. The database will maintain a complete and updated inventory of the number of specimens available to be used for the LHMP collaborative studies. Tracking and inventory of specimens for use in local studies will remain the responsibility of the local institution. The system will document specific specimen characterization data, as required by BioLINCC.

BioLINCC Guidelines are accessible at:

https://biolincc.nhlbi.nih.gov/static/guidelines/guidelines.html#_Toc236373459

2.4. Confidentiality and Security

The LHMP is aware of the need to ensure patient privacy and data security. All LHMP investigators, staff and project consultants who deal with data must comply with NIH human protection regulations.

The study manual of procedures includes a policy statement regarding the confidentiality of patient data. A key element of this policy is the recognition that the data are to be held in total confidence.

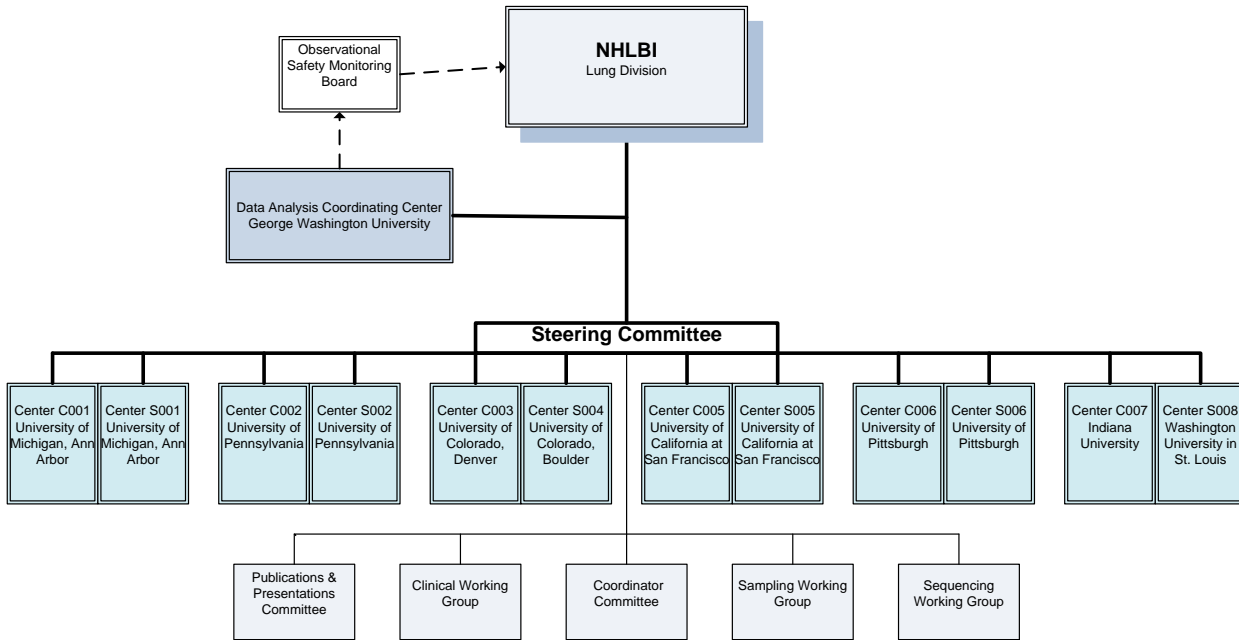
The LHMP Centers will be responsible for reporting adverse and serious adverse events (SAE) both locally and centrally to the project. The SAE Forms specific to each protocol and are to be submitted to the DACC. An SAE must be reported immediately without delay, with information added as it is provided. The DACC will forward reports of SAE to the NHLBI project officer and the Observational Study Monitoring Board (OSMB) chair for review and further action as warranted.

3. PROJECT ADMINISTRATION

3.1. Organization Overview

The Steering Committee is composed of the principal investigators of all the clinical centers and sequencing centers, the DACC, and the NHLBI project office. The Steering Committee is the primary decision making body for the study with overall responsibility for the design and conduct of study protocols. The NHLBI project office participates in all decision-making activities of the LHMP. NHLBI also appoints and oversees the activities of the OSMB.

Lung-HIV Microbiome Project Organizational Structure



3.2. Committees

3.2.1. Steering Committee

The Steering Committee is the overall governing body for LHMP. The committee consists of the Principal Investigators from each clinical center, sequencing center, the coordinating center, and NHLBI. The committee meets monthly via conference call and bi-annually in person. The representatives from the clinical centers and sequencing centers will include the Principal Investigators and Co-Investigators. Steering Committee voting membership shall consist of the principal investigators (i.e., cooperative agreement awardees), the NIH Project Scientist, and the Chairperson. Each full member will have one vote. Members of the Steering Committee will be required to implement policies approved by the Steering Committee.

3.2.2. Observational Safety Monitoring Board (OSMB)

The LHMP OSMB is appointed by the NHLBI to help assure the integrity of the project through monitoring data acquisition for comprehensiveness, accuracy, and timeliness; and monitoring other concerns such as participant confidentiality. An NHLBI staff member, who is not the primary program official, serves as the Board's Executive Secretary. The OSMB makes its recommendations to the Institute's Office of the Director.

The Board members reflect the necessary expertise for the LHMP. The OSMB membership will consist of a minimum of three members who will collectively provide representation in the relevant disciplines, e.g., pulmonary medicine, immunology, microbiology, biostatistics, and ethics.

The OSMB will assure the NHLBI that every effort is made to protect the safety of participants.

NHLBI Guidelines: <http://public.nhlbi.nih.gov/ocr/home/GetPolicy.aspx?id=8>

3.2.3. Publications Committee

The goal of the Publications Committee is to facilitate and coordinate collaborative dissemination of the LHMP results through high-impact publications, without delaying those groups that have already accumulated substantial data.

3.2.3.1. Publications and Presentations Policy

3.2.4. Working Groups

Three working groups focus on the task of developing uniform approaches in their area of expertise. These include the Clinical Working Group, the Sampling Working Group and the Sequencing Working Group. The Clinical Working Group developed the data collection forms and clinical procedures for the LHMP. The Sampling Working Group developed homogenous procedures for specimen collection and the documentation of the specimen's characteristics in accordance with the anticipated requirements of the BioLINCC repository. The Sequencing Working Group developed procedures for processing and analysis of sequencing data.

3.3. Data Analysis Coordination Center (DACC)

As the LHMP DACC, the Biostatistics Center, George Washington University participates in the planning and design of studies in all areas of LHMP collaborative research. This includes coordinating data acquisition and editing; designing and maintaining a central LHMP database; providing leadership in appropriate and efficient collaborative study design and data analysis.

4. APPENDICES

4.1. APPENDIX 1: Center C001 - University of Michigan, Ann Arbor

4.1.1. Study M008 - Understanding the Lung Microbiome in HIV-Infected and HIV-Uninfected Individuals

Study Web Site:

https://www.umms.med.umich.edu/umclinicalstudies/detail_pub_study.do?show=YES&id=5355&TYPE=F



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University of Michigan, Ann Arbor**

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Abbreviations Used

BAL	Bronchoalveolar Lavage
CBC	Complete Blood Count
CBSQ	Chronic Bronchitis Symptom Questionnaire
CD4	Cluster of Differentiation 4
COPD	Chronic Obstructive Pulmonary Disease
CRF	Case Report Form
DOB	Date of Birth
ELISA	Enzyme-Linked Immunoabsorbent Assay
GWU	The George Washington University
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HSP	Heat-Shock Protein
IRB	Institutional Review Board
LHMP	Lung HIV Microbiome Project
MMRC	Modified Medical Research Council Dyspnea Scale
NHLBI	National Heart, Lung, and Blood Institute
NIH	National Institutes of Health
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PFT	Pulmonary Function Testing
PT	Prothrombin Time
PTT	Partial Thromboplastin Time
RFA	Request for Applications
SOBQ	Shortness of Breath Questionnaire
UMHS	University of Michigan Health System
VAAHS	VA Ann Arbor Healthcare System

1 INTRODUCTION

“Understanding the Lung Microbiome in HIV-Infected and HIV-Uninfected Individuals” is a prospective study that will follow participants for two years at the University of Michigan Health System (UMHS) and the VA Ann Arbor Healthcare System (VAAAHS).

1.1 Parent Study Abstract

N/A

1.2 Specific Aims

The study has three specific scientific aims:

- 1.2.1.1 To employ molecular and advanced culture-based techniques for the characterization of the bacterial microbiota of the lower respiratory tract in HIV-infected and HIV-uninfected individuals longitudinally, and to compare the lung with the upper respiratory tract and gastrointestinal tract.
- 1.2.1.2 To compare microbiome data from currently smoking and non-smoking individuals (never- or former- smokers) and to correlate these data with measurements of pulmonary function and respiratory systems longitudinally.
- 1.2.1.3 To compare colonization with *Pneumocystis* in HIV-infected and HIV-uninfected individuals, and to correlate colonization with Microbiome data.

2 OVERVIEW, HYPOTHESIS, METHODS AND STUDY DESIGN

2.1 Overview

Like other organs and organ systems, the respiratory tract is continually exposed to the external environment. Although physical and other immunologic barriers exist to minimize exposure of the lower respiratory tract to microorganisms, it is entirely likely that the lung plays host to an indigenous microbiota.

The lung microbiota would be expected to have both beneficial and deleterious effects for the host. It is likely that the microbiome of the lung facilitates the hosts' welfare, providing antigenic challenges that result in protective immunity against a continual onslaught by potential pathogens. Since the beginning of the HIV epidemic in the 1980's, HIV-infected individuals have been known to be extremely susceptible to pneumonias caused by opportunistic and non-opportunistic organisms. Comparison of the Microbiome of HIV-infected and HIV-uninfected individuals, then, should be a productive way to begin to explore changes in the lung microbiome.

The use of highly active antiretroviral therapy (HAART) and of specific prophylaxis directed against respiratory pathogens has dramatically decreased the incidence of pneumonias in HIV-infected patients. Therefore, the lung microbiome of HIV-infected and HIV-uninfected individuals without active respiratory infections will be studied.

2.2 Primary Hypothesis

We hypothesize that the microbiota will differ significantly in patients with and without HIV infection, that smoking will alter the microbiota, and that the microbiota will be altered by colonization with *Pneumocystis*.

2.3 Methods and Study Design

2.3.1 Design Summary

This is a non-interventional cohort study with single time point cross-sectional comparisons and longitudinal analyses, with the primary study aims of: evaluating how the immune deficiency associated with HIV infection influences the lung microbiome and lung function, and evaluating changes in lung microbiota over time. The study also aims to characterize the microbiome of the lower respiratory tract, determine the relationship between the lower respiratory Microbiome and the upper respiratory and gastrointestinal tract microbiomes, evaluate the influence of smoking on the microbiome compared with the influence of smoking on pulmonary function and respiratory systems, and to evaluate how *Pneumocystis* infection influences HIV-infected and HIV-uninfected individuals.

2.3.1.1 Total Sample Size

The total sample size for the project is 80. The HIV-infected cohort will consist of 40 participants, and the HIV-uninfected cohort will consist of 40 participants. All participants will be enrolled at the University of Michigan Health System or the VA Ann Arbor Healthcare System.

2.3.2 Eligibility Criteria

2.3.2.1 Inclusion Criteria

HIV Infected Group

- Age ≥ 18
- Confirmed diagnosis of HIV infection
- No signs of respiratory infection at enrollment, such as:
 - Fever
 - Recent change in quantity or quality of sputum
 - Chest pain
 - Recent change in dyspnea
- Willing and able to sign the informed consent document

HIV Uninfected Group

- Age ≥ 18
- Negative HIV ELISA
- No signs of respiratory infection at enrollment, such as:
 - Fever
 - Recent change in quantity or quality of sputum
 - Chest pain
 - Recent change in dyspnea
- Willing and able to sign the informed consent document

2.3.2.2 Exclusion Criteria

HIV Infected Group

- Age < 18
- Pregnancy
- Signs or symptoms of respiratory infection at enrollment
- Unwilling or unable to sign the informed consent document
- Unstable heart disease
- Other systemic disease and unlikely to survive at least 2 years
- Mental incompetence
- Participation in another interventional protocol within the last 6 weeks
- Use of antibiotics for a lung infection within the last 4 weeks
- Renal Failure (creatinine >3)
- Child's Class C Cirrhosis

HIV Uninfected Group

- Age < 18
- Pregnancy
- Unwilling to have the HIV ELISA performed
- Signs or symptoms of respiratory infection at enrollment
- Unwilling or unable to sign the informed consent document
- Unstable heart disease
- Other systemic disease and unlikely to survive at least 2 years
- Mental incompetence
- Prednisone >20mg per day
- Participation in another interventional protocol within the last 6 weeks
- Asthma
- Cystic fibrosis
- Clinically significant bronchiectasis
- Lung cancer or any cancer not in remission for at least 5 years
- Other inflammatory or fibrotic lung disease
- Use of antibiotics for a lung infection within the last 4 weeks
- Autoimmune disease, i.e. RA, SLE, Autoimmune hepatitis, Crohn's disease or other
- HIV positive

2.3.3 Informed Consent Criteria

Informed consents for an NHLBI-funded study must include the following required elements. Sample language has been developed but is not required.

- A statement that allows for broad research use of specimens including research on lung disease, HIV, and other related illnesses. The statement must also specify that the participant will not receive results of any future research.
- A description of the NHLBI repository and who will have access to the specimens there.
- A list of who will have access to specimens, medical, and research information that includes the site staff, NHLBI/NIH, and the data analysis and coordinating center (GWU).
- A description of how confidentiality is protected. This description includes that data will be stored only in locked cabinets or on secured computers.
- A statement that specimens will be stored indefinitely.
- A statement that the participant has a right to change their mind at any time and that every effort will be made to destroy their samples but it may not be possible once samples are de-identified.
- A description of the risk of the bronchoscopy including death.

2.3.3.1 Stored Sample(s)

The informed consent must include a description of the risk of stored specimens and future testing such as paternity and other genetic information.

2.3.3 Secondary/Ancillary Research Questions

This project will also examine *Pneumocystis* colonization and its influence upon the bacterial microbiome. In collaboration with University College in London, real time PCR will be performed on respiratory secretions using amplification of HSP70. Colonization rates will be characterized over time among HIV-infected and HIV-uninfected individuals, and compared with dyspnea symptoms and sputum production. The data will be correlated with microbiome results, including the influence of *Pneumocystis* genotypes on microbial flora.

3 CORE PROCEDURES

3.1 Introduction

All participants will receive full and open explanation of the study purpose, design, and potential risks and benefits from a study investigator or coordinator. All participants will be asked to provide written informed consent and receive a copy of the signed consent form. Participants will be free to withdraw from the study at any time without fear of negative repercussions. Those participants experiencing significant clinical deterioration, as deemed by the attending physician, will be withdrawn and treated as deemed most appropriate.

3.2 Recruitment Strategies

Eligible participants with HIV Infection will be identified in the HIV clinics at the University of Michigan Health System and at the VA Ann Arbor Healthcare System. HIV providers at the two care facilities will participate in identification of participants and will work with the study coordinators to enroll these individuals. HIV-uninfected individuals will be identified from the general medicine clinics at the two care facilities and by advertising, with enrollment facilitated by the study coordinators.

3.3 Screening for Eligibility and Chart Abstraction

The study coordinators will screen for eligibility using the following personal information: Age, any recent history of respiratory infection, screen subject's CPRS cover sheet to look for allergies, active problems, and medications; Convenient location of residence. In order to complete enrollment, the study coordinators must verify that prospective subjects for the HIV-positive cohort have confirmed positive HIV tests. The study coordinators must also verify that prospective subjects for the HIV-negative cohort do not have positive HIV tests. If a subject lives too far from AAVAMC they would most likely not want to come back for the follow up visits. We would need to know this information before approaching a potential subject about the study.

3.4 Quality Assurance

Quality assurance for bronchoscopy will be conducted as part of routine procedures for the endoscopy units. Quality assurance for pulmonary function testing is routinely conducted per American Thoracic Society protocols and criteria.

3.5 Human Participants

3.5.1 Data/Observational Safety Monitoring Board

The LHMP OSMB is appointed by the NHLBI to help assure the integrity of the project through monitoring data acquisition for comprehensiveness, accuracy, and timeliness; and monitoring other concerns such as participant confidentiality. An NHLBI staff member, who is not the primary program official, serves as the Board's Executive Secretary. The OSMB makes its recommendations to the Institute's Office of the Director.

The Board members reflect the necessary expertise for the LHMP. The OSMB membership will consist of a minimum of three members who will collectively provide representation in the relevant disciplines, e.g., pulmonary medicine, immunology, microbiology, biostatistics, and ethics.

The OSMB will assure the NHLBI that every effort is made to protect the safety of participants.

NHLBI Guidelines: <http://public.nhlbi.nih.gov/ocr/home/GetPolicy.aspx?id=8>

3.6 Monitoring for Adverse Events

Subjects will be monitored by the PI and the study coordinators throughout enrollment. The investigators will meet on a monthly basis to review safety issues. Drs. Curtis and Bauman are pulmonologists who oversee invasive procedures and their monitoring. Drs. Cinti, and Riddell are infectious disease specialists who conduct HIV clinics. All adverse events will be

reported immediately to the IRB at the University of Michigan Health System or the VA Ann Arbor Healthcare System, as appropriate based on the site of recruitment.

4 STANDARD CLINICAL CENTER PROCEDURES

4.1 Overview

Each participant will be studied over a 24 month period, with rolling enrollment into the study. The clinical data collection period is 24 months, with visits occurring every six months. At the time of each evaluation, an interval medical history will be obtained. The physical exam will consist of vital signs (including oxygen saturation), and a brief cardiopulmonary exam. Participants will be queried about episodes of infection (including viral syndromes) and use of anti-infectives. Changes in antiretroviral regimens for the HIV-infected participants will be recorded. All participants will also undergo evaluation of dyspnea and of sputum production.

4.2 Forms

Consent forms will be completed by the study coordinators or investigators. Enrollment and screening forms will be completed by the study coordinators. Physical examination forms will be completed by the physician investigators. Procedure forms will be completed by the physician investigators.

4.3 Laboratory Specimen Collection

The clinical data collection period is 24 months, with visits occurring every six months. Participants will undergo evaluation as specified in the table below. Initially, Microbiome analysis will be performed on biologic samples at two time points, enrollment and month 18.

At enrollment and month 18, data will be compared from nasal swabs, oropharyngeal rinses, bronchoalveolar lavages, and gastric aspirates. At these time points, all specimens will be collected on the same day the participants undergo bronchoscopy, assuring that sampling from the nose, oropharynx, lung, and stomach occur concurrently. Participants will be brought to the endoscopy suite, where identity and consent will be confirmed. Clinical staff will verify that the patient has fasted since midnight, and will confirm the presence of a driver to transport the patient after the procedure. Participants will be monitored non-invasively with measurement of oxygen saturation, pulse, and blood pressure.

At months 6, 12, and 24, nasal swabs and oropharyngeal rinses will be collected in the examining rooms and stored for possible later analysis. If data from nasal swabs and oropharyngeal rinses track with the more invasive specimens, then the stored specimens can be analyzed at a later time.

Month	Visit	Test	Procedure
0	Intake Health Status Medications Tobacco MMRC/SOBQ/CBSQ/ Breathlessness, Cough and Sputum Scale	PFT CBC Basic Metabolic Panel Pregnancy Test Platelets PT/PTT HIV ELISA PA & lateral Chest X-ray EKG	Nasal swab Oral rinse BAL Gastric aspirate Blood Draw
6	Health Status Medications Tobacco MMRC/SOBQ/CBSQ/ Breathlessness, Cough and Sputum Scale	PFT	Nasal swab Oral rinse
12	Health Status Medications Tobacco MMRC/SOBQ/CBSQ/ Breathlessness, Cough and Sputum Scale	PFT	Nasal swab Oral rinse
18	Health Status Medications	PFT	Nasal swab

	Tobacco MMRC/SOBQ/CBSQ/ Breathlessness, Cough and Sputum Scale	CBC Basic Metabolic Panel Pregnancy Test Platelets PT/PTT HIV ELISA PA & lateral Chest X-ray EKG	Oral rinse BAL Gastric aspirate Blood Draw
24	Health Status Medications Tobacco MMRC/SOBQ/CBSQ/Breathlessness, Cough and Sputum Scale	PFT	Nasal swab Oral rinse

4.3.1 Pulmonary Function Testing (PFT)

Full pulmonary function testing will be performed according to American Thoracic Society criteria. Participants will undergo spirometry before and after administration of bronchodilators, measurement of lung volumes by plethysmography, and measurement of diffusing capacity for carbon monoxide.

4.3.2 Safety Laboratory Specimens for Bronchoscopy Visit

In preparation for each bronchoscopy, each participant will have a complete blood count (CBC) and measurement of coagulation parameters. No procedures will be performed on participants with a hemoglobin less than 10g/dL, a white blood cell count less than 5,000/mm³, or a platelet count less than 100,000/ mm³. No invasive procedures will be performed for participants with INR values greater than 1.4, a creatinine greater than 2.0, or with electrolyte abnormalities judged by the study investigators to increase the risk to the subject. In addition, each participant will have a chest X-ray and EKG performed prior to each bronchoscopy. No procedures will be performed on participants with an abnormal chest X-ray or EKG if the abnormality is judged by study investigators to be clinically significant and to increase the risk of the procedure. The HIV-infected participants will have current measurements of CD4 count and HIV load available, as this laboratory evaluation is part of their regularly scheduled care. Additionally, the HIV-uninfected participants will undergo HIV testing prior to each bronchoscopy, to confirm that these individuals are indeed HIV negative at the time of sampling. Pregnancy test performed on women of childbearing age.

4.3.3 Bronchoscopy Study Visit

4.3.3.1 Oropharyngeal Rinse

At the start of the bronchoscopy study visit, before any topical anesthesia has been applied, participants will gargle with 20 mL sterile, non-bacteriostatic saline. After gargling for 30 seconds, participants will expectorate the saline into a sterile container. The samples will be placed on ice and then transferred to the lab on ice for further processing. Triplicate 5 mL samples will be centrifuged at >15,000 x g in conical centrifuge tubes. The supernatant will be decanted and the pellet resuspended in 200 µL of sterile PBS. This will be transferred to a 2 mL bead-beating tube (MolBio) and then stored at -80° C.

4.3.3.2 Moderate Conscious Sedation and Topical Anesthesia

Participants will be placed on the bronchoscopy stretcher and baseline vital signs will be measured. Participants will be pre-medicated with diphenhydramine 25-50 mg IV. They will then inhale 2.5 mL of aerosolized 4% lidocaine to begin anesthesia of the upper airway. At the conclusion of the aerosolization, participants will be placed supine. Moderate conscious sedation will then be administered with midazolam and an opiate such as fentanyl or meperidine, titrated to patient comfort. Because of the brief nature of the procedure, deeper sedation will not be required. After insertion of the bronchoscope via the mouth, the vocal cords

will be anesthetized with four aliquots of 1 mL each of 4% lidocaine through the bronchoscope, followed by 1 mL aliquots of 2 % lidocaine after the bronchoscope passes the cords. Aliquots of 2% lidocaine will be used as needed in the tracheobronchial tree to alleviate cough.

4.3.3.3 Bronchoscopy with Bronchoalveolar Lavage

After airway inspection, bronchoalveolar lavage will be performed in the right middle lobe and lingual with the bronchoscope wedged in a segmental bronchus. Aliquots of sterile, non-bacteriostatic saline (30 mL each) will be instilled through the bronchoscope and manually suctioned back into the syringe, with the amount instilled and returned recorded. The target return will be 60 mL, with no more than 300 mL of saline (150 mL on each side of the lungs) used for any lavage. BAL samples from the two locations will be processed separately.

The samples will be placed on ice and then transferred to the lab on ice for further processing. Triplicate 15 mL samples will be centrifuged at $>15,000 \times g$ in conical centrifuge tubes. The supernatant will be decanted and the pellet resuspended in 200 μ L of sterile PBS, transferred to a 2 mL bead-beating tube, and stored at -80° C.

4.3.3.4 Gastric Aspirate

After both bronchoalveolar lavages are completed and while the subject is still sedated, the bronchoscope will be withdrawn to a position immediately superior to the glottis, where it will be stabilized at the mouth by the nurse with the vocal cords in constant view. The subject's head will be flexed gently, and an 18 gauge gastric tube will be introduced via the mouth. Passage of the tube posterior to the larynx into the esophagus will be observed directly to assure that the tube does not enter the glottis and lungs; the tube will be advanced to 40-45 cm based on the markings on the tube. Correct placement of the gastric tube in the stomach will be confirmed by auscultation of air introduced via a Toomey syringe. The bronchoscope will then be withdrawn from the patient.

Next, 50 mL normal saline will be infused and immediately withdrawn; the return will be collected and its volume recorded and the gastric tube will be removed immediately.

The samples will be placed on ice and then transferred to the lab on ice for further processing. Triplicate 10 mL samples will be centrifuged at $>15,000 \times g$ in conical centrifuge tubes. The supernatant will be decanted and the pellet resuspended in 200 μ L of sterile PBS. This will be transferred to a 2 mL bead-beating tube and then stored at -80° C.

4.3.3.5 Nasal Swab

Finally, while the subject is still sedated, the posterior nasopharynx will be swabbed gently with a single sterile swab introduced via either nares, which will be removed immediately.

The tip of the swab will be cut off and placed into a 2 mL sterile screw cap tube. The samples will be placed on ice and then transferred to the lab on ice for further processing. The swab will be placed in 200 μ L of sterile PBS and vortexed. The swab will be removed, taking care to squeeze out excess fluid. The fluid will be centrifuged at $15,000 \times g$ and then the pellet resuspended in 600 μ L of PBS, divided into three 2 mL bead-beating tubes and stored at -80° C.

After the nasal swab is collected, the participant's head will be elevated and a final set of vital signs will be recorded by the Endoscopy personnel. The participant will be transported to the recovery area on the stretcher, where vital signs will be monitored per Endoscopy Recovery Room protocol. Participants will be discharged from the recovery area when meeting the unit's criteria for discharge, and will be transported home by a driver.

5 OUTCOMES EVALUATION

5.1 Primary Outcome

The primary outcome measure for study will be measurement of the constituents and relative composition of microbiota data. Comparisons will be made within subjects (repeated measures over time) and between subject groups (HIV-infected versus non-HIV-infected).

5.2 Other Outcomes

Secondary outcome measures will correlate them microbiota data by collection site, smoking status of subject, and collected health information.

6 DATA COLLECTION AND MANAGEMENT

Data will be collected on a paper case report form (CRF), and then entered into a clinical database. The CRF contains the following information:

- Patient Registration Information (DOB, gender, year of HIV diagnosis)
- Demographic and family history
- Smoking history and symptom questionnaires
- History of respiratory infections and/or diseases (pneumonia, bronchitis, asthma, COPD)
- Medications (including anti-retrovirals, medications for prophylaxis, and interval use of anti-infective medications)
- Laboratory Data (including CD4 counts and HIV loads)

All medical and demographic information will be considered strictly confidential. At the time of entry into the database, all links between subject identity (as reflected in the LHMP Study ID Number) and clinical data will be maintained exclusively in a password-protected database separate from the participant's clinical record (in the case of VA subjects, on a computer drive within the VA firewall) and all paper copies will be stored in locked cabinets within locked rooms. Upon entry into the database, all missing or questionable data items will be queried on a data clarification form. The investigator will respond to each query and the completed data will be updated in the database. Only authorized study personnel will have access to the locked cabinets. The database that contains the link between subject ID and other Protected Health Information will be password-protected and accessible by authorized study personnel only.

6.1 Site Specific Data Collection Forms

This site will use the data collection forms developed by the consortium of grant awardees.

6.1.1. Breathlessness Cough And Sputum Scale

This is a standard questionnaire that will be administered using a paper form by the study coordinators. Like other data, these forms will be stored in locked cabinets accessible only to the study team.

6.1.2. Dyspnea Questionnaire

This is a standard questionnaire that will be administered using a paper form by the study coordinators. Like other data, these forms will be stored in locked cabinets accessible only to the study team.

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Note: April 4, 2012 Version updated on December 4, 2012 to address name changes and correct section 3.5.1.

4.2. APPENDIX 2: Center C002 - University of Pennsylvania

4.2.1. Study M0012 – Pennsylvania Lung Microbiome Project



**Human Respiratory Tract Microbiome in Health, HIV Infection, and HIV Lung Disease
Lung HIV Microbiome Project (LHMP)
University of Pennsylvania**

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Version 10: May 1, 2013

Sponsored by the National Heart, Lung and Blood Institute (NHLBI)
of the National Institutes of Health (NIH).



***HUMAN RESPIRATORY TRACT MICROBIOME IN HEALTH,
HIV INFECTION AND HIV LUNG DISEASE***

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List of Abbreviations

ARV	antiretroviral
ART	antiretroviral therapy
BAL	bronchoalveolar lavage
CF	cystic fibrosis
CFAR	Center for AIDS Research
COPD	chronic obstructive pulmonary disease
FEV-1	forced expiratory volume in one second
FVC	forced expiratory vital capacity
HIV	human immunodeficiency virus
IPF	interstitial pulmonary fibrosis
NP	nasopharynx
OP	oropharynx
OTU	operational taxonomic unit
Pc	<i>Pneumocystis jiroveci</i>
PCoA	Principal coordinates analysis
PFT	pulmonary function tests
PSB	protected specimen brush
RFLP	restriction fragment length polymorphism

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Study Summary

Title	Human Respiratory Tract Microbiome in Health, HIV infection and HIV Lung Disease
Short Title	Lung Microbiome, HIV Infection, and COPD
Protocol Number	810851
Phase	Not applicable
Methodology	Cross sectional, and prospective observational study of the effect of HIV infection and/or COPD on the microbiome of the lung
Study Duration	5 years
Study Center(s)	Single-center
Objectives	<ol style="list-style-type: none"> 1. Define the lower and upper respiratory tract microbiome in HIV-infected and healthy HIV-negative individuals 2. Determine the effect on the respiratory system microbiome of progressive immune deficiency and ART-associated immune reconstitution 3. Define the respiratory system microbiome patterns in HIV-infected and uninfected individuals with COPD, compared with those without COPD 4. Determine the effects of smoking and diet on respiratory system microbiome populations, and the relationship between gut and upper respiratory microbiomes
Number of Subjects	110
Diagnosis and Main Inclusion Criteria	<p><u>HIV study group</u></p> <ul style="list-style-type: none"> • Chronic HIV-1 infection. • If on antiretroviral therapy, have a HIV viral load ≤ 200 copies/mL for ≥ 6 months at the time of screening on a stable antiretroviral medication regimen <p><u>COPD study groups (both HIV+ and HIV-)</u></p> <ul style="list-style-type: none"> • Moderate / stage 2 COPD: FEV1/FVC < 70% FEV1 < 80% and $\geq 50\%$ predicted Incomplete reversibility with bronchodilators
Statistical Methodology	Microbiome populations will be analyzed using UniFrac principal coordinates analysis. They will be compared in terms of principal coordinates for microbiome compositions and also the proportions of particular lineages using two sample Wilcoxon rank-sum tests between two groups and nonparametric ANOVA among multiple groups. For longitudinal measurements, we will perform paired t-test and repeated measurement ANOVA to test changes in microbiomes summarized as the first several principal coordinates

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1 Introduction

This document is a protocol for a human research study. This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

1.1 Background

HIV-associated lung disease in the era of effective antiretroviral therapy (ART). There are now estimated to be 33 million people infected with HIV worldwide, including ~1 million in the US with another 40,000 new infections each year. Prior to effective ART, pulmonary opportunistic infections were a principal cause of mortality (especially acute pneumonia caused by *P. jiroveci*), and in the developing world where access to ART is still limited, tuberculosis is the most important cause of death. Widespread use of ART in the developed world has led to dramatic changes in patterns of lung disease in HIV infected individuals (reviewed in ^{2, 3}). However, widespread use of ART has markedly altered the course of disease in the developed world, where HIV/AIDS is for the most part managed as a chronic illness. Even in people who achieve effective and long-term viral suppression, however, bacterial pneumonia remains common and a frequent cause of hospitalization and mortality ⁴⁻⁶. Virulent pathogens such as *S. pneumoniae*, other encapsulated organisms and *S. aureus* are common but a broad range of other agents are seen ^{2 7-9}. In addition, upper and lower airway inflammatory and infectious processes such bronchitis and sinusitis are extremely common and remain a persistent problem in infected individuals ¹⁰⁻¹³. Thus, even in the context of effective therapy, pulmonary consequences of immune dysfunction persist ¹⁴. In addition to infectious complications, however, a number of other pulmonary processes are emerging as increasingly important causes of morbidity and mortality in treated individuals in the US. Included among these are an accelerated pattern of emphysema and COPD among HIV-infected smokers (discussed further, below) ^{15, 16}, lung cancer ^{17, 18} and pulmonary hypertension ¹⁹⁻²¹.

The respiratory tract microbiome in health and HIV infection. Microbial colonization of the upper airway (above the glottis including oropharynx and nasopharynx) has long been recognized as an important factor in lower airway colonization and infection by virulent pathogens in both normal and immunodeficient hosts, as well as by opportunists that lead to immunocompromised or nosocomial pneumonia ^{22, 23}. Many host factors may affect upper airway microbial patterns, including age, debility, immune status, antibiotics and other medications, oropharyngeal disease and salivary or other oral abnormalities ^{24, 25}. Upper airway colonization with gram negative bacilli is frequent in elderly, debilitated, intubated or hospitalized subjects, and represents an important predisposition to gram negative pneumonia ²⁶. In HIV infection, overgrowth *Candida* is a prominent manifestation of the altered upper respiratory tract microbiota, but HIV infected people also have higher rates of colonization by lower respiratory pathogens such as *S. pneumoniae* & *S. aureus* ²⁷⁻²⁹. Thus, the upper respiratory tract microbiome is altered in HIV infection, although systematic analysis has not been carried out. Importantly, while both host and microbial factors are critical, interactions among organisms within a community is a determinant that influences growth of pathogens ²², and thus studies that evaluate complex communities present are likely to reveal broader and more relevant insights than testing for individual specific organisms. Understanding the resident microbiome in health & HIV infection would therefore provide important information into the basis for respiratory infections in this population.

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The lower airway microbiome. In contrast to the upper respiratory tract, the normal lower respiratory tract (below the glottis) is generally thought to be sterile (based largely on conventional microbial techniques). However, the establishment of microbial populations in the lower respiratory tract is a characteristic of a broad range of pathological conditions that alter local or systemic host factors. Examples include defective mechanical defense such as intubation or tracheostomy; defective anatomical, mucociliary and innate defense such as bronchiectasis and cystic fibrosis; inflammatory states such as asthma; and, importantly, both COPD and cigarette smoking (discussed below). Surprisingly, given the importance of recurrent pulmonary infections and prevalence of chronic respiratory symptoms, *lower respiratory tract colonization in HIV infection has received scant attention*, with just one study using standard culture reporting low levels of colonization that were not different from uninfected controls³⁰. An important limitation of studies to date, however, is reliance on traditional microbiological techniques that are restricted to organisms that can be cultured with conventional methods and limits the complexity of populations that can be analyzed.

Cigarette smoke and the respiratory tract microbiome. One environmental factor with a well-established role in modifying airway colonization is cigarette smoke. Multiple studies show that, in contrast to non-smokers, smokers who are otherwise healthy have markedly increased rates of lower airway bacterial colonization^{31, 32}. Thus, in addition to causing direct lung injury and inflammation, smoking leads to airway colonization, which likely has a second deleterious role in perpetuating inflammation and lung damage in COPD (discussed further, below). Importantly, smoking has a markedly higher prevalence in HIV-infected people compared with the general US population, with 50%-60% reporting moderate to heavy cigarette use³³⁻³⁶, so the consequences of smoking on the respiratory tract microbiome are particularly relevant to HIV-infected individuals and synergistic with immunodeficiency in effects on the respiratory system.

COPD and the respiratory tract microbiome. COPD is characterized by expiratory airflow limitation and pathologically by alveolar destruction and enlargement (emphysema) along with chronic airway inflammation and remodeling. The pathogenesis of COPD and emphysema is multifactorial, involving genetic and acquired host susceptibility and environmental factors. Cigarette smoke is the principal inciting factor, with multiple mechanisms of pathogenesis involved. These mechanisms include cellular apoptosis, vascular injury, protease/antiprotease imbalance and mechanical injury due to stretch, but chronic inflammation appears to be a key component (reviewed in³⁷⁻⁴⁰). Inflammation is associated with recruitment of macrophages and neutrophils, elevated cytokines, proteases and other mediators, and persists even following smoking cessation. Understanding the factors that perpetuate inflammation has been an important goal in the field, and chronic airway microbial colonization is being increasingly appreciated as factor that perpetuates the vicious cycle of inflammation and injury³⁷. While it has been long recognized that acute exacerbations of COPD are often microbial in origin, it is also clear that the lower airway of patients with COPD are often chronically colonized by bacteria with recognized pathogenic potential as well as organisms of uncertain significance^{32, 41-43}. Furthermore, in many studies the presence of organisms correlated with markers of inflammation and lung injury^{41, 44-46}. Thus, in contrast to earlier notions that this chronic colonization was innocuous, accumulating evidence suggests that the lower respiratory tract microbiome in stable COPD patients may have a pathogenic role, perpetuating a cycle of airway inflammation and decline in lung function³⁷.

HIV-associated COPD. Early reports suggested an increased prevalence of COPD and emphysema in HIV-infected individuals^(15, 47, 48). While some changes were likely the result of previous infection (particularly with *P. jiroveci*) or IV drug use that can cause emphysema-like changes, it is reported that up to 40% of HIV-positive smokers without these factors may have COPD or emphysema by CT scan or pulmonary function studies¹⁵. Compared with HIV-

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uninfected smokers, COPD presents at an earlier age and with a shorter smoking history, suggesting an accelerated natural history. The reason for accelerated disease in HIV-infected smokers is not clear. One mechanism may relate to a state of chronic inflammation from HIV infection itself (both systemically and in the pulmonary compartment)⁴⁹⁻⁵². In addition, a novel hypothesis based on PCR detection suggests that chronic colonization by *P. jiroveci*, even in individuals without a history of prior acute Pc pneumonia, may be responsible for chronic inflammation and lead to progressive lung injury and emphysema in both HIV-associated and uninfected individuals⁵³⁻⁵⁶, discussed further below.

Unculturable organisms in COPD/emphysema: P. jiroveci. In addition to the “vicious cycle” hypothesis of chronic bacterial airway colonization in the pathogenesis and perpetuation of COPD/emphysema³⁷, emerging evidence raises the possibility that chronic colonization with unculturable organisms may play a role. In fact, these observations may provide a particularly important link between COPD and HIV infection. *P. jiroveci* (Pc) is a ubiquitous organism that causes acute infection only in settings of immunosuppression. Pc cannot be efficiently cultured from respiratory specimens in vitro, so identification traditionally relies on microscopy, which is relatively insensitive unless large numbers of organisms are present (as in acute Pc pneumonia). Application of high sensitivity PCR techniques, however, show that low level respiratory colonization with Pc may be common in both in HIV-infected individuals without a prior history of acute Pc pneumonia, and HIV-uninfected people with COPD (reviewed in⁵⁶). While Pc detection by PCR is rare in immunocompetent people without lung disease, it is seen in a small proportion of otherwise healthy smokers^{57, 58}. In otherwise healthy HIV-infected individuals it can be detected in a varying but significant proportion (up to 50-70% depending on the particular study, level of immunosuppression and type of specimen tested^{57, 59, 60}), and cigarette smoking was associated with a higher risk of colonization⁶¹. Among individuals without HIV infection, Pc colonization was detected in up to 40% of individuals with COPD^{53, 62}. Importantly, Pc colonization appeared to correlate with the degree of airway inflammation and severity of disease⁵³. For unclear reasons, antimicrobial prophylaxis used to prevent Pc pneumonia may not be effective at preventing or eradicating colonization⁶⁰. Complementing these correlative data are recent animal studies, showing in a murine model that cigarette smoke enhances Pc colonization and the two act synergistically to induce airway inflammation and emphysema⁶³, and in a SIV-infected rhesus macaque model that induction of Pc colonization leads to airway inflammation and parenchymal changes characteristic of COPD and emphysema⁶⁴.

Thus, these data offer provocative evidence that colonization with Pc may be a mechanism contributing to inflammation and lung injury in chronic HIV infection and COPD. While this mechanism does not account for all such cases, it provides strong evidence that the respiratory tract microbiome is altered in HIV infection, even if not evident through traditional culture methods, and that effects on the microbiome may be an important mechanism of injury. *It is unknown whether chronic colonization by other unculturable organisms (previously described or novel) occurs in HIV-infected individuals or in people with COPD, and whether they may be linked to lung disease.* A goal of this project is to use powerful new unbiased molecular techniques to describe the respiratory tract microbiome of HIV-infected individuals, and identify additional agents that may be associated with and possibly mechanistically linked to lung injury and COPD.

Unbiased molecular approaches to defining the human microbiome. Until recently, a critical limitation of studies to define the microbiology of the respiratory tract (and the entire microbiome) has been reliance on traditional methods that are restricted to culturable organisms and limits the complexity of populations that can be analyzed, or molecular approaches such as PCR directed against specific organisms of *a priori* interest. The new deep sequencing

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methods, however, enable an unbiased approach that can detect most or all organisms.

The pyrosequencing approach we employ utilizes the 454 Life Sciences/Roche method, which works as follows. For sequencing of a complex bacterial population, DNA samples from communities of interest are fragmented, ligated to DNA linkers, then mixed with beads that have bound on their surfaces oligonucleotides complementary to the linkers. This step is done in dilute solution so that on average a single DNA strand binds per bead. A dilute mixture of beads is then added to an oil-water emulsion, arranged so on average each aqueous droplet contains a single bead with a single bound strand. PCR amplification is then carried out in the emulsion. Each DNA strand is amplified and then binds by sequence complementarity to the bead, thereby creating beads that are each conjugated to DNA strands of a single homogenous sequence. Beads with bound DNA are then distributed on a picotiter plate at a density of ~400,000 beads per plate. A primer is then bound and polymerase used to extend a DNA chain. The 4 nucleotide triphosphates are sequentially flowed over the plate, and an enzyme system in the buffer directs incorporation of pyrophosphate (liberated by nucleotide addition) into ATP, which then activates luciferase enzyme in the buffer to produce light. A CCD camera records each flash from each well. Sequential application of the four nucleotides allows DNA sequences of 200-400 bp to be built up ~400,000 at a time, yielding 10^8 bp of sequence in a 1-day run. At this writing, new "Titanium" technology is just being implemented in the University of Pennsylvania sequencing center, which allows reads of 400 bp and increased numbers of reads per plate. We have extensive experience generating and analyzing 454 pyrosequencing data and have applied it to define low frequency HIV-1 resistance mutations, lentivirus integration sites in the human genome and, as discussed below, the composition of uncultured microbial communities^{1, 65-72}. Of note, Dr. Bushman is also the PI of a recent NIH Large Instrument grant that enabled purchase a 454 Life Sciences Sequencer, and directs the Deep Sequencing program of the Viral/Molecular Core of the Penn CFAR that supports use of this instrument and data analysis infrastructure for HIV/AIDS research. Our laboratory was one of the first to apply deep sequencing to microbiome analysis, and still one of the only ones to use the method to characterize microbiome changes accompanying lentivirus infection¹ so we describe these methods further under Preliminary Data.

Molecular techniques and the respiratory tract microbiome. Few studies so far have applied these powerful molecular methods to the respiratory microbiome. One study of bronchoalveolar lavage samples from patients with cystic fibrosis (CF, and control subjects with a mixed group of other diseases) carried out PCR amplification of bacterial 16S rRNA genes followed by cloning, RFLP analysis to identify unique clones and sequence determination⁷³. While many established CF pathogens were identified, dominant organisms were unexpectedly found in about half of CF patients that are not routinely identified by culture, including previously unsuspected candidate pathogens such as gram negative bacilli, anaerobes and a novel *Rickettsia* species. Control subjects revealed some known pathogens, but several harbored previously unsuspected organisms as the dominant species, such as *T. whipplei* (the agent of Whipple's disease) in a subject with pulmonary fibrosis (IPF). Although the pathogenic significance of agents identified in that study remain to be clarified, it shows that novel organisms and associations remain to be discovered within the respiratory tract. That study utilized individual clones of PCR products, so was limited to ~6,600 clones containing ~1,600 unique sequences, much smaller numbers than are accessible with 454 pyrosequencing. Nevertheless, it confirms that unbiased molecular techniques have the potential to uncover previously unsuspected microbiomal patterns in individuals with lung disease.

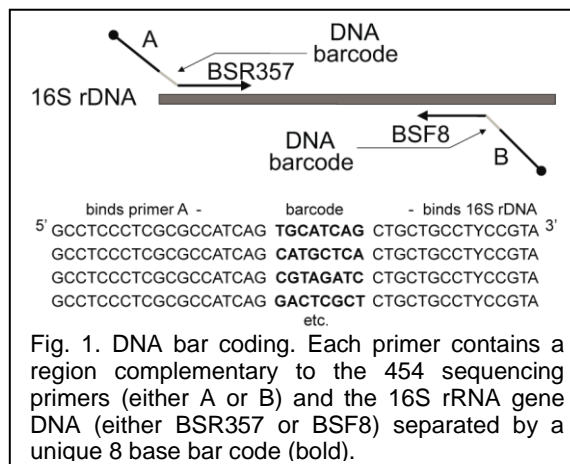
Preliminary Data

Introduction. This project is a collaboration among: Dr. Frederic Bushman, whose group has

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pioneered the use of deep sequencing techniques to define complex genetic populations (including microbiomal analysis) and is PI of a project in the Human Microbiome Roadmap program, and of director of the Deep Sequencing program in the Viral/Molecular Core of the Penn Center for AIDS Research (CFAR); Ron Collman, a pulmonologist and HIV viral pathogenesis researcher who is director of the CFAR Viral/Molecular Core and co-director of the Penn CFAR; Ian Frank, an HIV clinical investigator and clinician who directs the Clinical Core of the CFAR; and Hongzhe Li, a biostatistician with extensive expertise in statistical methodology. Here we first present an overview of deep sequencing methods that the Bushman group is using to profile uncultured microbial communities. We then describe studies in which these methods were used to analyze the vertebrate microbiome, including defining effects of disease (including lentiviral infection), diet and other factors. Later we describe the CFAR Clinical Core cohort led by Dr. Frank and studies it has supported, which will be utilized to carry out the research proposed, and lung-related studies carried out by Dr. Collman.



Development of a novel DNA bar coding strategy for multiplexing samples during 454 pyrosequencing. One of the challenges to exploiting deep sequencing technology is the cost of each run, which for the 454 method is in the range of \$10,000 for a single plate. To make analysis of large numbers of samples affordable, we and others developed a DNA bar coding strategy (Fig. 1), in which we tag each template with a unique eight-base bar code positioned at the start of the sequence read^{74 72}. Once the sequence data are generated, reads are sorted by bar code. Thus we are able to analyzed >100 different samples in a single 454 pyrosequencing plate, thereby speeding throughput while controlling costs. Published examples from our group include^{1, 67, 69, 70, 72, 75, 76}. This validated approach will greatly enhance our ability to carry out the amount of sequencing involved in these proposed studies in an efficient and affordable manner.

Characterizing bacterial populations using 16S sequencing. We will use pyrosequencing of 16S rRNA gene segments as one of our main methods for characterizing bacteria in microbiome samples. Near-universal PCR primers can be used that bind to highly conserved regions of 16S rRNA genes⁷⁷. The regions between primer binding sites are heterogeneous and contain phylogenetic information. Thus PCR amplification and sequencing allows an uncultured community to be profiled both for the types of sequences present and their relative abundances. However, several factors can introduce recovery biases⁷⁸⁻⁸¹, including PCR selection, in which some templates are amplified more efficiently than others, and PCR drift, which includes biases originating during the PCR reaction itself. Reconstruction experiments suggest that the influence of these factors can be diminished experimentally by 1) using high template concentrations, 2) performing fewer PCR cycles, and 3) mixing replicate PCR reactions. All of these steps are incorporated into our protocols, but biases likely remain, though these will generally only distort the composition of the amplicon population by a factor of a few fold. Often the question involves comparing two or more communities, and if biases are the same in all samples, they become irrelevant to detecting differences.

To validate application of 454 pyrosequencing to 16S analysis, we carried out several types of bioinformatics simulations^{1, 71}. First, we asked if different simulated amplicons generated the same clustering results as near-full-length sequences for three large-scale studies of the

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microbial communities, and showed that they did. Second, we repeated this analysis for short sequences (100-, 150-, 200- and 250-base reads) resembling those produced by pyrosequencing. Third, we used sequences from over 200 globally dispersed environments to test whether studies that used similar sequencing primers clustered together mistakenly, without regard to environment. The results showed that reads lengths accessible with 454/Roche pyrosequencing GS FLX did an excellent job of recapturing clustering known to exist in the 16S sequence samples studied, provided optimal primer pairs were chosen.

Analyzing pyrosequencing data for 16S rDNA gene segments. To work up 16S rDNA pyrosequence data, the sequence reads are trimmed to remove the primer and bar codes, then aligned on curated phylogenetic trees made from full length 16S sequences. This allows each sequence read to be associated with a bacterial taxa. To validate our methods, in a reconstruction study we took 16S sequences from a well established database of 16S sequences, extracted the short regions expected from the 454 reads, then inserted them into the Hugenholtz tree⁸² using Greengenes/NASt⁸³ and ARB⁸⁴. We found that placements were 93% correct at the phylum level, 91% at the class and order level, 84% at the family level, and 72% at the genus level. Two groups consistently behaved poorly, accounting for about 99% of the misplacements above the family level. From this we conclude that the 454/Roche sequence reads would indeed be useable for phylogenetic fingerprinting and comparing microbial communities to each other (the two poorly behaved groups would be the same in all samples and so have minimal effects on comparisons between samples). Several other groups have presented 454-based analysis of microbial communities and reached generally similar conclusions⁸⁵⁻⁸⁸.

We have made extensive use of Rob Knight's UniFrac program to compare microbial populations. UniFrac measures the distance between two populations in terms of the fraction of shared evolutionary history among organisms in those populations⁸⁹⁻⁹¹. Communities to be compared are aligned on a common phylogenetic tree. Our presently favored method relies on NASt⁸³ and ARB⁸⁴, and further optimization of this step is ongoing. UniFrac scores the fraction of the branch length that is shared between communities and the fraction that is unique. Thus a single number between 0 and 1 is generated summarizing the relative similarity between communities, even when the communities contain thousands of members (in our case, 16S sequence reads).

Note that we do not make phylogenetic trees from the pyrosequencing data itself. Instead, we align the pyrosequence reads with previously generated trees of full length 16S sequences. Thus we avoid potential errors due to forming trees with short sequences. In a related error control measure, often we condense our pyrosequence reads into operational taxonomic units (OTUs) composed of pyrosequence reads that are 97% or more identical, then perform the downstream analysis on OTUs. This allows us to use a consensus of many reads to improve the qualities of taxonomic placements.

To analyze a collection of communities, 16S sequences from each are arrayed on a common phylogenetic tree, then the UniFrac value is determined for all pairs of communities, thereby generating a distance matrix. The distance matrix is then used to cluster the UniFrac values using hierarchical clustering. The distance matrix can also be used to generate clustering maps using dimensionality reduction by PCoA (Principal Coordinates Analysis), a geometric technique that converts a matrix of distances between points in multivariate space into a projection that maximizes the amount of variation along a series of orthogonal axes. In a successful analysis of this type, multiple axes may be attributable to independent biological phenomena¹.

The UniFrac analysis can be carried out in either a weighted fashion, which takes into account

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the numbers of reads of each sequence, or unweighted, which focuses only on the types of sequences present and not their relative abundance. Since these two approaches generate different types of information, both are typically applied in each analysis. Clustering detected in the UniFrac PCoA analysis can be tested for statistical significance in several ways. For example, if the question is whether two clusters are well separated, this can be addressed by comparing the mean distances among all points within clusters to mean distances for all points between clusters. An empirical P value can then be computed by permuting the labels and recalculating the test statistic over many iterations. Several examples of UniFrac analysis are presented below.

We have also used multiple additional methods to compare communities using 16S rDNA sequence data. Estimates of community richness, diversity and evenness can be calculated (eg; Chao, Ace, Shannon, Simpson etc.) and used for community comparison. Enrichment of specific bacterial taxa were tested for statistical significance using Fisher's exact test, Chi Square, and ANOVA. These and other methods are discussed further in the Experimental Plan.

Analyzing shotgun metagenomic pyrosequencing data. Our second approach to analyzing the composition of microbial communities relies on metagenomic shotgun sequencing. In this method, DNA samples from uncultured microbial communities are fragmented by nebulization, ligated to linkers, and subject to 454 pyrosequencing. The resulting sequence reads are aligned with sequence databases, allowing the types of organisms present and their gene complement to be quantified. The metagenomic method provides an important second window on uncultured microbial communities that is complementary to the 16S rDNA sequence analysis. Bacteria transfer DNA between cells at remarkable rates. The relationship between a gene and a bacterial species is a statistical one, where a given gene will have a finite probability of being found in the genome of many different bacterial species. For bacteria of a single ostensible species, the gene complement can vary radically - E. coli K12 has a genome of 4.6 Mb, while that of O157:H7 has 5.4 Mb, and many of the genes that differ between the two are thought to affect phenotype ⁹². The 16S rDNA is fairly stable within a bacterial group, so it provides useable taxonomic information, but even the 16S rDNA has been proposed to be subject to lateral transfer on rare occasions ⁹³.

Our analytical approach is designed to clarify the most crucial aspects of the relationship between microbiome and disease against this fluid background. It could be that a collection of bacterial groups defined by 16S sequences is associated with disease. However, it could also turn out that specific bacterial genes (or pathways reported by genes) are most relevant, and these genes reside in different bacterial species from subject to subject! Our 16S analysis would identify the first trend involving specific types of bacteria, and the metagenomic analysis would identify the second. Metagenomic analyses have been used previously to compare the functional capabilities of microbial communities in habitats as diverse as acid mine drainage, a deep mine, soil, and the open ocean ⁹⁴⁻⁹⁷. An example of our use of metagenomic analysis is presented below, in which we carried out detailed metagenomic comparison of the effects of low fat or high fat diet on the murine gut microbiome (Hildebrandt et al., manuscript submitted).

The metagenomic method also allows detection of eukaryotic microbes or viruses. Shot-gun reads can be aligned to database sequences and the taxa extracted. MEGAN ⁹⁸ and our custom VHunter software allows convenient visualization of taxa and comparisons between samples ⁹⁸. This way we can identify sequences from any kingdom or from viruses, provided that their sequences are present in sufficient abundance in the starting sample. A considerable challenge, however, is posed by probable contamination of lung microbiome specimens with large amounts of human DNA. These issues are discussed in depth in the Research Plan.

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Effects of lentiviral infection and associated colitis on the macaque gut microbiome.

Accumulating evidence now indicates that along with viral replication, a principal factor driving CD4 T cell loss and immune deficiency in pathogenic HIV and SIV infection is chronic immune activation and, further, that microbial contents of the gut may be responsible, at least in part, for this chronic immune stimulation⁹⁹⁻¹⁰². Therefore, to better understand the gut microbiome in immunodeficiency virus infection, we took advantage of the SIV infection model in macaques to compare community composition from different gut anatomical sites in health, simian AIDS and GI disease¹. To profile the bacterial taxa present, we analyzed bacterial DNA from samples of stool, intestinal contents taken at necropsy, lower GI mucosa and upper GI mucosa. We amplified segments of the 16S rRNA gene as described above, determined the sequences using 454 pyrosequencing, then used these data to identify and quantify the types of bacteria present. A total of 100 bacterial communities were characterized using 141,000 sequence reads of ~260 nt, for a total of 37,000,000 bases of DNA sequence.

Over all the sequences analyzed in this study, 99.94% aligned with previously determined 16S rRNA gene sequences. In addition, 94 longer (near-full length) macaque bacterial 16S rRNA gene sequences from two communities were also determined by conventional Sanger sequencing to provide a check on the pyrosequencing data. The major types and relative numbers of taxa were closely similar in the Sanger and pyrosequencing analysis for each sample, indicating that the pyrosequencing data yielded an accurate reflection of the species present¹.

Summaries of the taxa present indicated considerable heterogeneity between individuals, and even within individuals over time (Fig. 2). The monkeys are fed standard chow, but for humanitarian reasons are given varied dietary supplements. In light of our murine data on diet (below), we now attribute much of the variation in community composition to variations in diet.

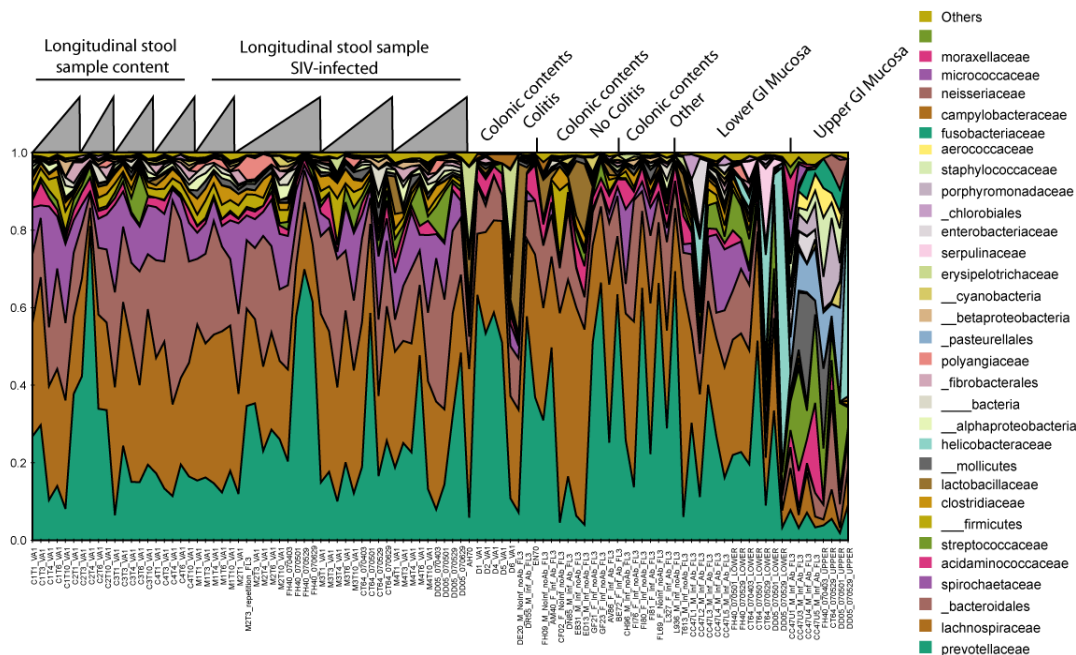


Fig. 2. Bacteria of the macaque gut microbiome determined using 454 pyrosequencing of 16S rDNA sequences and alignment with ARB. The designations for each monkey and sample are along the X-axis, the Y-axis indicates the percent of the community comprised by each type of bacteria. Bacterial taxa are listed at the right. Taxa corresponding to bacterial phyla are indicated with the triple underscore before the name, classes by a double underscore, orders by single underscores, and families by no underscore. Additional details are described in McKenna et al 2008¹

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Differences were also seen in communities from different anatomical sites, with upper GI tract samples showing notably different compositions. We also found that the macaque microbiota was distinct from other vertebrates studied previously (Fig. 3).¹

Analysis by disease state showed, for specimens of colonic contents taken at necropsy, animals with severe colitis (and associated treatment history) showed significant differences from healthy animals (Fig. 4). Similar results were seen for macaques with advanced colitis due to SIV infection (a common complication during simian AIDS), or colitis due to other causes. An analysis of the relative diversity, as reported by the Shannon Index, revealed that diversity was consistently lower in the communities from colitic animals. An analysis of the bacterial taxa specified some of the groups involved, and suggested mechanisms of pathogenesis. Unexpectedly, communities from males and females also differed. The physiological mechanism for the observed sexual dimorphism is unknown. These data provide an illustration of the use of UniFrac and principal coordinate analysis to detect and analyze multiple orthogonal sources of variation in 16S data sets.

In summary, this study indicated that colitis due to lentiviral infection and its treatment were associated with transitions in GI microbiota in the macaque. This is the first use of deep sequencing showing lentiviral infection alters the primate microbiome, thereby providing proof-of-concept for studies of the effects of HIV infection on the human lung microbiome.

Profound differences in the murine gut microbiome comparing high fat and normal chow diets. We have completed a study of the role of diet and host cell genotype on the gut microbiome as a collaboration among the laboratories of Bushman, Gary Wu, Rob Knight, and Rexford Ahima (Hildebrandt et al., submitted). We found that 1) switching mice to a high fat diet resulted in a massive change in microbiome composition, and 2) RELM-beta, a signaling molecule expressed in the colon in response to commensal gut bacteria, regulates both the response to a high fat diet and the composition of the gut microbiome. Using deep sequencing of 16S and metagenomic samples, we determined the composition of the microbiome in fecal pellets in wild-type and RELM-beta KO mice on both diets and documented radical differences. The diets caused profound changes in microbiome, and RELM-beta KO caused more modest but detectable changes (Fig. 5). Effects of a high fat diet included an increase in the *Firmicutes* and a corresponding decrease in *Bacteroidetes* and multiple changes in less abundant bacterial lineages.

Metagenomic analysis of samples from wild-type mice on

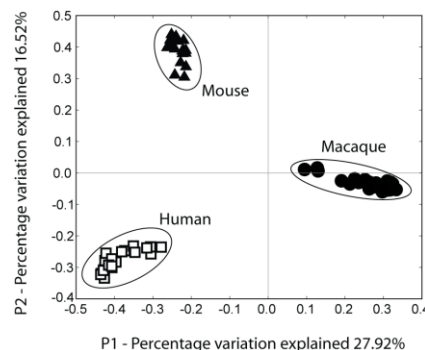


Fig. 3. Comparison of gut communities of macaques, mice & humans using UniFrac. The plot was generated using unweighted UniFrac. Mouse & human sequences were trimmed to match the macaque pyrosequence reads in length (264 nt) and location within the 16S rRNA gene. Differences among communities from different vertebrates was significant at $P < 0.001$ (t-test with permutation).

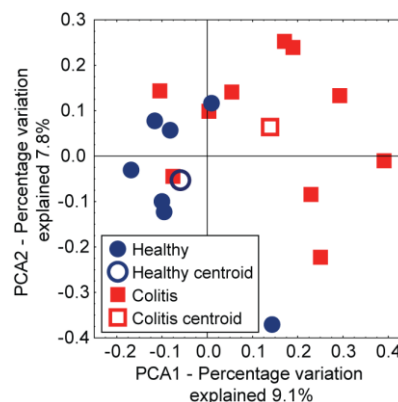


Fig. 4. Distinct composition of gut bacterial communities from colitic and healthy macaques. The analysis was restricted to samples of colonic contents taken at necropsy that allowed unambiguous assignment to the "colitis" or "healthy" categories. Communities were analyzed using unweighted UniFrac. Samples in colitis & healthy categories showed significant separation along the first principal coordinate ($P < 0.05$, t-test with permutation).

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the high fat and low fat diets was

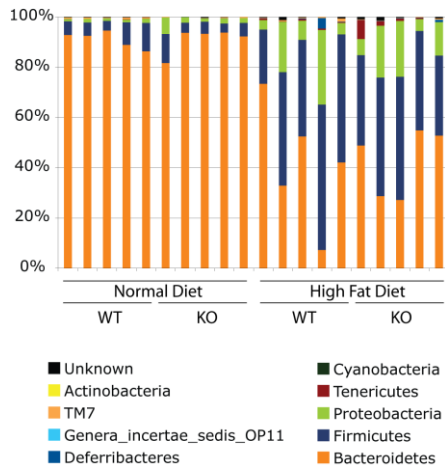


Fig. 5. Massive changes in murine gut communities between mice fed normal chow or a high fat diet. The figure shows the percentages of each community contributed by the indicated Phyla. Diet and genotype are indicated below the figure. KO indicates Relm-beta knockout, which had a modest but detectable effect on microbiome composition.

also carried out (Fig. 6). Samples were sheared by nebulization, ligated to linkers, and subjected to shotgun 454 sequencing. A total of 537,604 sequence reads were compared for high fat and normal chow diets for wild-type mice. Analysis using BLAST and MEGAN⁹⁸, carried out at the Penn Genome Frontiers Institute High-Performance Computing facility (<http://www.genomics.upenn.edu/computers.html>), allowed the taxa present to be extracted from the alignments. Crucially, the main changes in bacterial lineages specified by this analysis matched quite well with changes identified in the 16S data, providing a key check on data quality (compare Figure 5 to the top set of bar graphs in Figure 6). One important finding was that the high-fat diet samples showed sharply increased representation of murine DNA. Use of our 16S Taqman Q-PCR assay confirmed that bacterial sequences were a lesser proportion of the total in the high fat diet samples. These findings are consistent with the idea that the high fat diet causes increased sloughing of epithelial cells into the lumen of the gut, a potential contributor to the adverse effects of the high fat diet. A detailed analysis of gene content (Fig. 6) specified *types of genes* that changed significantly with the change in diet. In this study, we introduced software that allows

the changes in gene content to be assigned to specific bacterial taxa. Thus, within the *Proteobacteria*, lineages with more genes for signal transduction, cell motility and membrane transport increased in abundance on the high fat diet, while lineages with more genes for energy metabolism and nucleotide metabolism decreased. For *Bacteroidetes*, lineages rich in genes for amino acid metabolism, translation, and nucleotide metabolism decreased, while those with more genes for membrane transport and replication and repair increased. For the *Firmicutes*, lineages with more genes for membrane transport, transcription and cell motility increased in abundance on the high fat diet, while those with more genes for carbohydrate metabolism & energy metabolism decreased. Collectively these data document a community-wide change in metabolism that was distinctive for each Phylum accompanying the switch to high fat diet. While we recognize the obvious & important differences in the relationship between diet and gastrointestinal vs. respiratory tracts, based on the massive changes in gut community composition with dietary modification and known interrelationships between microbiota of the respiratory and GI tracts (at least upper GI), we believe it will be important to determine if diet also affects the respiratory microbiome.

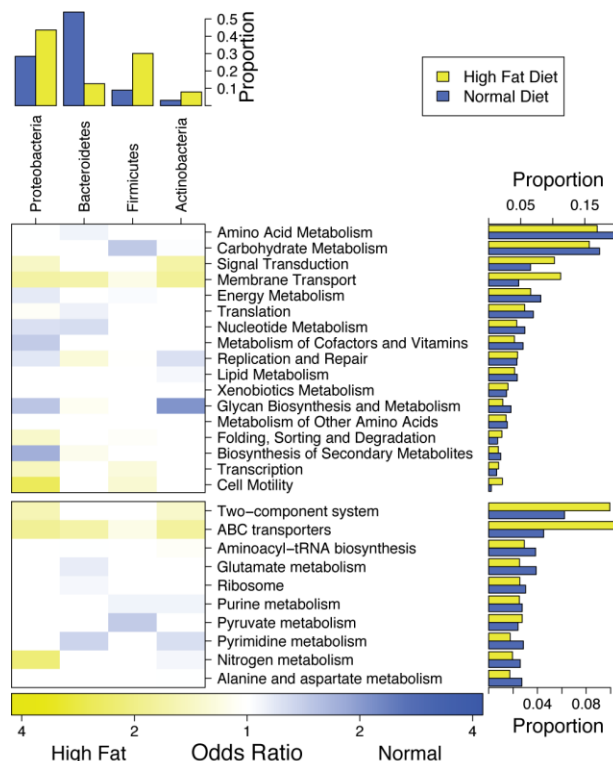


Fig. 6. Metagenomic analysis of DNA samples from mice on normal or high fat diets. The bacterial phyla are indicated along the top of the figure for the most abundant four bacterial phyla (95% of total), the functional categories in the column to the right. The colored tiles in the body of the figure indicate the changes in gene content within each Phylum (that is, changes in gene types are compared between diets considering only sequences from each Phylum). Functional classes were assigned using KEGG and SEED annotation.

Global spatial and temporal changes in gut microbial communities accompanying enteropathogenic *Citrobacter* infection. In collaboration with Dr. David Artis at the University of Pennsylvania, we investigated the response of the murine microbiota to infection with *Citrobacter rodentium*, which causes attaching effacing lesions with associated inflammation. Previously the response of the full gut microbial community to infection and the attendant immune response has been difficult to measure. We used DNA bar coding and 454 pyrosequencing to characterize 102,398 partial 16S rDNA sequences from 85 microbial communities, sampling tissue associated and luminal bacteria from the cecum, distal colon, and proximal colon (Fig. 7).

The deep sequencing data specified that *Citrobacter* was most abundantly associated with the cecal mucosa at day 9, then diminished in abundance. Notable changes were associated with both the mucosally-adhered and luminal microbiota at both day 9 and day 14 after infection. The temporal progression of infection resulted in altered communities in the infected samples at the two times analyzed but not in naïve controls. Alterations in abundance were seen for *Proteobacteria*, *Tenericutes*, *Deferribacteres* and others. The *Lactobacillus* group dropped in

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abundance during infection, which may be important for pathogenesis because members of this lineage modulate the composition of the gut microbiota by regulating pH and are used as probiotics. Thus deep sequencing discloses that

Citrobacter infection and clearance results in dynamic reshaping of the gut microbial community in space and time.

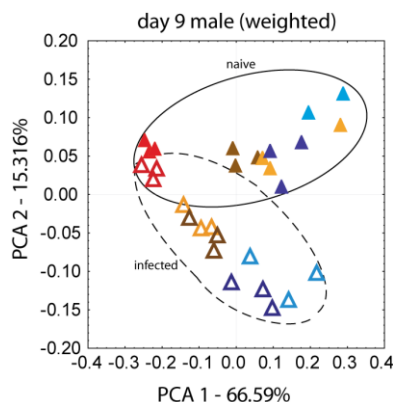


Fig. 7. Community-wide changes in bacterial populations accompany *Citrobacter rodentium* infection in mice. Points from naïve (filled symbols) and infected animals (open symbols) are enclosed in ovals. Orange indicates communities from intestinal contents, blue communities associated with the gut mucosa. Male mice were compared, because of sexual dimorphism in GI microbiota. Samples were taken 9 days after infection with *C. rodentium*. PCA 1 separates the communities by tissue of origin, and PCA 2 separates by infection status. Note that the effects of infection are most prominent in tissue-associated communities.

Massive alterations of the murine gut microbiome accompanying intensive antibiotic treatment.

Antibiotics are widely used in medicine and agriculture, but excessive use can result in perturbations of the normal microbial communities, in some cases resulting in colonization by pathogenic organisms such as *Clostridia difficile*. In collaboration with Dr. David Artis, we used 454 pyrosequencing to characterize the effects of the combination of vancomycin, gentamycin, metronidazole, ampicillin, and neomycin on the murine gut microbiome (unpublished data). Initially, quantitative PCR was used to analyze samples from antibiotic treated or control mice, showing that the numbers of detectable bacteria were reduced by ~100-fold with antibiotic treatment but not reduced to zero. We use DNA bar coding and pyrosequencing of 16S rDNA to generate 69,492 sequence reads from 62 gut microbial communities, revealing that massive changes in community structure affecting all lineages examined. The communities from antibiotic-treated animals were quite similar among animals. These data reveal that the mouse gut is not sterilized by quintuple antibiotic treatment, though bacterial densities are substantially reduced, and the bacterial community composition is profoundly altered.

Sequence-based approaches for identifying unknown pathogens in clinical samples.

The Bushman laboratory has carried out studies of samples from HIV patients designed to identify previously unknown pathogens (unpublished data), which is also one of the goals of the project proposed here. Serum was obtained from late stage AIDS patients, reasoning that such patients would be particularly likely to harbor previously unidentified organisms. A variety of methods were applied to extract nucleic acids. In one, serum samples were treated with DNase and RNase, so that only protected nucleic acid would survive (for example, nucleic acids within viral particles). Treated serum samples were pooled and subjected to ultracentrifugation. Pellet fractions were processed to isolate DNA or RNA. As controls, phage particles or HIV particles were doped into some samples to verify efficient recovery of viral particles. Nucleic acids were analyzed by PCR, using PCR primed with random hexamers with specially designed "tails" to allow nested PCR amplification. To analyze the data, we developed the VHunter software package. This tool aligned newly acquired sequences to databases of viral sequences, extracted the best matches, then placed them in user-configurable tables together with associated annotation. By this means, we were able to scan through newly determined reads efficiently to identify new viral sequences. Large numbers of HBV sequences were recovered, indicating that the method worked well, though no previously unknown microbes were identified (unpublished data). We propose to employ a variety of related strategies with the microbiome samples studied here to identify novel microbes as described below.

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The University of Pennsylvania Human Microbiome Project. U. Penn is the site of the Human Microbiome Demonstration Project "Diet, Genetic Factors, and the Gut Microbiome in Crohn's Disease", directed by PIs F. Bushman, J. Lewis & G. Wu. The project's overall goal is to analyze the role of the human microbiome in the pathogenesis of Crohn's disease, while controlling for effects of diet & human genetics. Its Specific Aims are:

1. To establish optimal methods to collect stool samples for assessment of fecal microbiome composition.
2. To determine the effect of dietary fat on the composition of the human microbiome in a prospective study of normal volunteers.
3. To define the human gut microbiome in patients treated for active Crohn's disease using elemental diet.
4. To analyze the interplay of human genetics (using genome-wide SNP data), gut microbiota, diet, and Crohn's disease.
5. To carry out metagenomic analysis of key samples from specific aims 1 through 4.

The Lung project proposed here will make extensive use of methods and infrastructure developed in the HMP Gut program. Examples include shared sample preparation methods, sample tracking procedures, clinical and molecular databases, bioinformatic analytical tools, and methods for multidimensional data analysis. We will also take advantage of samples available in the controlled diet intervention study (Gut study Aim 2), discussed in Aim 4 of this application. Our HMP Gut application received an outstanding score and we were notified that funding would be forthcoming; the start date was originally scheduled for April 1, 2009, but we just received word that the start date would be either May 1 or June 1, 2009. Based on information from our Program Officer, we expect to receive the notice of award shortly.

1.2 Investigational Agent

No investigational agents will be used in this study.

2 Study Objectives

2.1 Primary Objectives:

1. Define the lower and upper respiratory tract microbiome in HIV-infected and HIV-negative individuals through analysis of the lower and upper respiratory tract microbiome in HIV infected individuals at different levels of immune deficiency and normal healthy HIV-negative controls.
2. Define the stability of the upper respiratory tract microbiome by serial sampling over one year in a subset of individuals.
3. Evaluate the effect on the respiratory system microbiome of progressive immune deficiency and ART-associated immune reconstitution through a) longitudinal analysis of microbiome populations in untreated HIV-infected individuals, and b) longitudinal analysis of respiratory microbiome following ART initiation.
4. Define the respiratory system microbiome in HIV-infected and uninfected individuals with COPD by comparison of the lower and upper respiratory microbiome of HIV-infected and uninfected individuals with COPD, and through correlation of the respiratory microbiome with cellular, functional and imaging evidence of inflammation and lung injury by bronchoalveolar lavage cell analysis, pulmonary function and CT scanning.

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5. Evaluate the effects of smoking on the upper and lower respiratory microbiome populations in HIV-infected and uninfected nonsmokers and smokers.
6. Evaluate the effect of diet on the upper airway microbiome and the relationship between respiratory and gut microbiome by evaluation of subjects participating in the Human Microbiome Roadmap gut project.

2.2 Secondary Objective

Develop new methodologies based on metagenomic and other approaches to identify novel microbiome constituents not identifiable using 16S-based sequencing.

3 Study Design

3.1 General Design

This is a non-interventional cohort study with single time point cross sectional comparisons, and longitudinal analyses, with the primary study aims of evaluating how the immune deficiency associated with HIV infection influences the microbiome of the upper and lower respiratory tract and lung function, how COPD influences the microbiome of the upper and lower respiratory tract, how smoking influences the microbiome of the upper and lower respiratory tract, and the relationship between the microbiome of the upper respiratory tract and the gastrointestinal tract. There are three major groups of subjects as outlined below. The duration of subject participation and the study procedures vary by each group as outlined below and in Section 5.

3.1.1 Group 1

Group 1 subjects will have HIV infection with quantifiable viral loads, not on antiretroviral therapy. These subjects will participate in longitudinal analyses. There are two subgroups of subjects in Group 1.

3.1.1.1 Group 1A

Group 1A subjects (n=12) will have CD4+ T lymphocyte counts ≥ 400 cells/mm³, and will have decided together with their primary care providers to not begin antiretroviral therapy. There will be six subjects in Group 1A who are non-smokers and six who are smokers. These subjects will be followed longitudinally over a three year period.

3.1.1.2 Group 1B

Group 1B subjects (n=12) will have CD4+ T lymphocyte counts of 200-400 cells/mm³ and non-smokers or smokers. These subjects will be followed longitudinally over a three-year period.

Group 1 will have upper and lower respiratory tract microbiome analysis at entry and yearly for 3 years (4 time points), to assess the effect of HIV infection and progressive immune decline on the respiratory microbiome. To ensure that we can study both changes during disease progression and effects of ART immune reconstitution, we will include stable subjects with relatively high CD4 counts (≥ 400) who are likely to remain untreated for a substantial period of time (Group 1A), and subjects with CD4 counts in or near a range at which treatment is usually begun (< 400 , Group 1B). It is anticipated that some subjects in Group 1B will initiate ART shortly after enrollment into the trial.

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To evaluate the short term effects of the immune reconstitution that follows ART subjects will have microbiome sampling of the upper and lower respiratory tract 4 months following initiation of ART. Subjects in Group 1A who initiate ART during the course of the study will have lower and upper respiratory tract sampling before ART initiation, if not performed within the prior 6 months, and then 4 months following the initiation of therapy.

To evaluate the effect of smoking, we will include equal numbers of smokers and nonsmokers in Group 1A. Because microbiome stability over time is unknown, we will carry out a stability analysis on a subset of subjects (Group 1A; nonsmokers) who will have monthly upper airway sampling over a 12-month period.

Along with microbiomes, BAL will be evaluated for inflammatory cells by total and differential cell count and CD4/CD8 flow cytometry. BAL fluid will also be stored for potential future studies that may include analysis of soluble mediators, which although outside the scope of this study, is something that we hope to do in the future. We will also sample and store respiratory epithelial cell RNA for potential future studies of the relationship between lung microbiome populations and epithelial innate immune mediator expression, which is outside the scope of this study but something we hope to do in the future. Subjects will also undergo pulmonary function testing and chest CT at baseline and year 3, to provide quantitative data on lung function and COPD/emphysema.

3.1.2 Group 2

Group 2 subjects will have HIV infection and be on antiretroviral therapy with HIV viral loads suppressed below detectable levels. There are two subgroups of subjects in Group 2.

3.1.2.1 Group 2A

Group 2A subjects (n=10) will have COPD/emphysema. These subjects will be followed longitudinally over a three-year period.

3.1.2.2 Group 2B

Group 2B subjects (n=10) will have no lung disease, and will attempt to be matched to Group 2A by age, race, gender, and CD4+ count. These subjects will be followed longitudinally over a three-year period.

In the Penn Center for AIDS Research (CFAR) Clinical Cohort, most subjects with a diagnosis of COPD are on ART. Since this complication is emerging in the context of the ART era, we will focus our analysis of the effect of COPD on the pulmonary tract microbiome of HIV patients by studying HIV patients on stable ART with effective control of virus replication (undetectable HIV RNA level, <200 copies HIV-1 RNA/mL). Subjects with prior pneumocystis pneumonia (documented or treated empirically) will be excluded, to limit the contribution of this known cause of emphysema-like changes. A comparison group of ART-treated individuals without evidence of lung disease will attempt to be matched to the HIV/COPD group by race, gender, age (± 5 years) and CD4 count (± 100). In addition to serving as a comparison group to the ART-suppressed HIV/COPD subjects, this group will also enable us to generate microbiome data sets on subjects with HIV infection and established ART suppression, which will complement post-ART data from Group 1 subjects who initiate ART. These ART-suppressed individuals will undergo upper and lower respiratory tract sampling at entry and at year 3, along with quantification of lung disease by PFT and chest CT scan. As with Group 1, BAL samples will be analyzed for inflammatory cell composition and an aliquot stored for potential future soluble mediator studies, and epithelial cell RNA will be obtained and stored for future analysis.

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3.1.3 Group 3

Group 3 includes HIV-uninfected control populations. There are three subgroups of subjects in Group 3.

3.1.3.1 Group 3A

Group 3A subjects (n=10) will have COPD/emphysema, be current nonsmokers or smokers, and will attempt to be matched to Group 2A by age, race, gender, and FEV-1. These subjects will be followed longitudinally over a three-year period.

3.1.3.2 Group 3B

Group 3B subjects (n=20) will have not have COPD/emphysema, and will include smokers (n=10) and non-smokers (n=10). This group will be matched to Group 1A be current nonsmokers, and will attempt to be matched to Group 2A by age, race, and gender. These subjects will have a single evaluation at one cross sectional time point.

To evaluate the effect of smoking, we will include equal numbers of smokers and nonsmokers in Group 3B. Because microbiome stability over time is unknown, we will carry out a stability analysis on a subset of subjects (Group 3B; nonsmokers) who will have monthly upper airway sampling over a 12-month period.

3.1.3.3 Group 3C

Group 3C subjects (n=24) will be healthy subjects enrolled in an IRB approved protocol [IRB #809460; "Controlled feeding experiment of high and low fat diet and stool microbiome composition"; James D. Lewis, PI] evaluating the effect of diet on the stool microbiome. These subjects will have upper respiratory tract samples collected up to 5 time points over a 9-day study period.

To evaluate the effect of HIV infection on the lung microbiome in relationship to the presence of COPD, HIV-negative subjects with COPD/emphysema will be recruited. These subjects will attempt to be matched to the HIV/COPD group by race, gender, age (± 5 years) and lung function ($\pm 15\%$ FEV1 % predicted), and will also be studied by respiratory microbiome sampling, BAL cell analysis, PFT and CT scanning at entry and year 3. As with the other groups, an aliquot of BAL and epithelial cell RNA will be archived for potential future studies.

To evaluate the effect of HIV infection on the lung microbiome in the absence of COPD, HIV uninfected subjects without lung disease will be recruited. To evaluate effect of smoking on the respiratory microbiome (a critical question even apart from HIV infection^{31, 32}) we will include both smokers and nonsmokers. These subjects will attempt to be matched to Group 1A by race, gender, age (± 5 years), and smoking status. These healthy controls will be evaluated by upper and lower airway sampling at one time point, along with assessment of lung function by PFTs and CT scan. As with HIV-infected subjects (Group 1A), stability analysis will be carried out on a subset of healthy individuals with monthly upper airway sampling.

To evaluate the effect of diet on the upper respiratory microbiome, and also address the relationship between the microbiomes in the respiratory tract and gastrointestinal tract, a third HIV-negative group drawn from the Human Gut Microbiome Roadmap study will be co-enrolled into this project. Healthy volunteer subjects in the Human Gut Microbiome Roadmap study are admitted to the Penn Clinical & Translational Research Center for 9 days of evaluation, including microbiome sampling of stool following consumption of a normal, high fat or low fat diet. We will sample the upper respiratory microbiome on 'ad lib' diet upon entry, and up to 4 times on the defined diet.

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3.1.3.4 Group 3D

Group 3D subjects (n=12) will have not have COPD/emphysema, and will include smokers (n=6) and non-smokers (n=6). This group will attempted to be matched to Group 1A and 2A by age, race, gender and smoking status. These subjects will have a single evaluation at one cross sectional time point.

To evaluate the effect of smoking, we will include equal numbers of smokers and nonsmokers in Group 3D. Because microbiome stability over time is unknown, we will carry out a stability analysis on a subset of subjects (Group 3D; nonsmokers) who will have monthly upper airway sampling over a 12-month period.

3.2 Primary Study Endpoints

This is not an efficacy study. There is no pre-specified endpoint for this study. Rather, we will use an unbiased approach to determine the composition of the microbiome in the human upper and lower respiratory tract using state of the art methodologies including 16S rDNA informatics and metagenomic analysis (described below), under conditions of HIV infection, COPD, and/or smoking. The resultant data will be quantifiable using methods to characterized uncultured bacteria

3.3 Secondary Study Endpoints

Not applicable.

3.4 Primary Safety Endpoints

Not applicable. Adverse events associated with bronchoscopy are the clinically relevant safety issues in this study. Mechanisms to monitor those events are described in Section 7 below.

4 Subject Selection and Withdrawal

4.1 Inclusion Criteria

- Age ≥ 18
- Able to understand and provide informed consent
- Willingness to comply with study evaluations, including bronchoscopy, PFT, CT studies.
- Anticipated retention in local geographic area for duration of study.
- Laboratory:
 - Hemoglobin >10 (males); >9.5 (females) g/dL
 - ANC $>1000/\mu\text{L}$
 - Platelet count $>100,000/\text{mm}^3$
 - Serum creatinine <2.0 mg/dL (177 $\mu\text{Mol/L}$)
 - AST, ALT <3.0 times the ULN
 - Total bilirubin <5 times the ULN (unless in HIV group on atazanvir)

4.1.1 HIV study group

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General Inclusion Criteria

- Chronic HIV-1 infection.
- Have a Karnofsky Performance score of 80 or higher.
- Anticipated retention in geographic area for duration of study.
- CD4+ T cell count ≥ 200 cells/mm³

Suppressed Group

- Undetectable HIV viral load <200 copies HIV-1 RNA/mL for ≥ 6 months at the time of screening.
- On a stable antiretroviral medication regimen (no changes to treatment within 4 weeks of study entry) and be willing to continue on antiretroviral therapy for the duration of the study.

4.1.2 COPD study groups (both HIV+ and HIV-)

Inclusion Criteria

- Moderate / stage 2 COPD (based on ATS/ERS and GOLD ¹⁰³⁻¹⁰⁶)
FEV1/FVC <70%
FEV1 <80% and $\geq 50\%$ predicted
Incomplete reversibility with bronchodilators
(Subjects will not be required to have evidence of emphysema)

4.1.3 Participant in Human Gut Microbiome Project

- Meeting eligibility criteria and willingness to participate in IRB approved protocol, "Controlled feeding experiment of high and low fat diet and stool microbiome composition" (IRB##809460, See attached protocol – Amendment 1)

4.2 Exclusion Criteria

- Clinical evidence of upper respiratory tract infection in preceding 4 weeks.
- Received antimicrobial agent within preceding 3 months.
- History of cancer with the exception of successfully removed basal cell carcinomas or solid tumor successfully removed by surgery more than 5 years previously, without chemotherapy or radiation therapy.
- NYHA Class III or IV congestive heart failure or ischemic heart disease.
- Any medical condition considered to pose an increased risk of complications from study procedures as defined by the investigators
- Hypoxemia requiring supplemental oxygen.
- Systemic corticosteroids or other immune modifying agents.
- HIV seroconversion within the previous year
- History of *Pneumocystis jirovecii* pneumonia

4.3 Subject Recruitment and Screening

HIV patients, COPD patients, and participants in the stool microbiome study will be recruited through direct contact.

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HIV infected subjects will be recruited through the HIV clinical practices of the University of Pennsylvania by discussion with HIV providers; in addition self referrals will be accepted. The Adult/Adolescent Database of the Penn CFAR Clinical Core will be screened to identify potentially eligible subjects. Agreement of suitability of the patient to participate in the protocol will be obtained from the potential participant's HIV care provider. Clinical records and laboratory tests obtained for ongoing medical care will be used to determine the eligibility of subjects. A self referral will grant permission to the study team to contact one's treating health care provider. The participant and health care provider will determine if the self referral is suitable to participate in the research study.

HIV-negative subjects with COPD/emphysema will be recruited from the COPD cohort managed by Dr. Panettieri at the University of Pennsylvania Airways Biology Initiative or any UPHS outpatient clinics and its affiliated facilities. Clinical records, laboratory tests, and pulmonary function tests obtained as part of patients' ongoing medical care will be used to determine eligibility based upon PFT and other clinical criteria. Self referrals in this cohort will be accepted. A self referral will grant permission to the study team to contact one's treating health care provider. The participant and health care provider will determine if the self referral is suitable to participate in the research study.

The clinical coordinator for the "Controlled feeding experiment of high and low fat diet and stool microbiome composition" trial will notify the coordinator of this trial when subjects are planning to be studied. Permission for upper airway sampling has been added to the parent study.

Healthy HIV uninfected volunteers with and without COPD will be recruited through advertising. All advertisements will be approved by the U Penn IRB before use.

4.4 Early Withdrawal of Subjects

4.4.1 When and How to Withdraw Subjects

Participants may be withdrawn from the study at any time if the investigator or the participant believes that the participant's safety will be jeopardized by continued participation. Participants may withdraw consent at any time by discussing that request with the study coordinator or investigator.

4.4.2 Data Collection and Follow-up for Withdrawn Subjects

Data collected on subject up until their time of participation will be used. Cells will be stored for possible genetic analysis in the future. Participants who do not wish their cells to be used for genetic analysis or chose to modify their consent for use of these specimens must notify the investigators in writing.

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5 Study Procedures

The schedule of study procedures by research group are outlined in the accompanying table.

Table 1. Schedule of Events – Groups 1 and 2

	Scr	Day 0	Day 5	Day 9	MO 1	MO 2	MO 3	MO 4	MO 5	MO 6	MO 7	MO 8	MO 9	MO 10	MO 11	MO 12	Pre ART ₁	Post ART ₂	YR 2	URI ³	YR 3
Group 1 A and B																					
Medical History	X															X			X		X
Physical Exam	X	X														X	X	X	X		X
CBC, LFTs, crea	X															X			X		X
CD4+ T cell count	X							X								X	X		X		X
HIV-1 RNA level	X															X	X		X		X
PFTs	X ¹	X																			X
Chest CT scan		X																			X
Oral wash		X			1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	X	X	X	X	X	X
Nasal swab		X			1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	X	X	X	X	X	X
Throat swab		X			1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	1A ⁴	X	X	X	X	X	X
Bronchoscopy		X														X	X	X	X		X
Blood for storage		X														X	X	X	X		X
Urine pregnancy		X														X	X	X	X		
Urine Cotinine	X															X			X		X
Group 2 A																					
Medical History	X																				X
Physical Exam	X	X																			X
CBC, LFTs, crea	X																				X
PFTs	X ¹	X																			X
Chest CT scan		X																			X
Oral Wash		X																			X
Nasal swab		X																			X
Throat swab		X																			X
Bronchoscopy		X																			X
Cells for storage		X																			X
Urine pregnancy		X																			X
Urine Cotinine	X																				X
Group 2 B																					
Medical History	X																				X
Physical Exam	X	X																			X
CBC, LFTs, crea	X																				X

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HIV rapid test	X																			X
PFTs	X ¹	X																		X
Chest CT scan		X																		X
Oral Wash		X																	X	X
Nasal swab		X																	X	X
Throat swab		X																	X	X
Bronchoscopy		X																		X
Cells for storage		X																		X
Urine pregnancy		X																		X
Urine Cotinine	X																			X

Schedule of Events – Group 3

Group 3A																				
Medical History	X																			
Physical Exam	X	X																		X
CBC, LFTs, crea	X																			
HIV rapid test	X																			
PFTs	X ¹	X																		X
Chest CT scan		X																		X
Oral Wash		X																X		X
Nasal swab		X																X		X
Throat swab		X																X		X
Bronchoscopy		X																		X
Blood for storage		X																		X
Urine pregnancy		X																		X
Urine Cotinine	X																			X
Group 3B																				
Medical History	X																			
Physical Exam	X	X																		
CBC, LFTs, crea	X																			
HIV rapid test	X																			
PFTs	X ¹	X																		
Chest CT scan		X																		
Oral Wash		X					NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	
Nasal swab		X					NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	
Throat swab		X					NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	
Bronchoscopy		X																		
Cells for storage		X																		
Urine pregnancy		X																		
Urine Cotinine	X																			

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Group 3C																	
Medical History	X																
Physical Exam	X																
CBC, LFTs, crea	X																
HIV rapid test	X																
Oral Wash		X	X	X													
Nasal swab		X	X	X													
Throat swab		X	X	X													
Group 3D																	
Medical History	X																
Physical Exam	X	X															
CBC, LFTs, crea	X																
HIV rapid test	X																
PFTs	X ¹	X															
Chest CT scan		X															
Oral Wash		X			NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^
Nasal swab		X			NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^
Throat swab		X			NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^	NS^
Bronchoscopy		X															
Cells for storage		X															
Urine pregnancy		X															
Urine Cotinine	X																

1. Screening PFTs may consist of simple FEV-1 and FVC (spirometry) and participants with spirometry available within previous 6 months will not require screening PFTs.

NS^: Evaluations to be performed on six non-smokers in Group 3B or Group 3D.

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5.1 Screening Evaluations – All participants

Except as noted, all screening examinations should be completed within 90 days of baseline evaluations. Diagnostic tests performed for clinical purposes in the preceding 90 days may be used to satisfy screening evaluation requirements, except as noted below.

5.1.1 Medical History – All participants

Medical history will include date of birth, gender, race, ethnicity, past and current medical conditions, surgical history, current and past use of any medication in the preceding 6 months, including nutritional supplementation, smoking history, alcohol and recreational drug use.

5.1.2 Physical Examination- All participants

A complete physical examination will be performed, including height, weight, and all major body systems, documenting any abnormality.

5.1.3 Laboratory Evaluations – All participants

Blood (approximately 15 mL) will be taken for CBC and comprehensive metabolic panel.

5.1.4 CD4+ T Cell Count – Group 1 and 2 participants

Blood (approximately 5 mL) will be taken for CD4+ and CD8+ T lymphocyte counts.

5.1.5 HIV-1 RNA Level – Group 1 and 2 participants

Blood (approximately 5 mL) will be taken for HIV-1 RNA quantification.

5.1.6 HIV Rapid Test – Groups 2 and 3 participants

A rapid HIV test will be performed using the OraQuick ADVANCE rapid HIV 1/2 antibody test on a drop of blood obtained by fingerstick or oral fluid obtained by saliva collected by placing a swab inside the cheek. Pre- and post-test counseling will be provided. The test will be performed by trained HIV testers employed by the Infectious Diseases Division. Results will be given to potential participants within 30 minutes. Any positive test will be repeated. A potential participant with repeatedly positive tests will be referred to Dr. Ian Frank, Infectious Diseases Division, for further counseling and standard HIV antibody testing.

5.1.7 Pulmonary Function Tests (Spirometry) – All participants except Group 3C

FEV-1 and FVC measurements obtained for clinical evaluation of lung function at screening or within the preceding 6 months may be used to qualify participants.

5.1.8 Electrocardiogram (ECG) – All participants except Group 3C

An ECG will be performed for clinical evaluation of the heart performance at screening or within the preceding 2 months may be used to qualify participants.

5.1.9 Cotinine Testing – All participants except Group 3C

Urine cotinine test will be performed during the screening visit in all participants to verify smoking/non-smoker status.

5.2 Baseline Evaluations – All participants

All baseline evaluations will be performed within a two-week period. The upper respiratory tract sampling and bronchoscopy will be performed on the same day. Urine pregnancy testing will be performed on women who are not post-menopausal or surgically sterilized prior to bronchoscopy, and the bronchoscopy will not be performed if the participant is pregnant.

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Pulmonary function tests and the chest CT scan will be performed within a four-week period of the bronchoscopy.

5.2.1 Upper Respiratory Tract Sampling – All participants

Oral cavity, oropharyngeal (OP) and nasopharyngeal (NP) microbial populations are relevant to respiratory system pathogenesis, and have overlapping but not identical communities based on studies of specific respiratory pathogens¹⁰⁷⁻¹⁰⁹. Therefore, we will assess the oral cavity, OP & NP microbiomes. Oral cavity sampling will be done by having the subject swish and gargle with 10ml (1/3 ounces) of 1% saline for one minute. The subject will spit into a sterile container. This will be the oral wash microbiome sample. The NP/OP sampling will be done using flocked swabs (Copan Diagnostics, Murrieta CA), for both greater sample recovery and subject comfort^{107, 110}. One swab will be introduced into one nare and passed straight back until contact is made with the posterior nasopharynx, then immediately removed. A second swab will be introduced into the mouth and passed to the back of the oropharynx without contacting the tongue, buccal mucosa, or palate. The swab will be briefly brushed along the posterior oropharynx and then removed. For time points when both upper and lower respiratory tract microbiome sampling is planned, the upper tract samples will be collected before any nebulizer or anesthetic medications are applied in preparation for bronchoscopy. The above samples will be eluted in iced saline for processing.

5.2.2 Bronchoscopy – Groups 1, 2, 3a, 3b, 3d

Prior to the bronchoscopy a Listerine decontamination rinse will be performed. The subject will be asked to rinse mouth and gargle for 30 seconds. The bronchoscopy will be performed to characterize the lower respiratory tract microbiome by obtaining secretions via protected specimen brushing (PSB) for central airways^{42, 111, 112} and bronchoalveolar lavage (BAL) for distal airway and alveolar sampling^{31, 32, 42}. A critical issue in lower respiratory tract sampling is to minimize the potential for contamination by upper airway contents. For this reason, bronchoscopy will be carried out trans-orally using a sequential 2-scope procedure in which the glottis is anesthetized with one bronchoscope, and then a second clean bronchoscope is used to complete the procedure; this approach is demonstrated to have excellent success in sampling the lower respiratory tract without contamination^{41, 113}. After local anesthesia with the first scope, the clean bronchoscope is advanced through the glottis to a lobar bronchus, where a protected specimen brush (Conmed Corp., Utica, NY) is advanced, the plug expelled, and the brush gently drawn across the subsegmental bronchial mucosa several times. If visually evident lower airway secretions are present they will be included in the sample. The brush is then withdrawn and cut with scissors into iced saline. A second PSB is then done for standard respiratory tract culture. A third PSB will be done and placed into RNAlater, which will store epithelial cell RNA for potential later analysis. The scope is then re-positioned in the right middle lobe and wedged into position in a segmental or subsegmental orifice. Five sequential lavages using 30 ml room temperature saline are instilled; as previously described the first lavage return is discarded to further minimize any potential contaminants from passage of the scope^{31, 32}. An aliquot of lavage fluid is reserved for standard respiratory culture; the remainder is placed on ice and transported to the laboratory where it is strained through sterile gauze to remove mucus (in pilot studies an aliquot of this mucous will be reserved for extraction and 16S sequencing to determine if relevant microbiomal populations might be present within the mucous layer, as reported in cystic fibrosis¹¹⁴). An aliquot is then utilized for cell analysis (total count, differential and CD4/CD8 flow cytometry) and the remainder is processed for DNA extraction and microbiomal analysis.

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Note: The bronchoscopy procedure will be identical for group 3D except that only one bronchoscope will be used, which is identical to how bronchoscopy is done in clinical practice.

5.2.3 Chest CT Scan – Groups 1, 2, 3a, 3b

A chest CT scan will be performed to quantify severity of emphysema and depict its spatial distribution within the lung. CT quantitative densitometry scores correlate well with quantitative pathological scores¹¹⁵⁻¹²⁰. The loss of lung tissue associated with emphysema can be measured as a reduction in lung density using various parameters derived from the frequency distribution histogram of lung voxel attenuation values. The 'voxel index' or 'density mask' method calculates the proportion of low-attenuation lung voxels below a threshold value known to represent emphysema. We will apply computer-assisted approaches to high-resolution chest CT data sets to quantify the amount and distribution of emphysema. CT images will be acquired during full inspiration and full expiration using 64x0.6 mm collimation, a gantry rotation time of 0.5 sec., a kVp of 120, and an effective mAs of approximately 150-200 depending on subject's body habitus. Axial sections (1 & 5 mm) from inspiratory and expiratory data sets will be reconstructed for qualitative (visual) interpretation, as well as quantitative image analysis using semi-automated computer based techniques.

5.2.4 Pulmonary Functions Tests – Groups 1, 2, 3a, 3b, 3d

Lung function will be assessed and quantified by PFTs using Medgraphic Elite plethysmography units (Medical Graphics Corp., St. Paul, MN). Full PFTs including spirometry with bronchodilator response, lung volumes by plethysmography, and diffusion capacity for carbon monoxide (DLCO) will be done, providing maximum uniformity and comparability between studies. PFTs are interpreted using standard age, height, race and gender-adjusted reference values¹⁰⁵, and lung function and COPD severity documented using ATS/ERS and GOLD criteria^{104 103 106}. Quantification of disease severity for COPD will be by FEV1 and FEV1/VC, and emphysema by DLCO (corrected for hemoglobin but not alveolar gas volume¹⁰⁵).

5.2.5 Blood for Storage – All participants

Up to 50 mL of blood will be collected. 5 mL of serum and 5 mL of plasma will be stored. Peripheral blood mononuclear cells (PBMCs) will be isolated from 45 mL of blood and will be stored.

5.2.6 Urine Cotinine Test – All non-smoking participants

Urine cotinine test will be performed during the screening visit in all participants that are non-smokers.

5.2.7 Urine Pregnancy Test - Groups 1, 2, 3a, 3b, 3d

Urine pregnancy will be performed prior to bronchoscopy in women of child bearing potential.

5.3 Days 1 and up to 4 additional collections – Group 3C only

- Upper respiratory tract sampling

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5.4 Months 1 – 11 – Selected Participants in Groups 1A , 3B and 3D only

- Upper respiratory tract sampling

5.5 Month 12 – Group 1 only

All month 12 evaluations will be performed within a four-week period. The physical examination and phlebotomy will be performed on the same day. The upper respiratory tract sampling and bronchoscopy will be performed on the same day. Urine pregnancy testing will be performed on women who are not post-menopausal or surgically sterilized prior to bronchoscopy and the bronchoscopy will not be performed if the participant is pregnant.

- Targeted physical examination
- CD4+ T cell count
- HIV RNA level
- Upper respiratory tract sampling
- ECG
- Bronchoscopy
- Blood for storage
- Urine pregnancy test

5.6 Year 2 – Group 1 only

All month year 2 evaluations will be performed within a four-week period. The physical examination and phlebotomy will be performed on the same day. The upper respiratory tract sampling and bronchoscopy will be performed on the same day. Urine pregnancy testing will be performed on women who are not post-menopausal or surgically sterilized prior to bronchoscopy and the bronchoscopy will not be performed if the participant is pregnant.

- Targeted physical examination
- CD4+ T cell count
- HIV RNA level
- Upper respiratory tract sampling
- ECG
- Bronchoscopy
- Blood for storage
- Urine pregnancy test

5.7 Pre-Antiretroviral Therapy – Group 1A only

Participants in Group 1A that are beginning antiretroviral therapy more than 6 months after their previous bronchoscopy will have the following evaluations.

- CD4+ T cell count
- HIV RNA level
- Upper respiratory tract sampling
- Bronchoscopy
- Blood for storage
- Urine pregnancy test

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5.8 Post-Antiretroviral Therapy – Group 1A only

Participants in Group 1A who began antiretroviral therapy will have the following evaluations 4 months after the pre-antiretroviral evaluations. These evaluations will satisfy the corresponding evaluations at month 12, year 2 or year 3 if obtained less than six months from those time points.

- CD4+ T cell count
- HIV RNA level
- Upper respiratory tract sampling
- Bronchoscopy
- ECG
- Blood for storage
- Urine pregnancy test

5.9 Acute Respiratory Illness – Groups 1, 2, and 3a

Participants in group 1 who develop an upper respiratory illness will be asked to agree to a nose and throat swab prior to the initiation of antibiotics, and two weeks and two months following the initiation of antibiotics.

- Upper respiratory tract sampling

5.10 Year 3 – Groups 1, 2 and 3a

All year 3 evaluations will be performed within a four -week period. The physical examination and phlebotomy will be performed on the same day. The upper respiratory tract sampling and bronchoscopy will be performed on the same day. Urine pregnancy testing will be performed on women who are not post-menopausal or surgically sterilized prior to bronchoscopy and the bronchoscopy will not be performed if the participant is pregnant. Pulmonary function tests and the chest CT scan will be performed within a two-week period of the bronchoscopy.

- Complete physical examination
- CD4+ T cell count – Group 1 only
- HIV RNA level – Group 1 only
- Upper respiratory tract sampling
- Bronchoscopy
- ECG
- Pulmonary function tests
- Chest CT scan
- Blood for storage
- Urine pregnancy test

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6 Statistical Plan

6.1 Sample Size Determination

Sample size estimates were derived from calculations of the mean differences in UniFrac principal coordinates that can be detected in term of the sample standard deviation (SD) for each sample size combination for alpha level of 0.05 and power of 0.80. Table 2 outlines sample sizes required to detect differences across multiple groups. For the smallest sample sizes 6 vs. 6, we can detect a difference in means of the principal coordinates of 1.68 SD. Table 3 outlines the sample sizes required to detect differences within groups. For paired comparisons with 6 subjects in each group, we will have 80% power to detect a mean difference in principal coordinates of 1.43 SD at two different time points assuming a correlation of 0.5 among the pairs (Table 3).

Sample sizes	Detectable difference
12 vs 10	1.25 SD
12 vs 20	1.05 SD
6 vs 10	1.54 SD
6 vs 6	1.78 SD
10 vs 10	1.33 SD
10 vs 20	1.12 SD
12 vs 12	1.20 SD

Table 2. Detectable differences in means of the principal coordinates in term of sample standard deviation (SD) for various sample size combinations based on two-sample t-tests for alpha level of 0.05 & power of 0.80.

6.2 Statistical Methods

Microbiome populations will be analyzed using UniFrac principal coordinates analysis. They will be compared in terms of principal coordinates for microbiome compositions and also the proportions of particular lineages using two sample Wilcoxon rank-sum tests between two groups and nonparametric ANOVA among multiple groups with permutations to assess the statistical significance. Richness and diversity will be estimated for each community using the Chao, Ace and Shannon Indices will be compared by similar tests. For longitudinal measurements, we will perform pair t-test and repeated measurement ANOVA to test changes in microbiomes summarized as the first several principal coordinates. We will also perform exploratory analysis to cluster the microbiome populations based on principal coordinates in order to identify commonalities in microbiome composition in each niche among subjects within each group.

Sample sizes	Detectable difference
6 vs 6	1.43 SD
10 vs 10	1.00 SD
12 vs 12	0.90 SD

Table 3. Detectable differences in means of principal coordinates in term of sample standard deviation (SD) for various sample size combinations based on paired t-tests for alpha level of 0.05 & power of 0.80 assuming correlation of 0.5 among pairs.

Associating measurements of microbiome composition to multiple types of clinical data (Table 4 & 5, below) will require multidimensional data analysis. We have considerable experience with these approaches; in addition, we expect that further methods development will be ongoing throughout the lifetime of this award and our HMP project.

Table 4. Binary variables (yes/no)
HIV infected COPD
Smoker ART-suppressed

Table 5. Continuous variables (absolute level and change over time)
Level of HIV immunosuppression: CD4 count Lung inflammation (BAL): total cells; % neutrophils; % lymphocytes; CD4/CD8 COPD (lung function): FEV1 % predicted; FEV1/VC Emphysema (lung function & imaging): DLCO; CT emphysema score

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The phylogenetic-based method as implemented in UniFrac^{90, 91} is our main statistical and computational tool for comparing 16S microbiome data among different groups (eg; HIV-infected/suppressed vs non-suppressed groups, and HIV+ vs HIV- individuals in Aim 1; between microbiome measurements over time in Aim 2; or between groups with COPD vs without, and between smokers vs non-smokers in Aims 3 & 4). UniFrac measures the similarity among the microbiome community based on phylogenetic distances determined by the 16S rRNA gene sequences of different bacteria (UniFrac is also described in Preliminary Data). Results of metagenomic analysis will be summarized as Principal Components analyzed over the counts of SEED and KEGG gene types per sample. The significant principal components/coordinates can then be compared between two groups using the two sample t-tests or Wilcoxon rank sum tests or among multiple groups using ANOVA or Kruskal-Wallis nonparametric ANOVA.

For comparing microbiomes measured over time, we will use paired-t tests or repeated measurement ANOVA to account for the correlations of microbiome measurements over time. Realizing that the principal coordinates might not follow the required normal distributions, permutations will be used to obtain the p-values by permuting the sample labels. Alternatively, we can compare several principal coordinates simultaneously by performing nonparametric permutation test for association between the two groups and the microbiome compositions. Specifically, we can randomize the labels of the groups and compare all distances between points that both come from the same group to all distances between points from different groups using t-test. In such a permutation test, we can obtain a nonparametric distribution of the t statistic that takes into account the correlations introduced by the pair-wise distance matrix structures. Besides these formal tests, exploratory analysis, especially clustering analysis, will be employed to cluster the samples based on these principal coordinates in order to identify samples that have similar microbiome populations. These basic analyses will be mainly performed using the R package (<http://cran.r-project.org/>).

In another approach, we will survey collections of clinical variables, such as descriptors of lung inflammation, lung function, smoking, CD4 count, etc to determine which best predict the value of a continuous descriptor of microbiome composition, such as the first few principal coordinates describing 16S data or the principal components describing metagenomic data. Comparisons between single continuous clinical variables and microbiome descriptors can be analyzed by simple linear regression analysis for one coordinate or multivariate regression when multiple principal coordinates are considered. Analysis over many clinical variables simultaneously will be carried out using multiple linear models or machine learning methods such as classification and regression tree methods or random forests to identify those clinical parameters that best predict microbiome composition^{121, 122}.

We are also interested in the frequency of selected taxa. As an alternative method to summarize the microbiome, we will compute the proportion of the microbiome community represented by different taxa, such that a given taxa has a value ranging from 0% to 100%. The comparisons of these proportions can be done using the Fisher's exact tests. Relating the frequency of selected taxa in different microbiome populations to various clinical variables can be performed by multiple logistic regression analysis.

6.3 Subject Population(s) for Analysis

6.3.1 Objective: Define the lower and upper respiratory tract microbiome in HIV-infected and healthy HIV-negative individuals

The first goal here is to generate high quality data sets providing comprehensive and unbiased characterization of the lower and upper respiratory microbiome of HIV-infected and healthy individuals in this previously unexplored area (ie; reference data sets). We will also assess microbiome stability over time, an unknown but potentially critical factor for interpretation of such data, focused on upper respiratory tract because of easier accessibility. This study will also address the relationship between upper and lower respiratory microbiomes. The second goal is

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to compare and contrast the respiratory tract microbiome in HIV-infected subjects at different levels of immune deficiency, with and without viral suppression, with healthy HIV-negative individuals (cross-sectional analysis). This will focus on both microbiome diversity and composition.

Cross-sectional data on lower and upper respiratory tract microbiomes (BAL & PSB; OP & NP):

- *HIV-infected, not suppressed (Group 1A and 1B; N=24)*
- *HIV-infected, suppressed (Group 2B; N=10)*
- *Healthy individuals (Group 3B; N=20)*

Groups 1A (HIV, not suppressed, CD4 \geq 400) and 3B (healthy HIV-negative) will include both smokers and nonsmokers, which will be the focus of specific study in Aim 4.

Upper respiratory microbiome stability by OP & NP sampling on a monthly basis for one year:

- *Healthy individuals (Group 3B; N=6; nonsmokers)*
- *HIV-infected, not suppressed (Group 1A; N=6; CD4 \geq 400; nonsmokers)*

We will define microbiome populations in these groups to establish important reference data sets as to the unbiased culture-independent communities in each of the 4 interrelated niches in HIV-infected & healthy individuals. We will also ask the following specific questions: (a) are there commonalities in microbiome composition in each niche among subjects within groups? (b) are there consistent differences in the composition or diversity of the microbiome in a niche among subjects who are HIV-negative compared with those who are HIV-infected? (c) are there differences in the microbiome composition or diversity in HIV-infected subjects depending on degree of immunosuppression (continuous variable based on CD4 count) or with/without viral suppression? (d) how are microbiome populations within the 4 niches (2 upper and 2 lower respiratory) related? (e) are there stable or transient populations of the upper respiratory tract microbiome over a one year period?

6.3.2. Objective: Determine the effect on the respiratory system microbiome of progressive immune deficiency and ART-associated immune reconstitution.

We will examine how the microbiome changes over time as a function of immune status, both decline as disease progresses and improvement resulting from treatment (longitudinal study). Half of the non-suppressed infected individuals enrolled will have relatively high CD4 counts (\geq 400) who we expect will be followed for longer periods without need for ART. We expect that subjects with lower CD4 counts ($<$ 400) will begin ART shortly after enrollment, based on current practice. Microbiome evolution within each individual over time and upon ART initiation will be determined. While the primary focus here is on untreated infection & effect of ART initiation, we will also assess suppressed subjects on ART at entry & year 3, which will enable comparison of microbiomes at two time points in that group.

Longitudinal analysis with yearly sampling and immediately prior to and 4 months after ART initiation:

- *HIV-infected, not suppressed, CD4 \geq 400 (Group 1A; N=12) – delayed or no treatment*
- *HIV-infected, not suppressed, CD4 $<$ 400 (Group 1B; N=12) – early treatment*

Analysis at entry and at year 3:

- *HIV-infected, suppressed (Group 2B; N=10)*

Here we will ask: (a) how does the microbiome change over time in composition or complexity in each of the 4 niches within a subject as immune function declines? (b) how does the microbiome change in each niche upon ART initiation and viral suppression, and does it evolve either in composition or complexity towards a pattern seen in HIV-negative subjects?

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Microbiome populations will be analyzed using UniFrac analysis and will be compared by paired t-tests over two time points using the principal coordinates derived from the UniFrac sequences and by nonparametric repeated measurements ANOVA to compare the changes over time between early and delayed or no treatment. Again, permutations of sample labels will be used to assess the significance of these test statistics.

6.3.3 Objective: Define the respiratory system microbiome patterns in HIV-infected and uninfected individuals with COPD, compared with those without lung disease.

Individuals with COPD have frequent lower respiratory tract colonization by culture^{32, 41-46} and PCR evidence of Pc colonization^{37, 53, 56}, which are hypothesized to drive inflammation & lung injury. We anticipate that HIV infection also perturbs the respiratory microbiome, which we hypothesize is a mechanism of synergy contributing to accelerated COPD (particularly in combination with smoking, to be addressed in Aim 4). Our first goal here is to generate high quality reference data sets for comprehensive and unbiased characterization of the respiratory microbiome of individuals with COPD, both HIV-infected and uninfected, to complement studies in the literature based on standard microbiology^{32, 41-46}. Our second goal is to identify differences in respiratory tract microbiome populations in people with and without COPD, both HIV-infected and HIV-negative. Finally, we will determine how the respiratory microbiome is related to lung inflammation as assessed by BAL cell numbers and subsets, lung function as measured by PFTs, and emphysema as quantified by CT scanning.

Cross sectional analysis will be carried out on the following groups (most individuals with COPD in our HIV cohort are on ART, so we will focus on virologically suppressed subjects):

- *HIV-infected, suppressed, COPD (Group 2A; N=10)*
- *HIV-infected, suppressed, no lung disease (Group 2B; N=10)*
- *HIV negative, COPD (Group 3A; N=10)*
- *HIV negative, no lung disease (Group 3B; N=20)*

The first 3 groups above will also undergo microbiome sampling and PFT/CT at year 3 for longitudinal analysis.

In addition to microbiome sampling, BAL will be analyzed for total and differential cell count and lymphocyte CD3/CD4/CD8 populations (as we have done previously¹²³). Both smokers and individual with COPD have elevated BAL total cells, neutrophils, and CD8 lymphocyte populations compared to healthy nonsmokers¹²⁴⁻¹²⁷, and in some studies using conventional microbiological techniques, airway inflammatory markers were correlated with microbial colonization in these groups^{32, 41, 45, 128}. HIV infection is also associated with increased BAL inflammatory cells in approximately 60% of untreated individuals^{50, 129-132}, which decreases over time with effective ART^{123, 133}. This analysis will enable us to determine the relationship between lung inflammation and respiratory tract microbiome populations identified through unbiased deep sequencing approaches.

We will define the microbiomes in these groups to establish important reference data sets in each of the 4 interrelated niches in people with COPD, with and without HIV infection. We will also ask the following specific questions: (a) are there commonalities in composition of the respiratory tract microbiome among subjects with COPD compared to those without COPD? (b) do respiratory tract microbiome communities in individuals with COPD differ between those with and without HIV infection? (c) are lower respiratory tract microbiome patterns related to the degree of lung inflammation (based on BAL cells), dysfunction (PFT obstruction or diffusion capacity loss) or injury (emphysema by CT scan)? (d) are microbiome populations related to

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disease progression over time? Microbiome communities will be compared by Wilcoxon rank sum tests using the first several principal coordinates derived from the sequences data using UniFrac with permutations to assess their significance. Community composition will be compared using the proportions of specific organisms between two samples as detailed in Methods (section D.4.5.). Finally, this will also enable us to determine the relationship between lung disease in HIV-infected and uninfected subjects and the presence of novel or non-16S identifiable organisms carried out in the context of Aim 5 (discussed in that Aim).

6.3.4 Objective: Determine the effects of smoking and diet on respiratory system microbiome, and relationship between respiratory tract and gut.

We will address two environmental factors that we believe play important roles in the respiratory microbiome. Smoking is the principal inciting agent for COPD, and is associated with lower airway inflammation and colonization based on culture methods (as well as altered upper respiratory microbiota¹³⁴⁻¹³⁷). Given the very likely role of microbiome changes in mediating pathogenic effects of smoking in COPD, high prevalence of smoking in the HIV-infected population in the US³³⁻³⁶, and possible synergy between smoking and HIV infection, we believe this is critical to evaluate. Diet, on the other hand, clearly plays a major role in the gut microbiome (as we showed in rhesus macaques¹) but little is known about effects respiratory tract microbiota directly. However, it is clear from clinical studies that upper & lower respiratory tract colonization and infections are profoundly influenced by upper GI microbial populations¹³⁸⁻¹⁴¹. Therefore, by leveraging our Human Gut Microbiome project, we will determine if diet affects the respiratory microbiome. In addition, we will be able to address the important question of co-variation in microbiome populations in two distinct but potentially interrelated niches, the gut & respiratory tract. In the Gut study, healthy volunteers will be admitted to the Clinical and Translational Research Unit (CTRU) and placed on a defined high fat or low fat diet for 10 days, with serial analysis of fecal microflora and adherent colonic microflora obtained by colonoscopy and mucosal biopsy. Upper airway microbiome sampling will be taken upon admission (ad lib diet) and after 5 and 9 days of defined diet.

The following group will be studied in the smoking analysis (lower and upper respiratory sampling):

- *HIV-infected, not suppressed, CD4 \geq 400 (Group 1A; N=12) – 6 smokers and 6 nonsmokers*
- *HIV uninfected healthy controls (Group 3B; N=20) – 10 smokers and 10 nonsmokers*

The following group will be studied in the diet analysis (upper respiratory sampling plus gut sampling as part of the Gut Microbiome study; sampled prior to and during defined diet):

- *HIV uninfected controls (Group 3C; N=24) - ad lib diet followed by 12 on high fat & 12 on low fat diet*

We will ask the following specific questions: (a) how does the lower respiratory and upper respiratory tract microbiome of smokers differ from nonsmokers? (b) how does HIV infection modify the effect of smoking on the respiratory tract microbiome? (c) do changes in diet affect the upper respiratory tract microbiome? (d) are there shared patterns in microbiome changes among individuals placed on specific diets? (e) are there patterns in how the respiratory tract microbiome changes in response to diet that correlate with gut microbiome changes in response to diet? Microbiome populations will be compared between smokers and nonsmokers in HIV-infected but not suppressed groups and in HIV uninfected healthy controls using UniFrac analysis and two sample t-test or Wilcoxon rank-sum test using the first several principal coordinates with permutations to assess their statistical significance. Similarly the microbiome compositions will be assessed using UniFrac principal coordinate analysis and will be compared using two sample t-test or Wilcoxon rank-sum test using the first several principal coordinates

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with permutations to assess their statistical significance

7 Safety and Adverse Events

7.1 Definitions

Unanticipated Problems Involving Risk to Subjects or Others

Any incident, experience, or outcome that meets all of the following criteria:

- Unexpected in nature, severity, or frequency (i.e. not described in study-related documents such as the IRB-approved protocol or consent form, the investigators brochure, etc)
- Related or possibly related to participation in the research (i.e. possibly related means there is a reasonable possibility that the incident experience, or outcome may have been caused by the procedures involved in the research)
- Suggests that the research places subjects or others at greater risk of harm (including physical, psychological, economic, or social harm).

Adverse Event

An **adverse event** (AE) is any symptom, sign, illness or experience that develops or worsens in severity during the course of the study resulting from study procedures. Intercurrent illnesses or injuries will not be regarded as adverse events unless related to study procedures. Abnormal results of diagnostic procedures will not be considered to be adverse events unless the abnormality relates to complications of the study procedures.

Serious Adverse Event

Adverse events are classified as serious or non-serious. A **serious adverse event** is any AE that is:

- fatal
- life-threatening
- requires or prolongs hospital stay
- results in persistent or significant disability or incapacity
- a congenital anomaly or birth defect
- an important medical event

All adverse events that do not meet any of the criteria for serious will be regarded as **non-serious adverse events**.

Adverse Event Reporting Period

The study period during which adverse events must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up. For this study, the study treatment follow-up is defined as 30 days following the last administration of study procedures.

Preexisting Condition

A preexisting condition is one that is present at the start of the study. A preexisting condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

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General Physical Examination Findings

At screening, any clinically significant abnormality will be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event will also be recorded and documented as an adverse event.

Post-study Adverse Event

All unresolved adverse events will be followed by the investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit, the investigator will instruct each subject to report any subsequent event(s) that the subject, or the subject's personal physician, believes might reasonably be related to participation in this study. The investigator will notify the study sponsor of any death or adverse event occurring at any time after a subject has discontinued or terminated study participation that may reasonably be related to this study. The sponsor will also be notified if the investigator should become aware of the development of cancer or of a congenital anomaly in a subsequently conceived offspring of a subject that has participated in this study.

Abnormal Laboratory Values

A clinical laboratory abnormality will be documented as an adverse event if resulting from study procedures and any one of the following conditions is met:

- The laboratory abnormality is not otherwise refuted by a repeat test to confirm the abnormality
- The abnormality suggests a disease and/or organ toxicity
- The abnormality is of a degree that requires active management; e.g. change of dose, discontinuation of the drug, more frequent follow-up assessments, further diagnostic investigation, etc.

Hospitalization, Prolonged Hospitalization or Surgery

Any adverse event that results in hospitalization or prolonged hospitalization will be documented and reported as a serious adverse event if it results from any of the procedures performed during the course of the study. Any condition responsible for surgery will be documented as an adverse event if the condition meets the criteria for an adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an adverse event in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition. Surgery should **not** be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.
- Hospitalization or prolonged hospitalization required to allow efficacy measurement for the study.
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

7.2 Recording of Adverse Events

At each contact with the subject, the investigator must seek information on adverse events by specific questioning and, as appropriate, by examination. Information on all adverse events should be recorded immediately in the source document, and also in the appropriate adverse event module of the case report form (CRF). All clearly related signs, symptoms, and abnormal

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diagnostic procedures results should be recorded in the source document, though should be grouped under one diagnosis.

All adverse events occurring during the study period will be recorded. The clinical course of each event should be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. Serious adverse events that are still ongoing at the end of the study period must be followed up to determine the final outcome. Any serious adverse event that occurs after the study period and is considered to be possibly related to the study treatment or study participation should be recorded and reported immediately.

7.3 Reporting of Serious Adverse Events and Unanticipated Problems

Investigators and the protocol sponsor will conform to the adverse event reporting timelines, formats and requirements of the various entities to which they are responsible, but at a minimum those events that must be reported are those that are:

- related to study participation,
- unexpected, and
- serious or involve risks to subjects or others
(see definitions, section 8.1).

If the report is supplied as a narrative, the minimum necessary information to be provided at the time of the initial report includes:

- Study identifier
- Subject number
- A description of the event
- Date of onset
- Current status
- The reason why the event is classified as serious
- Investigator assessment of the association between the event and study treatment

7.3.1 Investigator reporting: notifying the study sponsor

Any study-related unanticipated problem posing risk of harm to subjects or others, and any type of serious adverse event, will be reported to the study sponsor by telephone within 24 hours of the event. To report such events, a Serious Adverse Event (SAE) form must be completed by the investigator and faxed to the study sponsor within 24 hours. The investigator will keep a copy of this SAE form on file at the study site. Report serious adverse events by phone and facsimile to:

Ian Frank, MD
Phone: 215-662-7419
Fax: 215-349-8011

In the event that Dr. Frank can not be contacted, SAEs will be reported to:

Ronald Collman, MD
Phone: 215-898-0913

Within the following 48 hours, the investigator will provide further information on the serious adverse event or the unanticipated problem in the form of a written narrative. This should include a copy of the completed Serious Adverse Event form, and any other diagnostic

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information that will assist the understanding of the event. Significant new information on ongoing serious adverse events should be provided promptly to the study sponsor

7.3.2 Investigator reporting: notifying the Penn IRB

This section describes the requirements for safety reporting by investigators who are Penn faculty, affiliated with a Penn research site, or otherwise responsible for safety reporting to the Penn IRB. The University of Pennsylvania IRB (Penn IRB) requires expedited reporting of those events related to study participation that are unforeseen and indicate that participants or others are at increased risk of harm. The Penn IRB will not acknowledge safety reports or bulk adverse event submissions that do not meet the criteria outlined below. The Penn IRB requires researchers to submit reports of the following problems within 10 working days from the time the investigator becomes aware of the event:

- Any adverse event (regardless of whether the event is serious or non-serious, on-site or off-site) that occurs any time during or after the research study, which in the opinion of the principal investigator is:

Unexpected (An event is “unexpected” when its specificity and severity are not accurately reflected in the protocol-related documents, such as the IRB-approved research protocol, any applicable investigator brochure, and the current IRB-approved informed consent document and other relevant sources of information, such as product labeling and package inserts.)

AND

Related to the research procedures (An event is “related to the research procedures” if in the opinion of the principal investigator or sponsor, the event was more likely than not to be caused by the research procedures.)

Reporting Process

Unanticipated problems posing risks to subjects or others as noted above will be reported to the Penn IRB using the form: “Unanticipated Problems Posing Risks to Subjects or Others Including Reportable Adverse Events” or as a written report of the event (including a description of the event with information regarding its fulfillment of the above criteria, follow-up/resolution and need for revision to consent form and/or other study documentation).

Copies of each report and documentation of IRB notification and receipt will be kept in the Clinical Investigator’s study file.

Reporting Deaths: more rapid reporting requirements

Concerning deaths that occur during the course of a research study, the following describes the more rapid reporting requirement of the Penn IRB for specific situations:

- Report the event within 24 hours when the death is unforeseen (unexpected) and indicates participants or others are at increased risk of harm.
- Report the event within 72 hours, for all other deaths, regardless of whether the death is related to study participation.

For reportable deaths, the initial submission to the Penn IRB may be made by contacting the IRB Director or Associate Director. The AE/Unanticipated Problem Form is required as a follow up to the initial submission.

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7.4 Medical Monitoring

It is the responsibility of the Principal Investigator to oversee the safety of the study at his/her site. This safety monitoring will include careful assessment and appropriate reporting of adverse events as noted above, as well as the construction and implementation of a site data and safety-monitoring plan (see section 9 Auditing, Monitoring and Inspecting). Medical monitoring will include a regular assessment of the number and type of serious adverse events.

8 Data Handling and Record Keeping

8.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the subject is alive) at the end of their scheduled study period.

8.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

8.3 Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. All data requested on the CRF must be recorded. All missing data must be explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, write "N/D". If the item is not applicable to the individual case, write "N/A". All entries should be printed legibly in black ink. If any entry error has been made, to correct such an error, draw a single straight line through the incorrect entry and enter the correct data above it. All such changes must be initialed and dated. DO NOT ERASE OR WHITE OUT ERRORS. For clarification of illegible or uncertain entries, print the clarification above the item, then initial and date it.

8.4 Records Retention

It is the investigator's responsibility to retain study essential documents for at least 2 years after the last approval of a marketing application in their country and until there are no pending or

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contemplated marketing applications in their country or at least 2 years have elapsed since the formal discontinuation of clinical development of the investigational product. These documents should be retained for a longer period if required by an agreement with the sponsor. In such an instance, it is the responsibility of the sponsor to inform the investigator/institution as to when these documents no longer need to be retained.

9 Study Monitoring, Auditing, and Inspecting

The investigator will permit study-related monitoring, audits, and inspections by the EC/IRB, the sponsor, government regulatory bodies, and University compliance and quality assurance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.). The investigator will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.).

Two independent safety officers, consisting of one faculty member from each of the infectious diseases and pulmonary divisions have been assigned the role of monitoring adverse events. The safety officers will receive quarterly reports of all adverse events. In addition, the officers will receive reports of serious adverse events within 72 hours of the investigators becoming aware of the event. The safety officers are empowered to temporarily halt the study while any safety concerns are investigated. Additionally, the safety officers will have the authority to review the study documents at any time and for any reason to assure the safety of the participants.

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable University compliance and quality assurance offices.

10 Ethical Considerations

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted independent Ethics Committee (EC) or Institutional Review Board (IRB), in agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the EC/IRB concerning the conduct of the study will be made in writing to the investigator and a copy of this decision will be provided to the sponsor before commencement of this study. The investigator should provide a list of EC/IRB members and their affiliate to the sponsor.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the EC/IRB for the study. The formal consent of a subject, using the EC/IRB-approved consent form, must be obtained before that subject undergoes any study procedure. The consent form must be signed by the subject or legally acceptable surrogate, and the investigator-designated research professional obtaining the consent.

11 Study Finances

11.1 Study Funding

This study is being funded by a grant from the National Institutes of Health.

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11.2 Conflict of Interest

Any investigator who has a conflict of interest with this study (patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, etc.) must have the conflict reviewed by a properly constituted Conflict of Interest Committee with a Committee-sanctioned conflict management plan that has been reviewed and approved by the study sponsor prior to participation in this study. All University of Pennsylvania investigators will follow the University conflict of interest policy.

11.3 Subject Stipends or Payments

Subjects will be compensated \$250 for each sampling of both upper and lower respiratory tracts, or \$25 for each sampling of only the upper respiratory tract, \$50 for having each chest CT scan, and \$50 for undergoing each pulmonary function testing.

12 Publication Plan

Neither the complete nor any part of the results of the study carried out under this protocol, nor any of the information provided by the sponsor for the purposes of performing the study, will be published or passed on to any third party without the consent of the study sponsor. Any investigator involved with this study is obligated to provide the sponsor with complete test results and all data derived from the study.

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4 Addendum

4.1 List of Protocol changes

4.1.1 Changes made in Protocol Version 7

- Footer: Updated protocol version number and date
- Pg 1: Change total enrollment goal to 110
- Pg 18: Description of group 3D
- Pg 23: Add Group 3D to chart
- Pg 24: Cotinine test to include all participants with the exception of Group 3C
- Pg 26: Single scope procedure for group 3D
- Pg 27: Removed PFT and chest Ct from 12mth and year 2 visits. It was in error that these 2 evaluations were in the protocol.

4.1.2 Changes made in Protocol Version 8

- In reviewing the protocol/consents a few procedures were omitted from the consent or timetable in the protocol. All group 1A participants that are actively participating in the study will be re-consented at their next visit.
- Modifications:
 - Footer: Updated protocol version number and date
 - Pg 21: Updated the timetable to include medical history and cotinine test collection at annual visits (depending on group).

4.1.3 Changes made in Protocol Version 9

- We are requesting a modification to the HIV(-) 1-scope consent form(Group D) because few of the participants in the HIV(-) 2-scope cohort were able to attend monthly visits. We would like to follow 6 HIV (-) non-smoking participants for 12 months from either cohort. At the monthly visit, we would collect samples identical to the other cohorts. This does not change the total number of participants being followed for monthly visits.
- Protocol changes:
 1. Updated version and date
 2. Pg 18 explanation of monthly visits, 23 updated table, 27 updated monthly visits

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4.1.4 Changes made in Protocol Version 10

- Updated version and date
- Pg 15: Inclusion of recruiting cigarette smokers in the 1B cohort
- Pg 17: Inclusion of recruiting cigarette smokers in the 3A cohort
- Pg 20: Inclusion of recruitment from locations other than the ABI

4.1.5 Changes made in Protocol Version 10 Received 5/1/2013

- COPD Inc criteria changed to use either FEV1/FVC <70% or FEV1/SVC <70%. This change will allow more of the screenfail potential participants to be eligible for the study.
- This applies to the following groups:
 - M012-C: Group 2A HIV+ on HIV Therapy, COPD/Emphysema
 - M012-E: Group 3A HIV-, COPD/Emphysema, Former Smokers/Current Non-Smokers

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4.3. APPENDIX 3: Center C005 - University of California at San Francisco

The Lung Microbiome in Cohorts of HIV-Infected Persons (Lung MicroCHIP) Studies M009, The Options Project (Options), M010, Observational Study of the Consequences of the Protease Inhibitor Era (Scope), M011, The International HIV-Associated Opportunistic Pneumonias Study (IHOP)

Options Study Web Site: <http://labs.ucsf.edu/options/>



**The Lung Microbiome in Cohorts of HIV-Infected Persons (Lung MicroCHIP) of the Lung HIV Microbiome Project (LHMP)
University of California, San Francisco**

Prepared by the

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Abbreviations Used

AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral Therapy
BAL	Bronchoalveolar Lavage
CHIP	Cohorts of HIV-Infected Persons
CD4	Cluster Differentiation
DSMB	Data Safety Monitoring Board
GWU	The George Washington University
HIV	Human Immunodeficiency Virus
IHOP	International HIV-associated Opportunistic Pneumonias
NHLBI	National Heart, Lung, and Blood Institute
NIH	National Institutes of Health
OI	Opportunistic Infection
OSMB	Observational Safety Monitoring Board
SCOPE	Study of the Consequences of Protease-inhibitor Era
UCSF	University of California, San Francisco

1. INTRODUCTION

The Lung Microbiome in Cohorts of HIV-Infected Persons (Lung MicroCHIP) is an interventional cohort study that will follow participants for up to two years at the University of California, San Francisco, San Francisco General Hospital in San Francisco, United States and Mulago Hospital in Kampala, Uganda.

1.1. Parent Study Abstract

The Options, SCOPE, and IHOP studies each focus on a different stage of HIV/AIDS. Combined, the cohorts span the spectrum of HIV infection and disease and provide an unparalleled opportunity to study the lung microbiome at all stages of HIV/AIDS.

1.1.1. Options

The Options study is an ongoing longitudinal cohort study of adults with acute HIV infection (defined as within one month of HIV infection), early HIV infection (within 6 months of HIV infection), and HIV-uninfected controls (present with similar risk factors for HIV and signs/symptoms suggestive of acute HIV but turn out to be HIV-negative).

1.1.2. SCOPE

The SCOPE study is an ongoing longitudinal cohort study of HIV-infected adults spanning the entire spectrum of HIV/AIDS. It includes participants who are long-term non-progressors (individuals who are HIV-infected but whose infection does not progress to AIDS), participants who are antiretroviral naïve (never received antiretroviral therapy), participants who have received ART but are currently untreated, participants who are receiving ART and have a detectable viral load, and participants who are receiving ART and have an undetectable viral load.

1.1.3. IHOP

The IHOP study is an ongoing longitudinal cohort study of HIV-infected adults admitted to San Francisco General Hospital and Mulago Hospital with acute pneumonia.

1.2. Specific Aims

This study has five specific aims:

1. To compare the lung microbiome in participants with and without HIV infection.
2. To determine whether the degree of HIV-mediated immunosuppression is related to the lung microbiome of HIV-infected participants without acute illness or pneumonia.
3. To determine the effect of initiation of antiretroviral therapy and opportunistic pneumonia prophylaxis on the lung microbiome of HIV-infected participants over time.
4. To determine the effects of opportunistic pneumonia and accompanying pneumonia treatment on the lung microbiome of HIV-infected participants over time.
5. To correlate lung microbiome composition and function with HIV-associated morbidity and mortality.

2. OVERVIEW, HYPOTHESIS, METHODS AND STUDY DESIGN

2.1. Overview

Opportunistic pneumonias remain a leading cause of illness in HIV-infected persons. There is increasing evidence that respiratory infections are caused by a complex polymicrobial community and changes within the community rather than simply by a single microbial species. The technology to study these communities exists and will be used in this study. The results will provide an important foundation towards understanding the lung microbiome in HIV-infected persons and other immunocompromised hosts.

2.2. Primary Hypothesis

The microbiome of the lung is shaped by HIV infection, the degree of HIV-mediated immunosuppression, and the use of antiretroviral and antimicrobial therapies. The microbiome of the lung changes between periods of health and pneumonia, and the presence of specific microbes or microbial consortia within the lung is associated with the development of HIV-associated pulmonary complications and mortality.

2.3. Methods and Study Design

2.3.1. Design Summary

This is an interventional longitudinal cohort study, where we will analyze specimens and clinical data cross-sectionally and longitudinally, with the primary study aims of comparing the lung microbiome of HIV-infected and HIV-uninfected participants, evaluating whether the degree of HIV-mediated immunosuppression is related to the lung microbiome of HIV-infected participants without acute illness or pneumonia, determining the effect of initiation of ART and opportunistic pneumonia prophylaxis on the lung microbiome of HIV-infected participants over time, determining the effects of opportunistic pneumonia and accompanying pneumonia treatment on the lung microbiome of HIV-infected participants over time, and correlation of lung microbiome composition and function with HIV-associated morbidity and mortality.

2.3.1.1. Total Sample Size

The total sample size for the project is 228, as totaled from the groups described below. The groups will be created using participants from three established cohorts of HIV-infected and HIV-uninfected individuals (Options, SCOPE, IHOP). Participants will be enrolled at San Francisco General Hospital in San Francisco, California, United States or at Mulago Hospital in Kampala, Uganda.

2.3.1.1.1. Aim 1, Options Cohort

Aim 1 participants will be recruited from the Options cohort at the time of enrollment into Options, and will participate in a cross-sectional study. There are two subsets of participants in Aim 1. The HIV-infected group (n=21) will have acute or early HIV infection. The HIV-uninfected group (n=21) will have risk factors and symptoms/signs suggestive of acute HIV, but will have been found HIV-negative upon testing.

2.3.1.1.2. Aim 2, SCOPE Cohort

Aim 2 participants will be recruited from the SCOPE cohort, and will participate in a cross-sectional study. These participants will be HIV-infected (n=86) and will be enrolled across the spectrum of CD4 cell counts. A minimum of ten participants will be enrolled in each of the following CD4 strata: >500 cells/ μ L, 401-500 cells/ μ L, 301-400 cells/ μ L, 201-300 cells/ μ L, 101-200 cells/ μ L, and \leq 100 cells/ μ L. Overall, there will be two subsets of participants in Aim 2. Participants with CD4 >200 cells/ μ L are expected to comprise 50% and participants with CD4 \leq 200 cells/ μ L are expected to comprise 50%.

2.3.1.1.3. Aim 3, Options and SCOPE Cohorts

Aim 3 participants will be recruited from the pool of participants established for Aims 1 and 2 and will be followed longitudinally over a two year period. There are two subsets of participants in Aim 3. Those participants recruited from the Options cohort will not initially be on ART at the time of enrollment, but will initiate ART within 6 weeks of enrollment and remain on this therapy for 2 years. Those participants recruited from the SCOPE cohort will not initially be on ART at the time of enrollment, but will initiate ART within the first year and remain on this therapy for 2 years. Those participants whose CD4 cell counts fall below specific thresholds will also initiate opportunistic infection (OI) prophylaxis.

2.3.1.1.4. Aim 4, IHOP Cohort

Aim 4 participants will be recruited from the IHOP cohort, will be HIV-infected and will have recovered from acute pneumonia. There will be two subsets of participants in Aim 4: those from San Francisco General Hospital in San Francisco, California (n=50) and those from Mulago Hospital in Kampala, Uganda (n=50). These participants will be followed longitudinally over a one year period.

2.3.1.1.5. Aim 5, Lung MicroCHIP Cohort

Aim 5 participants will be recruited from those in Aims 1-4 and will be followed observationally over a two year period. Any participant in Aim 1-4 that has undergone a bronchoscopy will be eligible for this group.

2.3.2. Eligibility Criteria

2.3.2.1. Inclusion Criteria

General Inclusion Criteria (for all groups in Aims 1-5)

- Participation in Options, SCOPE, and/or IHOP
- Willingness to undergo bronchoscopy within 4 weeks of enrollment
- Absence of acute respiratory illness/pneumonia (except for acute HIV infection)

Aim 1: Additional Inclusion Criteria

- No current use of antiretroviral or antimicrobial therapy

Aim 2: Additional Inclusion Criteria

- None

Aim 3: Additional Inclusion Criteria

- No current use of antiretroviral or antimicrobial therapy
- Participant must initiate and remain on antiretroviral therapy and/or OI prophylaxis at 6 months, 1 year, and 2 years

Aim 4: Additional Inclusion Criteria

- No current use of antiretroviral or antimicrobial therapy
- Recovered from acute pneumonia and remain well for one year

Aim 5 Additional Inclusion Criteria

- All participants from Aims 1-4 who undergo a bronchoscopy

2.3.2.2. Exclusion Criteria

For participants in groups established for Aims 1-5, the only exclusion criterion is a medical contraindication to bronchoscopy.

2.3.3. Informed Consent Criteria

Informed consents for an NHLBI funded study must include the following required elements. Sample language has been developed but is not required.

- A statement that allows for broad research use of specimens including research on lung disease, HIV, and other related illnesses. The statement must also specify that the participant will not receive results of any future research.
- A description of the NHLBI repository and who will have access to the specimens there.

- A list of who will have access to specimens, medical, and research information that includes the site staff, NHLBI/NIH, and the data analysis and coordinating center (GWU).
- A description of how confidentiality is protected. This description includes data will be stored only in locked cabinets or on secured computers.
- A statement that specimens will be stored indefinitely.
- A statement that the participant has a right to change their mind at any time and that every effort will be made to destroy their samples but it may not be possible once samples are de-identified.
- A description of the risk of the bronchoscopy including death.

2.3.3.1 Stored Sample(s)

The informed consent must include a description of the risk of stored specimens and future testing such as paternity and other genetic information.

2.3.4. Secondary/Ancillary Research Questions

Additional goals that this study hopes to achieve include: comparison of the upper respiratory (oral cavity) and lower respiratory (lung) tracts; comparison of the microbiome of the lower respiratory tract to conventional microbiologic results; comparison of the microbiome of the lower respiratory tract in persons in San Francisco and Kampala; and to establish a specimen and clinical database for use in future studies.

3. CORE PROCEDURES

3.1. Introduction

The purpose of this study will be to identify, recruit, and enroll subjects who will undergo the specific research procedures outlined below. All study procedures will be monitored and audited by the Data Safety Monitoring Board.

3.2. Recruitment Strategies

Participants will be recruited without regard to age, gender, or race/ethnicity. At the time of enrollment into Options, SCOPE, or IHOP, the Options, SCOPE, and IHOP study personnel will approach eligible participants regarding co-enrollment in Lung MicroCHIP. Lung MicroCHIP study personnel will then be provided with the names and contact information of interested participants. These participants will have a screening visit, at which time the study will be described and written, informed consent will be obtained.

3.3. Screening for Eligibility and Chart Abstraction

A bronchoscopy for clinical indications at San Francisco General Hospital, inpatients that are enrolled into the IHOP study and are having a bronchoscopy will also be enrolled into the Lung MicroCHIP study.

At time of enrollment into Options, subjects will be screened for eligibility for the Lung MicroCHIP study. If they fit criteria, they will be asked if they would like to participate in the Lung MicroCHIP study. The contact information of interested subjects will be given to the Lung MicroCHIP study team who will contact the subjects directly to set up an initial visit.

Individuals who participate in SCOPE will be screened for eligibility for the Lung MicroCHIP study. If they fit criteria, they will be asked if they would like to participate in the Lung MicroCHIP study. The contact information of interested subjects will be given to the Lung MicroCHIP study team who will contact the subjects directly to set up an initial visit.

At Mulago Hospital, patients who have had a cough for 2 or more weeks are eligible for enrollment. We will not review the medical records for the study participants at Mulago Hospital.

3.4. Quality Assurance

Standardized and optimized protocols will be developed for sample collection, handling, and shipping. Extensive staff training will be done at sample collection sites. Prior to shipping,

samples will have documented inspections. Analytically, standardized arrays will be implemented.

3.5. Human Subjects

3.5.1. Data/Observational Safety Monitoring Board

The Lung HIV Microbiome study has a Lung HIV Microbiome DSMB/OSMB to monitor all sites. The DSMB/OSMB committee formally meets twice a year, has regular conference calls, and is available on an ad hoc basis to review urgent matters.

3.6. Monitoring for Adverse Events

Any adverse events/serious adverse events will be reported to the UCSF CHR as per UCSF guidelines. A monthly conference call will take place on the third Wednesday of every month with the coordinating center. Enrollment and any adverse events will be discussed.

4. STANDARD CLINICAL CENTER PROCEDURES

4.1. Overview

At enrollment into Lung MicroCHIP, participants will complete a questionnaire that will collect information on patient demographics, medical history, cigarette smoking, and current medications. The questionnaires will be provided in the predominant languages of the HIV-infected population at each site (English in San Francisco; English and Luganda in Kampala), and will be administered by research personnel fluent in those languages. In addition, at enrollment participants will undergo a complete medical history, physical examination, and selected laboratory testing to assess suitability for bronchoscopy. For all specific aims, participants will receive a comprehensive physical examination before bronchoscopy, and we will collect oral (tongue scraping, oral wash, gingival plaque), BAL, protected brush, and blood specimens at study enrollment. For specific Aims 3 and 4 (longitudinal studies), collection of oral, BAL, protected brush, and blood specimens will be repeated at the specified follow-up visits (3, 6, 12, and/or 24 months).

	Study Enrollment	3 months	6 months	12 months	24 months
Aim 1: Options Cohort					
HIV-infected	X				
HIV-uninfected	X				
Aim 2: SCOPE Cohort					
CD4 > 200 cells/ μ L	X				
CD4 < 200 cells/ μ L	X				
Aim 3: Options & SCOPE Cohorts					
Start Antiretrovirals	X		X	X	X
Start OI Prophylaxis	X		X	X	X
Aim 4: IHOP Cohort					
San Francisco Cohort	X	X		X	
Kampala Cohort	X	X		X	
Aim 5: Lung MicroCHIP Cohort (Observational Aim)					

4.2. Forms

Dr. Laurence Huang and his colleagues will interview the study participants and if there is information that the participant cannot recall (e.g. past date of diagnosis, past antibiotic use), the researchers will review the participant's medical record for the information.

4.3. Laboratory Specimen Collection

4.3.1. Bronchoscopy with BAL and Protected Brush

Bronchoscopy will be performed according to standard procedures, after tongue scraping and oral wash have been performed. Bronchoalveolar lavage will be performed in a subsegment of the right middle lobe by instilling five 50 mL aliquots of 0.9% sterile saline and removal by suction. Protected brush specimens will be obtained from the right upper or middle lobes using a sterile cytology brush to sample airway epithelium. Protected brush sampling will be repeated up to four times.

4.3.2. Oral and Dental Examination

The oral and dental exam will include inspection of oral soft tissues (tongue, palate, all mucosal surfaces) and teeth (confirm presence/absence, un-restored carious lesions, and dental restorations). The periodontal exam will include Silness & Løe Plaque Index, probing depth, clinical attachment level, presence/absence of bleeding on probing, and collection of supra- and sub-gingival plaque samples.

4.3.3. Tongue Scraping

The dorsal surface of the tongue will be scraped using a clean tongue scraper (Breath Rx tongue scraper) to collect microbial and host cells. The procedure will be repeated approximately two to three times until there is no further accumulation of cellular debris. The collection will be placed into a sterile specimen cup with 10 mL sterile 0.9% Sodium Chloride.

4.3.4. Oral Wash

Following tongue scraping subjects will gargle and “swish” 10 mL sterile 0.9% sterile Sodium Chloride for 60 seconds. The sample will be collected and combined with tongue scraping into a sterile specimen cup.

4.3.5. Blood

Two tubes of blood specimens (16-20 mL) will be obtained by venipuncture. Serum specimen will be processed using the same lab procedures as the IHOP study. The specimen will be centrifuged at 1400G for 10 minutes; the serum will be aliquoted into 1-mL cryovials, labeled and stored at -80°C.

5. OUTCOMES EVALUATION

5.1. Primary Outcome

The primary outcome will be the specific operational taxonomic units identified by the PhyloChip, MycoChip and ViroChip.

5.2. Other Outcomes

The other outcomes will be predictors of morbidity and mortality.

6. DATA COLLECTION AND MANAGEMENT

6.1. Site Specific Data Collection Forms

The Lung HIV Microbiome Program forms (the consent, demographics, pulmonary questionnaire, diagnosis, and BAL form) will be used for all San Francisco cohorts (Options, SCOPE and IHOP). The demographics, pulmonary questionnaire, and diagnosis form will be administered by an interviewer during the pre-bronchoscopy screening visit. The consent form will be filled out by one of the researchers and the BAL form will be administered by the physician performing the bronchoscopy and by the laboratory technician processing the BAL and brushed cells. The information will be stored on the MIDAS database. In San Francisco, we will also fill out our own questionnaire which includes questions about past antibiotic use, CD4 count, viral load and the results and outcomes of the bronchoscopy. The information will be collected before and after the bronchoscopy and will be stored on a RedCap database. The

questionnaire that we will administer before, during and after the bronchoscopy in Kampala, Uganda will include clinical and demographic variables and the information will be stored on our server.

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8. Addendum

8.1. List of changes to UCSF Center Protocol

- Formatting changes through out
- Page iii: CD4 stands for Cluster Differentiation
- Page 1 & 2: Changed “Non-interventional” to “interventional”
- Page 2: Section 2.3.1.1. Please add United States to San Francisco, California.
- Page 4: Section 3.3. First sentence should read “...a bronchoscopy for clinical indications...”
- Page 5: Do not offer questionnaires in Spanish in San Francisco
- Page 5: Section 4.1 specified specific Aims 3 and 4
- Page 6: Section 4.3.1 Protected brush sampling is repeated four times
- Page 6: Section 6.1 Storage of all LHMP forms on the MIDAS database, not Epi Info. Additional clarification of data collection for site specific data collection forms and storage.

4.4. APPENDIX 4: Center C006 - University of Colorado, Denver

4.4.1. Studies M003 – Alterations in Lung Microbiome in Acute and Chronic HIV Infection and M014 Longitudinal Studies of HIV-1 Nef and Pulmonary Arterial Hypertension



Alterations in Lung Microbiome in Acute and Chronic HIV Infection of the Lung HIV Microbiome Project (LHMP)

**Longitudinal Studies of HIV-1 Nef and Pulmonary Arterial Hypertension
University of Colorado, Denver**

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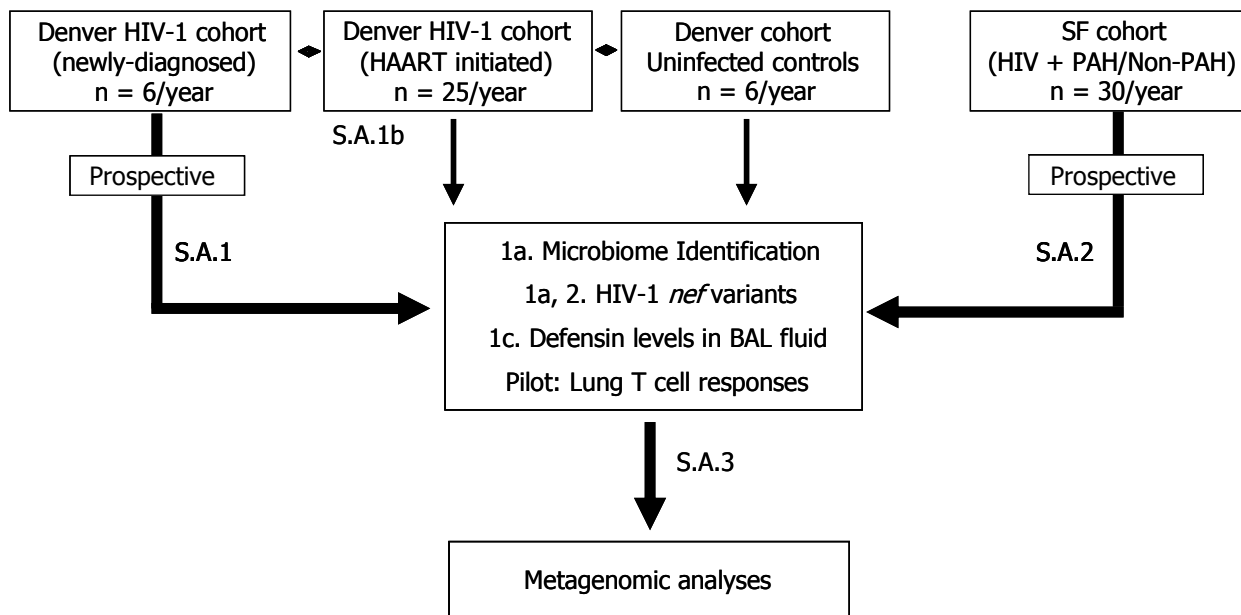
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Abbreviations Used

ARV	Antiretroviral
BAL	Bronchoalveolar Lavage
CBC	Complete Blood Count
DNA	Deoxyribonucleic Acid
DSMB	Data Safety Monitoring Board
EIA	Enzyme Immunoassay
ELISA	Enzyme-Linked Immunoabsorbent Assay
GWU	The George Washington University
HAART	Highly Active Antiretroviral Therapy
HBV	Hepatitis B Virus
HIV or HIV-1	Human Immunodeficiency Virus
IRB	Internal Review Board
LHMP	Lung HIV Microbiome Project
NHLBI	National Heart, Lung, and Blood Institute
NIH	National Institutes of Health
PAH	Pulmonary Arterial Hypertension
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PEP	Post-Exposure Prophylaxis
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
S.A.	Specific Aim
ZDV	Zidoduvine (also Azidothymine)

1. INTRODUCTION

The Alterations in Lung Microbiome in Acute and Chronic HIV Infection project (the project) consists of two prospective cohorts recruited at the University of Colorado Denver and the University of California San Francisco. The Denver cohort will be seen twice in 48 weeks. The two cohorts will be compared to determine alterations in the lung microbiome through different phases of HIV infection.



1.1. Parent Study Abstract

N/A

1.2. Specific Aims

The project has the following three specific aims:

1. Determine alterations in the lung microbiome and innate immune defensins in HIV-1-infected patients compared to age-matched HIV-1 seronegative control subjects.
2. Determine whether immune-modulating allelic variants of HIV-1 Nef are associated with chronic immune activation and alteration of the lung microbiome of HIV-1-infected patients with and without PAH. Through our currently funded “Longitudinal studies of HIV-associated lung infections and complications” study, we have access to longitudinal blood samples as well as BAL fluid and cells from patients with HIV-related PAH (HRPAH).
 - a. **Aim 2a.** Determine the microbiota in the lungs of chronically infected HIV-1 patients with and without PAH.
 - b. **Aim 2b.** Correlate specific bacteria and HIV-1 genomic variants identified in Aim #2a with markers of inflammation in chronically infected HIV-1 patients with and without PAH
3. Once bacterial subpopulations of interest have been identified, metagenomic studies will be performed to quantify the bacterial lineages, genes, and encoded functional properties associated with lung complications of HIV-1 infection.

2. OVERVIEW, HYPOTHESIS, METHODS AND STUDY DESIGN

2.1. Overview

Whether host defense is capable of maintaining a sterile lower airway microenvironment or whether a microbiota exists in the “undiseased” human lung remain important unanswered questions. The lung has been recognized as one of the main targets of infectious and non-infectious complications of human immunodeficiency virus type 1 (HIV-1) infection. Despite the introduction of highly active antiretroviral therapy, HIV-1 continues to persist in reservoir sites. Defects in adaptive and innate immune responses increase the risk for the development of pneumonias caused by both pathogenic and opportunistic microorganisms in HIV-1 infected individuals. These infections may increase the rate of HIV-1 replication and accelerate the course of HIV-1. Through our funded “Longitudinal Studies of HIV-Associated Lung Infections and Complications” program, we initiated a systematic analysis of HIV-1 *nef* sequences in the lung and blood using high-throughput sequencing platforms. These studies revealed that HIV-1 genomic variants containing *nef* alleles with mutations in functional domains may be associated with the pathogenesis of HIV-1-induced chronic lung diseases (e.g., pulmonary arterial hypertension (PAH, which has a prevalence of 2,000-fold higher in the HIV vs uninfected population). With the advent of even more highly advanced high-throughput sequencing technology, new data sets of sufficient quality and depth will be generated that will allow inferences to be made as to how progression of HIV-1 disease influences the lung microbiota and affects the development of HIV related acute and chronic lung complications.

2.2. Primary Hypothesis

Immunodeficiency associated with HIV-1 infection leads to a broadening of the lung microbiome and that the inflammatory response to increased microbiota and HIV-1 variants will result in non-infectious chronic lung complications **such as pulmonary arterial hypertension (PAH).**

2.3. Methods and Study Design

2.3.1. Design Summary

This is a prospective, observational cohort study. Two cohorts (A and B) will be enrolled in parallel. (See [Section 8 - Addendum – Revisions to Protocol](#) for the revised list of Cohorts)

Cohort A

Cohort A1 will consist of 18 subjects with primary HIV-1 infection (including acutely and recently infected individuals as defined below). Cohort A2 will consist of 18 HIV-1 seronegative healthy sex, smoking and age matched controls. At the time of study entry and after 12 months, 50 mL of blood will be obtained from each subject. At each time point, HIV-1 viral load and CD4⁺ T cell count will be determined, blood will be collected for cytokine and flow cytometry assays, bronchoscopy with BAL and protected brush will be performed in the right upper, middle, and lower lobes for a total of three brushes and an oral or nasal swab will be obtained.

Cohort B

Cohort B will consist of 75 subjects who have been recommended to initiate HAART by their health care provider. 24 to 48 hours prior to the initiation of HAART blood will be collected for CD4⁺ T cell count, plasma HIV-1 RNA, flow cytometry, cytokine measurements and bronchoscopy will be performed. Repeat evaluations will be performed as soon as possible after two consecutive plasma HIV-1 RNA measurements are documented to be below the limits of detection (<50 copies/mL). The study will not provide antiretroviral drugs. Antiretroviral drugs will be prescribed by the patient’s health care provider and obtained through local treatment programs.

After approximately 24 weeks, a repeat HIV-1 viral load will be performed to document a treatment response (defined as <50 copies of HIV-1 RNA/mL). It is estimated that >90% of

HAART-treated HIV-1-infected subjects will have a clinical response to HAART. Those subjects with documented viral suppression will undergo a second blood draw and bronchoscopy with BAL and brushing. The samples will be processed and handled as described above allowing a comparison of the bacterial microbiome in the lung before and after the suppression of HIV-1 replication.

Cohort for the Longitudinal Studies of HIV-1 Nef and Pulmonary Arterial Hypertension

The cohort for the Longitudinal Studies of HIV-1 Nef and Pulmonary Arterial Hypertension (Study code M014) consists of chronically infected individuals from San Francisco, after diagnosis of HRPAAH by right heart catheterization or at risk of developing HRPAAH by serial echocardiographic screens, and who are being prospectively followed.(as part of Dr. Flores' funded "Longitudinal Studies of HIV-Associated Lung Infections and Complications" R01).

2.3.1.1. Total Sample Size

The project will have a total sample size of 111. The Denver cohort will recruit 37 participants each year: 6 who newly diagnosed HIV positive, 25 who are HIV positive and HAART initiated, and 6 who are HIV negative controls. The San Francisco cohort will recruit 30 participants each year who are HIV positive, both normotensive, and hypertensive as established through echocardiographs or right heart catheterizations. Acellular BAL fluid and cells from UCSF will be processed for DNA extraction.

2.3.2. Eligibility Criteria

2.3.2.1. Inclusion Criteria

- 18-75 years old
- Newly diagnosed (acutely or recently infected) or ready to initiate HAART
 - Acute HIV-1 infection defined as a high risk HIV exposure within the prior 30 days, detectable plasma HIV-1 RNA levels of at least 2,000 copies/ ml by RT-PCR, and either a negative ELISA, a positive ELISA but negative or indeterminate Western Blot but with a documented negative ELISA or plasma HIV-1 RNA level within the prior 30 days.
 - Recent HIV-1 seroconversion defined as those with a high risk HIV-1 exposure in the past four to six months and a positive ELISA and Western Blot but with either a documented negative ELISA or plasma HIV-1 RNA level within the prior 31-90 days or a non-reactive detuned ELISA (either a 3A11-LS or a IgG-Capture BED-EIA assay).
- Healthy controls: HIV-1 seronegative with no high-risk HIV-1 exposure in the prior 30 days and no chronic medical illness.
- ARV drug-naïve (defined as ≤ 10 days of ART at any time prior to entry*).
 - For the UCSF PAH cohort, patients will have undergone either echocardiographs or right heart catheterizations; individuals with PASP > 30 mmHg, or mPAP > 25 mmHg will be enrolled as hypertensive. Individuals with PASP < 20 mmHg will be enrolled as normotensives. Individuals with known non-infectious causes of pulmonary hypertension will be excluded from the studies.
 - The San Francisco cohort will consist of approximately 10-15 HIV-1-infected patients diagnosed with HRPAAH by heart catheterization and 15-20 HIV-1-infected normotensive controls enrolled in our Longitudinal studies grant. The median duration of HIV-1 infection in the San Francisco cohort is 15 years.

*The only exceptions are:

- Use of ARV drugs as part of post-exposure prophylaxis (PEP) provided the subject did not acquire HIV-1 infection from the event that required PEP.
- ART use during pregnancy that resulted in virologic suppression based on the assay available at the time and was not complicated while on therapy either by detectable HIV-1 RNA following suppression or the development of resistance. Women who received ZDV monotherapy prior to the availability of viral load testing will still be considered eligible as long as ZDV was not taken for more than 12 weeks. ART must have been stopped within 4 weeks of delivery and can not have been received within 6 months of screening.
- Therapy with an investigational ARV drug that was not an INI, NRTI, NNRTI, or PI. The total duration of this therapy could not have exceeded 24 weeks and cannot have been received within 6 months of screening.
- Receipt of ARV drugs while HIV-uninfected, with documentation of negative HIV-1 serology at least 90 days after completion of ARV drugs.

NOTE: Prior use of low dose adefovir for treatment of HBV is allowed.

2.3.2.2. Exclusion Criteria

- Positive pregnancy test (for women of childbearing age)
- Weight less than 110 lbs. (for venipuncture)
- Positive lung washing or biopsy cultures for fungi or mycobacterial disease
- Severe room air hypoxemia (precluding transbronchial lung biopsy and/or BAL), e.g., PaO₂ <45 mmHg (Denver altitude 5,280 feet)
- Antibiotics within 30 days of enrollment
- Patient inability to participate in the study, such as inability to undergo venipuncture and BAL procedures

2.3.3. Informed Consent Criteria

Informed consents for an NHLBI funded study must include the following required elements. Sample language has been developed but is not required.

- A statement that allows for broad research use of specimens including research on lung disease, HIV, and other related illnesses. The statement must also specify that the participant will not receive results of any future research.
- A description of the NHLBI repository and who will have access to the specimens there.
- A list of who will have access to specimens, medical, and research information that includes the site staff, NHLBI/NIH, and the data analysis and coordinating center (GWU).
- A description of how confidentiality is protected. This description includes that data will be stored only in locked cabinets or on secured computers.
- A statement that specimens will be stored indefinitely.
- A statement that the participant has a right to change their mind at any time and that every effort will be made to destroy their samples but it may not be possible once samples are de-identified.
- A description of the risk of the bronchoscopy including death.

For participants who only speak or read Spanish, a Spanish version of the informed consent document will be available. For these participants, investigators or study nurses who are fluent in English and Spanish will review the information in the consent form.

2.3.3.1. Stored Sample(s)

The informed consent must include a description of the risk of stored specimens and future testing such as paternity and other genetic information.

2.3.4. Secondary/Ancillary Research Questions

The project will also be looking at the effects of HIV-1 infection on bacterial and viral adaptive immune responses in the lung, specifically if the lung microbiome is in part limited by antigen-specific T cell responses and that immunodeficiency associated with HIV-1 infection will result in alterations of the microbiome in HIV-1-infected subjects. The goal is to delineate the effects of HIV-1 infection on the host adaptive immune response to lung microbes. This will be done by examining the frequency, function and coregulatory receptor expression patterns on HIV-1 Gag- and Nef- (HRPAH-associated mutants vs. wild type sequences) specific T cells in the lung and blood of subjects with primary and chronic HIV-1 infection and by analyzing T cell responses to bacterial antigens detected in the lung by 16s rDNA pyrosequencing analysis in primary and chronic HIV-1-infected subjects pre- and post-HAART.

3. CORE PROCEDURES

3.1. Introduction

Participants will first discuss the study with an investigator or research nurse in person. If willing to participate, informed consent will be obtained. It will be explained that the study will be of no benefit to them and if they decide not to participate their medical care will not be affected. Participants will be informed that they can withdraw from the study at any time.

3.2. Recruitment Strategies

All participants will be recruited from the regions surrounding the participating universities. Participants with primary and chronic HIV-1 infection will be recruited from the Infectious Disease Clinics at the University of Colorado Hospital and Denver Health Medical Center, and by IRB approved advertising. Individuals interested in the study will contact the investigator and an appointment will be set up where discussion and consent will occur.

3.3. Screening for Eligibility and Chart Abstraction

Referrals are received from various clinics in the Denver area, which are then followed up by phone to see if the participant might qualify for the study. If it seems like they might qualify, the participant comes into the clinic for a screening visit which includes consent reading and signing. A Release of Medical Information form is signed by the subject so records may be obtained and data abstracted.

3.4. Quality Assurance

One study coordinator is involved with the study who has been doing research for 17 years and has had extensive training in HIV research. Data undergoes quality assurance before it is entered into the data system. Equipment calibration and maintenance is covered by the University Facilities Department.

3.5. Human Subjects

3.5.1. Data/Observational Safety Monitoring Board

The Lung HIV DSMB will serve to monitor the overall conduct of the study. An internal DSMB made up of the investigators and study staff will hold bi-monthly telephone DSMB conferences.

3.6. Monitoring for Adverse Events

Dr. Campbell will review all clinical adverse events that are temporally related to phlebotomy or bronchoscopy performed in this study. An independent safety officer will be identified. Dr. Campbell will meet with the safety officer twice yearly to review enrollment, retention and adverse events.

Drs. Laurence Huang and Priscilla Hsue at UCSF will monitor and review all clinical adverse events that are temporally related to phlebotomy or bronchoscopy. An independent safety officer and an NHLBI-centered DSMB will oversee safety of these studies.

All adverse events and unanticipated problems reporting will be completed by the Principal Investigator according to the COMIRB requirements to maintain compliance with Federal and Institutional Policies. The Principal Investigator is responsible for the accurate documentation, investigation and follow-up of all possible study-related adverse events. Reportable events that are unexpected and have a reasonable possibility of relatedness to the study and/or procedure(s) will be submitted to the COMIRB within 5 working days of the event or knowledge of the event.

4. STANDARD CLINICAL CENTER PROCEDURES

4.1. Overview

Cohort A2 (HIV-1 seronegative, healthy controls)

Evaluations	Screening	Enrollment	~12 months
Informed Consent	X		
Medical/Medication History	X		
Vital Signs		X	X
Pulse oximetry		X	X
HIV-1 antibody	X		X
Storage of PBMC & plasma		X	X
Urine Pregnancy Test		X	X
Bronchoscopy		X	X
Listerine Oral Wash		X	X
Oral or Nasal swab		X	X
Stool specimen		X	X
Oral Wash		X	X

Cohort A1 (Acute or recent HIV-1 infection)

Evaluations	Screening	Enrollment	~12 months
Informed Consent	X		
Medical/Medication History	X		
Vital Signs		X	X
Pulse oximetry		X	X
HIV-1 viral load	X		X
CD4+ cell count	X		X
Storage of PBMC & plasma		X	X
Urine Pregnancy Test		X	X
Bronchoscopy		X	X
Listerine Oral Wash		X	X
Oral or Nasal swab		X	X
Stool specimen		X	X
Oral Wash		X	X

Cohort B (HIV-1 infection who are about to initiate antiretroviral therapy)

Evaluations	Screening	Enrollment (24-48 hours prior to initiation of HAART)	~24 weeks
Informed Consent	X		
Medical/Medication History	X		
Vital Signs		X	X
Pulse oximetry		X	X
HIV-1 viral load #1	X		X
HIV-1 viral load #2			X ¹
CD4+ cell count	X		X
Storage of PBMC & plasma		X	X ¹
Urine Pregnancy Test		X	X ¹
Bronchoscopy		X	X ¹
Listerine Oral Wash		X	X ¹
Oral or Nasal swab		X	X ¹
Stool specimen		X	X ¹
Oral Wash		X	X ¹

¹only if <50 copies of HIV-1 RNA/mL

Cohort C for M014 (HIV-1 infection with/without pulmonary hypertension from UCSF)			
Evaluations	Screening	Enrollment After suspicion of PAH	~24 weeks
Informed Consent	X	X	X
Medical/Medication History	X	X	X
Vital Signs	X	X	X
Pulse oximetry		X	X
HIV-1 viral load #1	X		X
HIV-1 viral load #2	X	X	X ¹
CD4+ cell count	X	X	X
Storage of PBMC & plasma	X	X	X ¹
Urine Pregnancy Test			
Bronchoscopy	X	X	X ¹
Listerine Oral Wash		X	X ¹

4.2. Timing of Evaluations

Cohort A1 and A2

During the screening visit, study subjects will sign the informed consent, be asked questions about their medical history, and obtain a container to be used for at home stool specimen collection (to be collected within 48 hours of study entry). At the time of screening, approximately 20 mL of blood will be drawn for HIV-1 viral load and CD4+ T cell count (or HIV-1 antibody if HIV negative).

At the time of enrollment, 50 mL of blood will be obtained from each subject for plasma and PBMC storage. At the 12 month visit, 70 mL of blood will be obtained from each subject.

In addition, an oral wash and bronchoscopy with BAL and protected brush will be performed on all subjects at enrollment and after 12 months. Pulse oximetry on room air at rest will be performed on all subjects prior to bronchoscopy. If pulse oximetry is less than 90%, bronchoscopy will not be performed.

Cohort B

During the screening visit, study subjects will sign the informed consent, be asked questions about their medical history, and obtain a container to be used for at home stool specimen collection (to be collected within 48 hours of study entry).

Initial bronchoscopy and other evaluations will be performed 24 to 48 hours prior to the initiation of HAART as recommended by the subject’s health care provider.

Pulse oximetry on room air at rest will be performed on all subjects prior to bronchoscopy. If pulse oximetry is less than 90%, bronchoscopy will not be performed.

At the time of screening, 20 mL of blood will be drawn for HIV-1 viral load and CD4+ T cell count. Bronchoscopy with BAL and protected brush, as well as a 50 ml blood draw will be performed at the time of enrollment.

After approximately 24 weeks, a repeat HIV-1 viral load will be performed to document a treatment response (defined as <50 copies of HIV-1 RNA/mL). Those subjects with documented viral suppression will undergo a second blood draw and bronchoscopy with BAL and brushing.

4.3. Forms

For specific case report forms, please see section 6.1.

4.4. Laboratory Specimen Collection

At each blood draw for Cohorts A and B, 20-70 mL of blood will be obtained from each subject. At screening and each subjects' third visit (if eligible), HIV-1 viral load and CD4+ T cell count will be determined. Each sample will be assigned a unique identification number. All samples (blood, tissue or BAL fluids) will be transported to the laboratory by laboratory personnel. All specimens will arrive at the laboratory with a Case Report Form that contains the subject identification number, visit date, time of sample acquisition, and a list of tests to be run or specimen to be processed.

DNA will be extracted from a portion of blood, with the majority of blood being ficoll-prepped for the isolation and cryopreservation of PBMCs.

Prior to bronchoscopy, subjects will gargle with 5 mL of Listerine® mouthwash, for 60 seconds, and discard the liquid.

The subject will then gargle with 5-10 mL of a saltwater solution (1% saline) for 1 minute. The saline should be swished around the oral cavity for 30 seconds, followed by a throat gargle. After 1 minute, the subject will spit the wash into a sterile container. The container will be kept on ice or frozen at -20°C until processed.

Bronchoscopy with BAL and protected brush will also be performed on Cohorts A and B. In brief, aliquots of 60 ml of room temperature sterile normal saline will be infused into the medial and lateral segments of the right middle lobe and gently aspirated by hand using a syringe attached to the suction port of the bronchoscope until free flow of lavage effluent ceases. A total of 300 mL of saline will be instilled into the segments and the aspirated volume pooled. The BAL will be processed as previously described.¹⁰⁰⁻¹⁰²

Due to the potential for upper airway contamination when passing the bronchoscope through the nares and/or mouth, an oral or nasal swab will be obtained in all subjects and will serve as an internal control. In addition, as a quality control measure, the effectiveness of the cleaning and disinfection of the bronchoscope will be assessed in a subset of patients by flushing the working channel with sterile normal saline and extracting DNA from the flushed saline.

4.4.1. BAL

BAL is collected according to the BAL laboratory procedures as developed by the LHMP sampling working group.

4.4.2. Blood

About 14 teaspoons of blood are drawn at each visit. Lab tests include HIV-1 antibody, on HIV negative subjects, viral load, CBC, CD4+, storage of PBMC and plasma

4.4.3. Oral Swab

A cotton swab is gently rubbed on the inside of the participant's mouth to gather samples.

4.4.4. Nasal Swab

A cotton swab is gently rubbed on the inside of the participant's nose to gather samples.

4.4.5. Other

A stool specimen will be gathered by the subject by collecting a smear from used bathroom tissue or drilling a cotton swab into the center of the largest individual piece or depositing a sample into the sterile container provided. This will be collected within 48 hours of the first bronchoscopy.

5. OUTCOMES EVALUATION

5.1. Primary Outcome

N/A

5.2. Other Outcomes

N/A

6. DATA COLLECTION AND MANAGEMENT

6.1. Site Specific Data Collection Forms

6.1.1. Physical Exam Form

Basic anthropometrics (height, weight, blood pressure) and respiratory symptoms are collected on the Physical Exam Form as well as antibiotic use for the last six months. A form is filled out at each visit. Research nurse completes forms at each visit after meeting with the participant. Forms are completed at screening, enrollment and at the last visit.

6.1.2. Pulmonary Conditions/Disease Form

A list of pulmonary conditions and diseases are checked yes if the participant has ever been diagnosed with the disease.

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8. Addendum: Revisions to Protocol

8.1. Addition of New /Cohort

Cohort A1 – Acute or recent HIV-1 infection

Cohort A2 – HIV-1 seronegative, healthy controls

Cohort B1 – HIV-1 infection who are antiretroviral therapy naïve

Cohort B2 – HIV-1 infection who are on stable antiretroviral therapy

8.2. Inclusion Criteria for new cohort, B2

Cohort B2 – HIV-1 infection diagnosed by a previous positive antibody or PCR test at least 6 months prior to study entry and not meeting the inclusion criteria for Cohort A1.

and

- ARV drug-experienced defined as ≥ 12 months of continuous treatment with a minimum of three ART drugs prior to study entry. Changes in therapy for toxicity or tolerance reasons, but not for virologic breakthrough, during the 6-month period are acceptable..
- HIV-1 RNA < 50 copies/mL within 30 days prior to study entry.
- No plasma HIV-1 RNA ≥ 50 copies/mL in the past 6 months.

8.3. Design Summary for new cohort, B2

Cohort B2 will consist of 50 subjects who are on stable antiretroviral therapy and have HIV-1 RNA < 50 copies/ml. At study screening blood will be collected for CD4+ T cell count and plasma HIV-1 RNA. At study entry approximately 60 mL of blood will be collected for flow cytometry, cytokine measurements and bronchoscopy will be performed. Only one bronchoscopy will be performed for participants in Cohort B2.

8.4. Total Sample Size

The University of Colorado Denver has increased the total sample size to 136 for their site (18 for each A cohort and 50 for each B cohort).

8.5. Updated Schedules of Events

Cohort A2 (HIV-1 seronegative, healthy controls)

Evaluations	Screening	Enrollment	~12 months
Informed Consent	X		
Medical/Medication History	X		
Vital Signs	X	X	X
Pulse oximetry	X	X	X
HIV-1 antibody	X		X
Storage of PBMC & plasma		X	X
Urine Pregnancy Test		X	X
Bronchoscopy		X	X
Listerine Oral Wash		X	X
Oral or Nasal swab		X	X
Stool specimen		X	X
Oral Wash		X	X

Cohort A1 (Acute or recent HIV-1 infection)

Evaluations	Screening	Enrollment	~12 months*
Informed Consent	X		
Medical/Medication History	X		
Vital Signs	x	X	X
Pulse oximetry	x	X	X
HIV-1 viral load	X		X
CD4+ cell count	X		X
Detuned assay (if necessary)	X		
Storage of PBMC & plasma		X	X
Urine Pregnancy Test		X	X
Bronchoscopy		X	X
Listerine Oral Wash		X	X
Oral or Nasal swab		X	X
Stool specimen		X	X
Oral Wash		X	X

*or second bronchoscopy ~24 weeks after starting ARV (min 3 ARV drugs) and HIV-1 RNA \leq 50 copies/mL within 30 days prior to second bronchoscopy

Cohort B1 (HIV-1 infection who are not on antiretroviral therapy)

Evaluations	Screening	Enrollment	~24 weeks*
Informed Consent	X		
Medical/Medication History	X		
Vital Signs	x	X	X
Pulse oximetry	x	X	X
HIV-1 viral load #1	X		X
HIV-1 viral load #2			X
CD4+ cell count	X		X
Storage of PBMC & plasma		X	X
Urine Pregnancy Test		X	X
Bronchoscopy		X	X
Listerine Oral Wash		X	X
Oral or Nasal swab		X	X
Stool specimen		X	X
Oral Wash		X	X

*or second bronchoscopy ~24 weeks after starting ARV (min 3 ARV drugs) and HIV-1 RNA \leq 50 copies/mL within 30 days prior to second bronchoscopy. If subject does not start ARVs, then only 1 bronchoscopy

Cohort B2 (HIV-1 infection who are on stable antiretroviral therapy)

Evaluations	Screening	Enrollment
Informed Consent	X	
Medical/Medication History	X	
Vital Signs	X	X
Pulse oximetry	X	X
HIV-1 viral load #1	X	
CD4+ cell count	X	
Storage of PBMC & plasma		X
Urine Pregnancy Test		X
Bronchoscopy		X
Listerine Oral Wash		X
Oral or Nasal swab		X
Stool specimen		X
Oral Wash		X

8.6. Updated Timing of Evaluations

Cohort A1 and A2

During the screening visit, study subjects will sign the informed consent, be asked questions about their medical history, and obtain a container to be used for at home stool

specimen collection (to be collected within 48 hours of study entry). At the time of screening, approximately 20 ml of blood will be drawn for HIV-1 viral load and CD4+ T cell count (or HIV-1 antibody if HIV negative). For Cohort A1 a detuned assay might be needed (included in the 20 ml).

At the time of enrollment, approximately 60 ml of blood will be obtained from each subject for plasma and PBMC storage. At the 12 month visit, approximately 70 ml of blood will be obtained from each subject.

In addition, an oral wash and bronchoscopy with BAL and protected brush will be performed on all subjects at enrollment and after 12 months or, if ARV treatment is started prior to 12 months, ~24 weeks after starting ARV treatment. Pulse oximetry on room air at rest will be performed on all subjects prior to bronchoscopy. If pulse oximetry is less than 90%, bronchoscopy will not be performed.

Cohorts B1 and B2

During the screening visit, study subjects will sign the informed consent, be asked questions about their medical history, and obtain a container to be used for at home stool specimen collection (to be collected within 48 hours of study entry).

Pulse oximetry on room air at rest will be performed on all subjects prior to bronchoscopy. If pulse oximetry is less than 90%, bronchoscopy will not be performed.

At the time of screening, 20 ml of blood will be drawn for HIV-1 viral load and CD4+ T cell count. Bronchoscopy with BAL and protected brush will be performed. Approximately 60 mL of blood will be drawn at the time of enrollment.

Cohorts A1 and B1 only: If patients in Cohorts A1 or B1 initiate antiretroviral therapy, approximately 24 weeks after initiation of antiretroviral therapy, a repeat HIV-1 viral load will be performed to document a treatment response (defined as <50 copies of HIV-1 RNA/ml). Those subjects with documented plasma HIV-1 RNA \leq 50 copies/mL will undergo a second blood draw and a second bronchoscopy with BAL and brushing. If plasma HIV-1 RNA is not \leq 50 copies/mL, repeat plasma HIV-1 RNA in ~ 12 weeks. If repeat plasma HIV-1 RNA is not \leq 50 copies/mL, do not perform second bronchoscopy.

4.5. APPENDIX 5: Center C007 - University of Pittsburgh

4.5.1. Pathogens of Obstruction/Emphysema and the Microbiome (POEM) which incorporates Study Numbers: M001 – Multicenter AIDS Cohort Study (MACS) and M002 –Women’s Interagency HIV Study (WIHS)

MACS Study Web Site: <http://www.statepi.jhsph.edu/macs/macs.html>

WIHS Study Web Site: <http://statepiaps.jhsph.edu/wihs/>



**POEM Study of the Lung HIV Microbiome Project (LHMP)
University of Pittsburgh**

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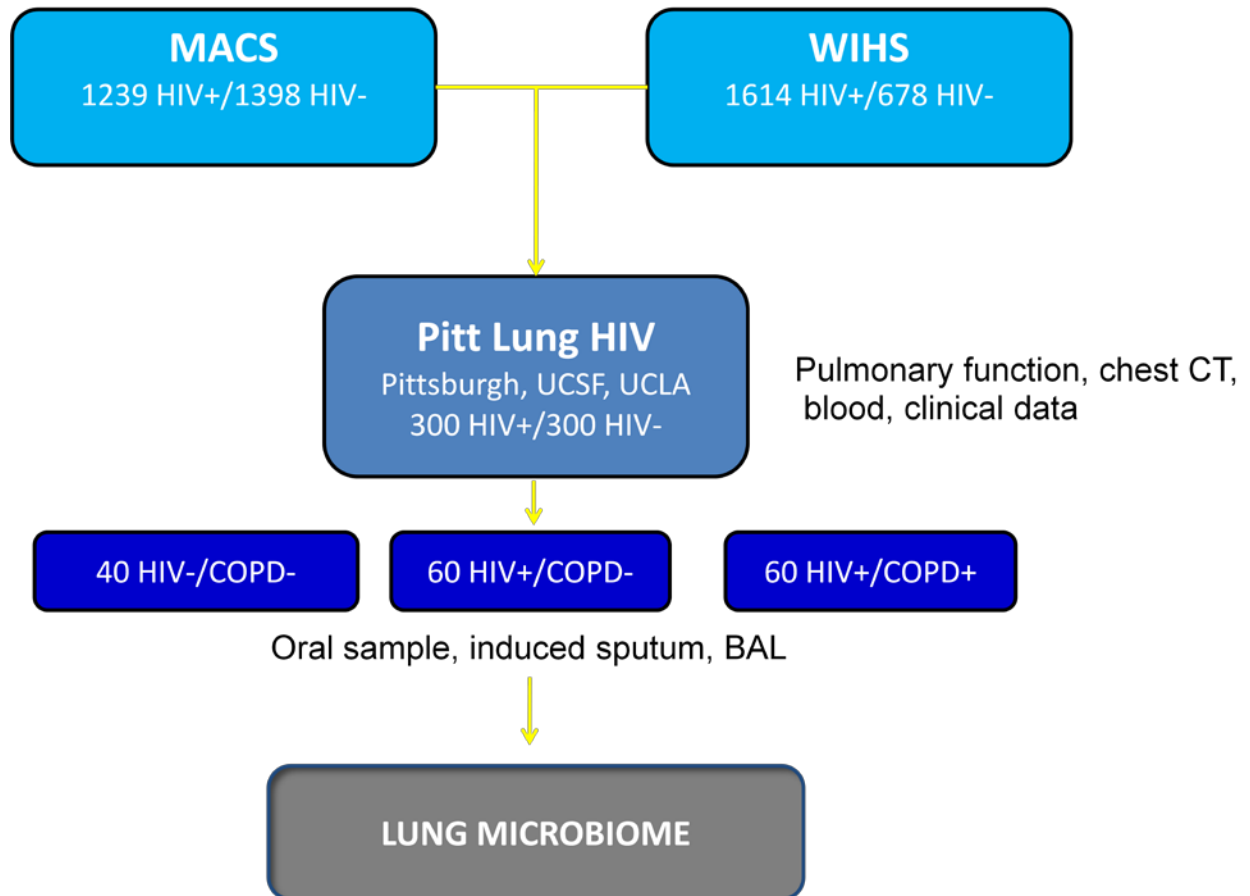
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Abbreviations Used

AIDS	Acquired Immune Deficiency Syndrome
COPD	Chronic Obstructive Pulmonary Disease
CT	Computed Tomography
DNA	Deoxyribonucleic Acid
GWU	The George Washington University
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
LHMP	Lung HIV Microbiome Project
MACS	Multicenter AIDS Cohort Study
NHLBI	National Heart, Lung, and Blood Institute
NIH	National Institutes of Health
PBMC	Peripheral Blood Mononuclear Cell
PFT	Pulmonary Function Testing
POEM	Pathogens of Obstruction/Emphysema and the Microbiome
RNA	Ribonucleic Acid
WIHS	Women's Interagency HIV Study

1. INTRODUCTION

Pathogens of Obstruction/Emphysema and the Microbiome (POEM) in HIV is composed of participants in the Pittsburgh Lung HIV which is a prospective cohort study of selected subjects from ongoing studies of pulmonary disease and COPD in HIV+ and HIV- men and women from the MACS and WIHS studies.



1.1. Parent Study Abstracts

1.1.1. Multicenter AIDS Cohort Study (MACS)

[The Multicenter AIDS Cohort Study \(MACS\)](#) is an ongoing prospective study of the natural and treated histories of HIV-1 infection in homosexual and bisexual men conducted since 1984 by sites located in Baltimore, Chicago, Pittsburgh and Los Angeles. Data from the MACS have been the basis of more than 780 publications in peer reviewed journals. In 1987, the [Department of Epidemiology of the Johns Hopkins School of Public Health](#) was awarded a contract to be the Center for the Analysis and Management of the MACS Data (CAMACS).

Starting in 1987 CAMACS has:

- Participated in the planning and design of studies in all areas of MACS research.
- Coordinated data acquisition by closely monitoring the development of forms used as data collection instruments and by establishing strict guidelines for data transfer through development of codebooks and design of data structures.
- Standardized procedures for editing and updating the data, in close collaboration with the performance sites.
- Established procedures for data management and organized the database to be conducive to efficient analysis.

- Provided data analysis leadership in collaboration with investigators of different centers.
- Developed relevant epidemiological and biostatistical methodology which has been published in peer reviewed journals.

Data and specimens collected at semiannual visits include: detailed questions covering sexual practices, HIV related symptoms, and utilization of health services, demographic and psychosocial characteristics, a quality of life survey, a physical examination, a detailed form on medications used as prophylaxis and/or treatment, a neuropsychological screening and examination, blood samples to measure hematologic variables including a complete enumeration of T-cell subsets and viral load measurements and the allocation of samples to be sent to the National Repository. These specimens are invaluable for helping to understand the pathogenesis of HIV-1 infection.

1.2. Women's Interagency HIV Study (WIHS)

The Women's Interagency HIV Study (WIHS) was established in August of 1993 to investigate the impact of HIV infection on women in the U.S. The core portion of the study includes a detailed and structured interview, physical and gynecologic examinations, and laboratory testing. The WIHS participants are also asked to enroll in various sub-studies, such as cardiovascular, metabolic, physical functioning, and neurocognition. New proposals for WIHS sub-studies are submitted for approval by various scientific investigators from around the world.

2,625 women (2,056 HIV+; 569 HIV-) were enrolled in 1994/1995, including women previously diagnosed with clinical AIDS, or women with low CD4+ cell counts. In 2001/2002, an additional 1,143 women (254 HIV+ HAART-naïve; 484 HIV+ HAART-experienced; 406 HIV-) were enrolled into the WIHS.

WIHS participants have follow-up visits at six-month intervals. The interview includes surveillance on socio-demographic, medical, obstetric, gynecological and contraceptive history, as well as alcohol, tobacco and other drug use and sexual behaviors. The medical history includes an extensive medication inventory utilizing medication photographs that are updated regularly to include newly available therapies.

Plasma, serum, lymphocyte, tissue, genital secretion and urine samples are placed in a central repository (maintained by SeraCare, Inc.), and indicated tissues are placed in the AIDS Cancer Specimen Resource.

1.3. Specific Aims

The POEM project has the following three specific aims:

1. To compare the microbial community in the respiratory tract of participants with and without HIV infection.
2. To test the hypothesis that the respiratory microbiome in subjects with HIV associated COPD differs from that in HIV+ participants without COPD and is related to COPD progression.
3. To test the hypothesis that the microbiome is associated with immune activation.

2. OVERVIEW, HYPOTHESIS, METHODS AND STUDY DESIGN

2.1. Overview

2.2. Primary Hypothesis

POEM is looking to compare the respiratory microbiome of participants without HIV to those with HIV to those with HIV and COPD.

2.3. Methods and Study Design

2.3.1. Design Summary

POEM is a prospective cohort study of selected participants from ongoing studies of pulmonary disease and COPD in HIV+ and HIV- men and women. Participants who are HIV-

negative will make-up a one-time cross-sectional cohort. Those who are HIV positive will have an additional follow-up at 36 months.

2.3.1.1. Total Sample Size

The total sample size is expected to be 160 participants, broken down as follows: 40 HIV- participants without COPD, 60 HIV+ participants without COPD, and 60 HIV+ participants with COPD.

2.3.2. Eligibility Criteria

2.3.2.1. Inclusion Criteria

- Between the Age of 18 and 75
- By participant's report, s/he is:
 - HIV positive OR
 - HIV negative and at high risk
- Recruited via:
 - Pitt Men's Study / MAC
 - Women's Interagency Health Study

2.3.2.2. Exclusion Criteria

- Younger than 18 years old
- Older than 75 years old
- Pregnancy or positive pregnancy test at screening
- Experiencing an increase in shortness of breath
- Cough or fever at time of visit
- Acute onset of shortness of breath, cough, fever
- Heart condition such as tachycardia (fast heart rate), angina (chest pain), or arrhythmias (irregular beats)
- MI, CVA or cardiovascular event within last 3 months
- Eye or abdominal surgery within last 3 months
- Active TB by documentation or self-report
- subjects with an upper or lower respiratory tract infection
- Individuals with a primary diagnosis of vocal cord dysfunction, or those with significant or uncontrolled systemic diseases will be excluded.

2.3.3. Informed Consent Criteria

Informed consents for an NHLBI funded study must include the following required elements. Sample language has been developed but is not required.

- A statement that allows for broad research use of specimens including research on lung disease, HIV, and other related illnesses. The statement must also specify that the participant will not receive results of any future research.
- A description of the NHLBI repository and who will have access to the specimens there.
- A list of who will have access to specimens, medical, and research information that includes the site staff, NHLBI/NIH, and the data analysis and coordinating center (GWU).
- A description of how confidentiality is protected. This description includes that data will be stored only in locked cabinets or on secured computers.
- A statement that specimens will be stored indefinitely.
- A statement that the participant has a right to change their mind at any time and that every effort will be made to destroy their samples but it may not be possible once samples are de-identified.
- A description of the risk of the bronchoscopy including death.

2.3.3.1. Stored Sample(s)

The informed consent must include a description of the risk of stored specimens and future testing such as paternity and other genetic information.

2.3.4. Secondary/Ancillary Research Questions

POEM is also looking at how the respiratory microbiome changes as COPD and HIV progress three years after the initial sample is taken.

3. CORE PROCEDURES

3.1. Introduction

Subjects will be asked to participate depending on the outcome of the PFT's and CT scans performed earlier. HIV status and smoking history will also be considered.

There will be two visits for the bronchoscopy participants. The first visit will be for pre testing; screening procedures, oral wash, tongue scraping, sputum induction and blood work.

The second visit is for the bronchoscopy; oral wash, tongue scraping with rinse, bronchoscopy and monitoring. Fiberoptic bronchoscopy will be performed using the ATS recommendation and institutional protocols for conscious sedation. Visits one and two will be no more than 30 days apart.

3.2. Recruitment Strategies

Subjects will be recruited from the Pitt Men's Study and the Women's Interagency Health Study (WIHS) programs. We will present the study to the primary providers at these programs who will then inform subjects about the study. The investigators will explain the study and invite any interested and qualified subject who would like to participate in this study to participate. No cold calling will be performed.

3.3. Screening for Eligibility and Chart Abstraction

A medical record review for accuracy of past medical history, a urine pregnancy test for women of child bearing potential and a physical exam to determine any contraindication to bronchoscopy will be completed. In addition lab work will be performed as per standard pre-op care.

3.4. Quality Assurance

Spirometry: equipment is calibrated each day there is a testing subject. A log of the date and the personnel doing the calibration is kept, as well as the calibration wave forms. This conforms to ATS standards.

CT Scans: The phantom protocol is scanned onto each sites CT equipment every 6 months to ensure standardization of all CT scans.

Data Entry: All data is double entered. It is entered in the main database, and then in a dummy data base. The data is compared and all discrepancies are looked up by hand in the hard copy chart and corrected. Data ranges are also checked.

3.5. Human Subjects

3.5.1. Data/Observational Safety Monitoring Board

The Lung HIV DSMB will serve to monitor the overall conduct of the study. An internal DSMB made up of the investigators and study staff hold bi-monthly telephone DSMB conferences.

3.6. Monitoring for Adverse Events

4. STANDARD CLINICAL CENTER PROCEDURES

4.1. Overview

4.2. Forms

Clinical data will be collected on an interview administered questionnaire. This includes demographics, previous pneumonia and other lung diseases, medication use, other co morbidities, and respiratory symptoms.

Some laboratory values and clinical/demographic data will be taken from MACS/WIHS testing and will not be available to investigators without written permission from MACS and/or WIHS.

For specific case report forms, please see section 6.1.

4.3. Laboratory Specimen Collection

4.3.1. BAL

BAL is collected according to the BAL laboratory procedures as developed by the LHMP sampling working group.

4.3.2. Pulmonary Function Testing

Pulmonary function testing is performed according to the PFT laboratory procedures as developed by the Longitudinal Lung HIV study.

4.3.3. Chest CT

CT scans are performed according to the CT laboratory procedures as developed by the Longitudinal Lung HIV study.

4.3.4. Blood

Eight tablespoons of blood are drawn. Blood is separated into plasma, serum, DNA, RNA, and PBMCs. The plasma is analyzed for flow cytometry, chemokines, and cytokines. One tube of blood is also tested for carboxyhemoglobin.

4.3.5. Oral Wash

Salt water is gargled for one minute and spit into a container to provide an approximately 10 mL sample. This sample is checked for cells or genes associated with emphysema, pneumocystis, and other lung diseases.

4.3.6. Tongue Scraping and Rinse

A tongue blade will be used to scrape the participant's tongue. This sample is analyzed for bacteria on the tongue.

4.3.7. Induced Sputum

Participants will breathe in and out mists of salt water (3%-5% saline) with a mouthpiece for 20 minutes. During this time, they will actively cough and spit out their saliva ("spit") and sputum into two separate cups every 4 minutes. Lung function will be frequently measured during the procedure. The sample will be analyzed for the presence of pneumocystis, bacteria and viruses, number of cells, and amounts of chemicals called mediators. These samples will also be used to measure any chemical compounds, cells or genes, produced that are involved in emphysema, pneumocystis colonization, or other lung diseases.

5. OUTCOMES EVALUATION

5.1. Primary Outcome

N/A

5.2. Other Outcomes

N/A

6. DATA COLLECTION AND MANAGEMENT

6.1. Site Specific Data Collection Forms

6.1.1. BAL Pre-Testing Form

6.1.2. BAL Testing Form

7. REFERENCES

Multicenter AIDS Cohort Study. < <http://www.statepi.jhsph.edu/mac/mac.html>>. Updated July, 2009. Visited April, 2010.

Women's Interagency HIV Study. < <https://statepiaps.jhsph.edu/wihs/>>. Visited April, 2010.

8. ADDENDUM: INCLUSION/EXCLUSION CRITERIA FOR LHMP PARTICIPANTS

8.1. Inclusion of children in research

No children will be included as they would not be expected to develop emphysema.

8.2. Inclusion:

1. Subject is Male / Female, 18years of age or older.
2. Subject who have been previously determined to be HIV positive or negative through testing, or have a high risk for infection as indicated by their participation in the Pitt Men's study (MACS), WIHS, or by their attendance at the UPMC HIV/AIDS program.

8.3. Exclusion:

1. Subject is experiencing acute onset of shortness of breath, cough, fevers or heart conditions problems such as tachycardia, angina or arrhythmias.
2. Female subject has told us she is pregnant (this might affect pulmonary function values; we will not require pregnancy testing).
3. Subject has had an MI, CVA, or cardiovascular event within the past 3 months.
4. Subject has had eye or abdominal surgery within past 3 months.
5. Active TB by documentation or self reported will be an exclusion criterion to the study.
6. Subjects will be excluded from the study if they are unable to sign consent, weigh > 300 pounds due to technical difficulties with the CT/EBCT scanner, or have been exposed to approximately 10 rads in the previous 12 months (i.e., 2 diagnostic CT scans or 4 cardiac caths or other fluoroscopic exams).

Received March 14, 2011

4.6. APPENDIX 6: Center C008 - Indiana University

4.6.1. Study Numbers: M004 – Analysis of Immunologic Responses in the Lung, and M006 – Examination of HIV Associated Lung Emphysema (EXHALE)



**Lung Microbiome and Pulmonary Inflammation/Immunity in HIV
Infection of the Lung HIV Microbiome Project (LHMP)
Indiana University**

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Abbreviations Used

ACTG	AIDS Clinical Trials Group
AIDS	Acquired Immune Deficiency Syndrome
BAL	Bronchoalveolar Lavage
CD4	Cluster of Differentiation 4
COPD	Chronic Obstructive Pulmonary Disease
CT	Computed Tomography
DNA	Deoxyribonucleic Acid
DSMB	Data Safety Monitoring Board
EXHALE	Examination of HIV Associated Lung Emphysema
GCWU	Genomic Center at Washington University
GWU	The George Washington University
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
ICRC	Indiana Clinical Research Center
ICTSI	Indiana Clinical and Translational Scientific Institute
IRD	Immune Reconstructive Disease
IRIS	Immune Reconstitution Syndrome
IU-IDRC	Indiana University Infectious Diseases Research Clinic
LHMP	Lung HIV Microbiome Project
NHGRI	National Human Genome Research Institute
NHLBI	National Heart, Lung, and Blood Institute
NIH	National Institutes of Health
OLD	Obstructive Lung Disease
RFA	Request for Applications
RNA	Ribonucleic Acid
TREC	T-Cell Receptor Excision Circles
VA	Veterans Affairs

1. INTRODUCTION

1.1. Parent Study Abstracts

1.1.1. ACTG 723

ACTG 723 (Effect of Highly Active Antiretroviral Therapy (HAART) on Viral Burden and Immune Function in the Lungs of HIV-Infected Subjects) was a prospective study designed to assess the effect of HAART on the lung HIV viral load and immune function. Subjects were studied with blood work and bronchoscopy with bronchoalveolar lavage (BAL) before and at 1 month and 6 months after beginning HAART. The specific aims were as follows: (1) To evaluate whether HIV viral load in bronchoalveolar lavage cells and fluid correlates with viremia; (2) To evaluate whether HAART reduces HIV viral load in bronchoalveolar lavage fluid and cells; and (3) To evaluate whether HAART reduces the intensity of lymphocytic alveolitis in HIV-infected subjects. This project has been closed since 2007. We will be utilizing frozen stored BAL fluid from this study for Microbiome analysis as part of the Lung HIV Microbiome Project.

1.1.2. Cross-Sectional Studies

We have been performing research bronchoscopies at Indiana University since 1989 to support various research projects, including NIH funded research. We routinely store extra BAL fluid. As such, we have accumulated a large number of frozen BAL from well characterized HIV-infected patients and normal volunteers. We will be using these samples for Microbiome analysis, limiting the samples to only those obtained from 2000 to present. Using the same IRB approved protocol, we will continue to acquire BAL, oral washes, and bronchial washes for prospective studies under the auspices of the Lung HIV Microbiome Project.

1.1.3. EXHALE

The EXHALE study (PI - Kristina Crothers, M.D.) will provide a source of prospective lung samples for the Lung HIV Microbiome Project. The focus will be on obtaining samples from normal and HIV-infected subjects, both smokers and nonsmokers. The abstract for the project is as follows:

As life expectancy of HIV-positive (HIV+) patients has increased for those receiving highly active antiretroviral therapy (HAART), chronic complications from smoking contribute substantially to morbidity and mortality. We have demonstrated that chronic obstructive lung disease (OLD) is an important problem and is more prevalent in HIV+ compared to HIV-negative (HIV-) patients, after adjusting for smoking and other confounding factors. Smoking is also a risk factor for other diseases that are increased in HIV, namely lung cancer, bacterial pneumonia, tuberculosis, and Pneumocystis pneumonia. We hypothesize that HIV+ patients have enhanced susceptibility to the consequences of smoking. Those who smoke and have HIV may be more predisposed to lung injury as a result of increased oxidative stress, which is known to be elevated in the setting of HIV, as well as in smoking and OLD. Our first aim is to 1) compare the incidence, risk factors, and outcomes for lung disease in HIV+ to HIV- patients. We will determine the independent risk for lung disease conferred by HIV infection, and to what extent the risk of lung complications is related to smoking or to concomitant OLD. These analyses will be conducted within an ongoing, large, multicenter, prospective observational study of HIV+ and HIV- subjects, the Veterans Aging Cohort Study that has recently had funding renewed through 2011. Studies for Aims 2 and 3 will focus in-depth on a smaller group of subjects to understand clinical and pathophysiologic differences in OLD between HIV+ and HIV- patients. We hypothesize that OLD progresses more rapidly in HIV+ compared to HIV- smokers and that the accelerated progression is related to increased oxidative stress. We will obtain baseline and serial evaluations of pulmonary function, peripheral blood, exhaled breath condensates, and bronchoalveolar lavage specimens in HIV+ and HIV- current and former smokers with or at risk for OLD. Additional aims are to 2) compare risk factors for progression of

OLD in HIV+ and HIV- smokers, and 3) compare markers of oxidative stress and progression of OLD in HIV+ and HIV- smokers. The studies proposed are poised to increase our understanding of lung diseases in HIV+ patients and to yield results that can be used directly to improve patient care. Furthermore, insights into reasons for susceptibility to OLD and accelerated progression of OLD can improve care for both HIV+ and HIV- patients.

1.1.4. IRIS

The IRIS study (PI – Kenneth Knox, M.D.) will provide a source of prospective and stored lung samples for the Lung HIV Microbiome Project. This project, which has been open since 2007, has prospectively studied HIV-infected subjects starting HAART with the aim of documenting the frequency and immune correlates of the Immune Reconstitution Syndrome over a 1-3 year follow-up period. The project is closed to enrollment, but longitudinal follow-up is continuing. The abstract for the project is as follows:

Most patients with Human Immunodeficiency Virus (HIV) infection will have a pulmonary complication at some point during the course of their disease. The advent of highly active antiretroviral therapy (HAART) has dramatically decreased the incidence of pulmonary infections. Although infections still predominate, some patients have a paradoxical worsening after initiation of HAART despite evidence of a recovering immune system. This phenomenon is termed immune restoration disease (IRD) and can affect up to 25% of patients who initiate HAART. IRD can be fatal and frequently mimics active pulmonary infection. As such, it can be a difficult diagnosis and contribute to morbidity and excessive clinical testing. Early pulmonary IRD is usually caused by latent or unrecognized infections at the time HAART is initiated. These infectious agents provide the substrate for the immunopathological response in IRD. However, late pulmonary IRD frequently manifests as sarcoidosis and is caused by unknown antigens. Stratifying people at risk for IRD and assessing their long term outcome is thus increasingly important, especially as the number of patients treated with HAART increases worldwide. We hypothesize that pulmonary IRD is clinically underrecognized and mediated by unbalanced Th1 responses. We propose to address this hypothesis by performing sequential CT scans, clinical assessment and bronchoscopies to harvest lung T cells in patients starting HAART therapy. Cytokine measurements, flow cytometry, and TREC analysis will be performed on lung and blood T cell subsets to assess if naive or memory cells are responsible for Th1-mediated IRD. Specific aims include: 1. To define a large, well-characterized clinical cohort of HIV-infected subjects for longitudinal study of IRD. 2. To determine the mechanisms of CD4 repopulation of the lung and 3. To define predictors of early and late pulmonary IRD based on clinical and immunological parameters. This work will have significant implications on clinical issues in HIV infected subjects, including predicting who is at risk for IRD and the 3 year outcome of patients with IRD. These studies also will provide the immunologic foundation for treatment strategies. Public Health Statement: As more patients worldwide are treated with anti-HIV drugs, the "side effects" of these drugs require study. Patients who are at risk for these therapy-related problems need to know if their long term health is affected.

1.1.5. Stored Samples

See above.

1.2. Specific Aims

The project has the following four specific aims:

1. To compare the respiratory microbiome between HIV-infected participants and normal volunteers and to determine if the microbiomes in HIV-infected participants are affected by:
 - A. CD4 count
 - B. History of prior opportunistic infection
 - C. Smoking status

- D. The presence and absence of chronic obstructive pulmonary disease.
- 2. To directly correlate the relationship between the respiratory microbiome and the pulmonary inflammatory milieu in HIV-infected participants and normal volunteers.
 - A. Cellular phenotype
 - B. Chemokine and cytokine concentrations
 - C. Antibody levels
- 3. To determine if highly active antiretroviral therapy alters the pulmonary microbiome and local inflammatory response.
- 4. Compare the respiratory microbiome in HIV-infected participants and normal volunteers from the oropharynx to the alveoli.

2. OVERVIEW, HYPOTHESIS, METHODS AND STUDY DESIGN

2.1. Overview

2.2. Primary Hypothesis

We hypothesize that the lung inflammatory and immunologic environment in HIV-infected subjects is directly related to the host microbiome rather than the pulmonary HIV burden. We further speculate that the immunodeficiency seen in HIV-infected subjects leads to a more diverse pulmonary microbiome compared to uninfected subjects, and that HAART returns the pulmonary microbiome towards normal.

2.3. Methods and Study Design

2.3.1. Design Summary

2.3.1.1. Total Sample Size

Total sample size will be 271. Participants will come from cross sectional studies, ACTG 723, and Knox studies.

Cross-Sectional Studies (Stored Samples)	HIV+	Smoker	31
		Non-Smoker	25
	Normal	Smoker	10
		Non-Smoker	40
Cross-Sectional Studies (Prospective Samples from EXHALE)	HIV+	Smoker	24
		Non-Smoker	24
	Normal	Smoker	24
		Non-Smoker	24
Longitudinal Studies (Stored and Prospective Samples from IRIS)	HIV+	ACTG 723	29
		IRIS Study	40

2.3.2. Eligibility Criteria

Cross Sectional Studies

To be eligible for participation subjects had to have

1. Confirmed HIV infection or normal volunteer
2. Age > 18 years
3. Free of respiratory tract symptoms or infection in the past 30 days

ACTG 723 and IRIS

To be eligible for participation subjects had to have

1. Confirmed HIV infection
2. No prior exposure to HAART
3. Age > 18 years
4. Free of respiratory tract symptoms or infection in the past 30 days
5. A CD4 lymphocyte count of ≤ 500 cells/ μ L and plasma HIV RNA copies of ≥ 5000 copies/mL within 30 days prior to study entry.

EXHALE

To be eligible for participation subjects had to have

1. Confirmed HIV infection or normal volunteer
2. Age > 18
3. Free of respiratory tract symptoms or infection in the past 6 weeks
4. Enrollment in VACS at the Atlanta, Bronx, or Los Angeles VAMC
5. Diagnosis of obstructive lung disease based on chart review, OR airflow obstruction by screening spirometry.

2.3.3. Informed Consent Criteria

Informed consents for an NHLBI funded study must include the following required elements. Sample language has been developed but is not required.

- A statement that allows for broad research use of specimens including research on lung disease, HIV, and other related illnesses. The statement must also specify that the participant will not receive results of any future research.
- A description of the NHLBI repository and who will have access to the specimens there.
- A list of who will have access to specimens, medical, and research information that includes the site staff, NHLBI/NIH, and the data analysis and coordinating center (GWU).
- A description of how confidentiality is protected. This description includes that data will be stored only in locked cabinets or on secured computers.
- A statement that specimens will be stored indefinitely.
- A statement that the participant has a right to change their mind at any time and that every effort will be made to destroy their samples but it may not be possible once samples are de-identified.
- A description of the risk of the bronchoscopy including death.

2.3.3.1. Stored Sample(s)

The informed consent must include a description of the risk of stored specimens and future testing such as paternity and other genetic information.

2.3.4. Secondary/Ancillary Research Questions

N/A

3. CORE PROCEDURES

3.1. Introduction

Clinical Procedures

1. Bronchoscopy
2. Collection of oral washes
3. Blood draw

Scientific procedures

1. 16s ribosomal RNA sequencing
2. Metagenomic analysis

3. Flow cytometry analysis of BAL cell phenotype
4. Cytokine and chemokine assays
5. Measurement of HIV viral load
6. Measurement of BAL immunoglobulin concentrations

3.2. Recruitment Strategies

3.2.1. Prospective samples

The primary resource for patient recruitment at Indiana University is through the Indiana University Infectious Diseases Research Clinic (IU-IDRC). The site is located in our Indiana Clinical Research Center (ICRC) space with Indiana University Hospital. The ICRC is part of the Indiana Clinical & Translational Scientific Institute (ICTSI). The IU-IDRC provides care for over 2000 patients in the Indianapolis area (metropolitan area~ 1.5 million) and is a recognized as a referral center for HIV/AIDS clinical research in the state of Indiana. The majority of referrals to this site are from HIV Clinics within the University Hospital and the Indianapolis metropolitan area. The IU-IDRC has enrolled both treatment naïve and treatment experienced subjects in a variety of clinical trials, totaling close to 200 subjects per year.

A second major source of patient referral is from the Infectious Disease clinic at Wishard Memorial Hospital in Indianapolis. Wishard Hospital is the metropolitan county hospital and an integral part of the Indiana University Medical Center. Wishard Hospital provides care to a large number of African-Americans, women and a growing number of Hispanic individuals. The Wishard clinic is the main site for care for newly diagnosed patients in Indiana. Two other sources of HIV-infected subjects on the Indiana University campus include the Indianapolis Veterans Administration Hospital and community networks with private physicians who routinely refer to the IU-IDRC.

HIV-Infected Subjects Available for Study at Indiana University			
University Hosp	Wishard Hosp	VA Hosp	Community Referral
300	600	200	500

A third source of subjects will come from a cohort of prospectively studied HIV-infected patients with COPD as part of the NHLBI RFA “Longitudinal Studies of HIV-Associated Lung Infections and Complications” [R01 HL090342-01 - Examination of HIV Associated Lung Emphysema (EXHALE); PI – Kristina Crothers]. The recruitment goal in this study is 96 HIV+ and 96 HIV- subjects with COPD divided evenly between three sites, all within the Veterans Affairs Hospital system. Each of the three sites in the EXHALE study has about 300-450 HIV+ and 300-450 HIV- patients potentially eligible for participation.

Recruitment Goal of the EXHALE Study				
Subjects	COPD-		COPD+	
	Current smoker	Non-smoker	Current smoker	Non-smoker
HIV+	24	24	24	24
HIV-	24	24	24	24
Total	48	48	48	48

The final source of prospective samples comes from an ongoing prospective trial to examine clinical predictors of pulmonary immune reconstitution syndrome [R01 HL083468 “Pulmonary CD4 T-Cell Repopulation in Immune Reconstitution Syndrome (IRIS)”; PI - Kenneth Knox, M.D.]. In this study subjects are followed prospectively for a total of three years after starting HAART.

3.2.2. Banked Specimens

Since 1999 we have taken a more prospective longitudinal study approach in HIV-infected subjects as part of our role as the principal site of a completed multicenter study sponsored by the AIDS Clinical Trials Group (ACTG). ACTG 723 was a project examining the effect of HAART on HIV viral loads in the lung. Naive HIV infected subjects underwent bronchoscopy with BAL before initiating HAART and then again after 4 weeks and 6 months of therapy. Subjects without HIV detectable in the lung at baseline were not eligible for continuation in the study. If HIV was still detectable in acellular BAL fluid or in BAL cells at 6 months, then the subject was invited to undergo a final bronchoscopy after one year of therapy. Much of the preliminary data describes our experience in this trial.

Cross Sectional Studies	HIV +	Smoker	31	56
		Nonsmoker	25	
	Normal	Smoker	10	50
		Nonsmoker	40	
Longitudinal Studies	HIV+	ACTG 723	29 subjects with entry, 1 mth, & 6 mth f/u. 7 subjects with 1 year f/u	94
		Knox Study (Ongoing)	34 subjects with entry, 1 mth, and 1 year follow-up by end March 2010	102

As a result of our experience we have built a large repository of 302 BAL samples which are well characterized and available for immediate analysis. These samples will allow us to immediately begin to compare lung BAL microbiomes in the following groups: (1) Untreated HIV-infected subjects versus uninfected controls, (2) Effect of HAART on the lung microbiome, and (3) Effect of smoking on the lung microbiome (in normal and HIV-infected subjects).

3.3. Screening for Eligibility and Chart Abstraction

Obviously screening for banked BAL samples is not necessary. Subjects at Indiana University are screened by research nurses in the Infectious Disease clinics who are funded either by institutional grants or principal investigator grants. For example, the IRIS study funds a nurse coordinator to recruit subjects and enter data into the study website.

Screening for subjects from the EXALE study is performed by paid study personnel who screen existing VACS survey data and perform medical record review. Patients who meet inclusion criteria based on chart review are asked to directly enroll in EXHALE. Subjects who do not have clear diagnoses of COPD by chart review are asked to first perform screening spirometry. Then, if eligible, these subjects are asked to enroll in the full EXHALE study.

3.4. Quality Assurance

16s rRNA sequencing is the primary scientific procedure to be performed in this project. These studies will be performed at the Genomic Center at Washington University (GCWU). The GCWU has a long history of producing sequences scrutinized by external reviewers. Our most stringent test was the Human Genome Project, where an external group specializing in this activity checked sequence that was produced. BACs were re-sequenced and discrepancies were further analyzed, with a required an error rate of 10^{-4} or less and gaps only allowed after a series of methods were applied. The GCWU sequenced over 20% of the Human Genome to this scrutiny. Other projects have ranged from the mouse genome, to microbial genomes, to metagenomes. The GCWU has made all data and assemblies publicly available. We believe continued large scale funding of GCWU by the NIH-NHGRI is an indicator of the quality of this data.

3.5. Human Subjects

3.5.1. Data/Observational Safety Monitoring Board

Data Safety Monitoring Boards are in place for all studies obtaining prospective samples in this project

1. Indiana University – chair, Dr. Chadi Hage (Indiana University).
2. IRIS – chair, Dr. Mark Wewers (Ohio State)
3. EXHALE – this project is part of the NHLBI Longitudinal HIV consortium, which has its own DSMB.

3.6. Monitoring for Adverse Events

Adverse events are reported directly to the DSMB at Indiana University and to the IRIS DSMB. They are reviewed every 6-12 months.

4. STANDARD CLINICAL CENTER PROCEDURES

4.1. Overview

1. Forms for bronchoscopies at Indiana University not part of the IRIS study are filled out by Brandon Day, clinical coordinator in Dr. Twigg's lab.
2. Forms for bronchoscopies performed under the IRIS study are filled out by paid research personnel on the grant (Jeff Waltz, Beth Zwickl)
3. Forms for bronchoscopies performed under the EXHALE study are filled out by research nurse personnel at the VA study sites.

4.2. Forms

We will be using the forms developed by the Sampling and Clinical Working groups of the LHMP. Specifically, we will be using the:

1. LHMP Demographics form
2. LHMP Pulmonary HIV Questionnaire
3. LHMP Diagnosis form
4. LHMP BAL form

4.3. Laboratory Specimen Collection

4.3.1. BAL

We will be performing bronchoscopy with BAL. Specimens we will be obtaining include:

1. Whole BAL fluid
2. Acellular BAL fluid
3. BAL cell pellets
4. Bronchial washes
5. Oral washes

4.3.2. Blood

We will be performing phlebotomy to obtain blood for:

1. Serum
2. Peripheral blood mononuclear cells

4.3.3. Other

N/A

5. OUTCOMES EVALUATION

5.1. Primary Outcome

N/A

5.2. Other Outcomes

N/A

6. DATA COLLECTION AND MANAGEMENT

6.1. Site Specific Data Collection Forms

6.1.1. Research Volunteer Information

The RVI form collects demographics and information on the BAL procedure and blood collection. As mentioned section 4.2, we will be using the LHMP forms developed by the Clinical Working Group. At Indiana University we have an additional database (IRB approved) where we store information on subjects who may want to undergo bronchoscopy for research in the future. This database serves as a rapid source of research volunteers. Basic demographic information on these subjects include age, sex, race, and smoking status. This information is collected by the research coordinator in my lab, Brandon Day.

7. REFERENCES