Rationale and Design of the Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis Study
Alpha-1 Protocol

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Abstract

Severe deficiency of alpha-1 antitrypsin has a highly variable clinical presentation. The Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis α_1 Study is a prospective, multicenter, cross-sectional study of adults older than age 35 years with PiZZ or PiMZ alpha-1 antitrypsin genotypes. It is designed to better understand if microbial factors influence this heterogeneity. Clinical symptoms, pulmonary function testing, computed chest tomography, exercise capacity, and bronchoalveolar lavage (BAL) will be used to define chronic obstructive pulmonary disease (COPD) phenotypes that can be studied with an integrated systems biology approach that includes plasma proteomics; mouth, BAL, and stool microbiome and virome analysis; and blood microRNA and blood mononuclear cell RNA and DNA profiling. We will rely on global genome, transcriptome, proteome, and metabolome datasets. Matched cohorts of PiZZ participants on or off alpha-1 antitrypsin augmentation therapy, PiMZ participants not on augmentation therapy, and control participants from the Subpopulations and Intermediate Outcome Measures in COPD Study who match on

FEV_1 and age will be compared. In the primary analysis, we will determine if the PiZZ individuals on augmentation therapy have a difference in lower respiratory tract microbes identified compared with matched PiZZ individuals who are not on augmentation therapy. By characterizing the microbiome in alpha-1 antitrypsin deficiency (AATD), we hope to define new phenotypes of COPD that explain some of the diversity of clinical presentations. As a unique genetic cause of COPD, AATD may inform typical COPD pathogenesis, and better understanding of it may illuminate the complex interplay between environment and genetics. Although the biologic approaches are hypothesis generating, the results may lead to development of novel biomarkers, better understanding of COPD phenotypes, and development of novel diagnostic and therapeutic trials in AATD and COPD.

Clinical trial registered with www.clinicaltrials.gov (NCT01832220)

Keywords: lung; microbiome; phenotype; bronchoalveolar lavage; COPD; emphysema

Alpha-1 antitrypsin deficiency (AATD) is a genetic condition that predisposes individuals to early-onset pulmonary emphysema and airway obstruction, which are often indistinguishable from usual chronic obstructive pulmonary disease (COPD). Its presentation and severity can vary remarkably between individuals. Prominent features of AATD COPD include basilar-predominant panacinar emphysema, airway reactivity, radiographic bronchiectasis, and a prominent interaction with environmental factors. The extent to which other modifying response genes influence individual gene expression and clinical disease phenotypes remains unknown in AATD.

Alpha-1 Antitrypsin (AAT) is the most abundant serum and lung antiprotease and has a variety of biologic activities that influence lung homeostasis. Prominent among these are roles in neutrophil elastase inhibition, antiprotease activities against cathepsins, involvement in the complement cascade, and interaction with Toll-like receptors.

Infections have long been thought to play a role in exacerbations and progression of traditional COPD, but they have not been as well studied in AATD. Given the structural and immunologic alterations seen in AATD, it is possible that local microbial factors may also contribute to variability in disease expression and severity. Culture-independent metagenomic techniques that employ direct sequence-based identification of microbial agents (microbiome studies) have expanded the ability to detect bacteria and viruses in the lung and relate them to disease processes.

Although AATD has classically been described as a disease with prominent emphysema, a large body of evidence suggests that airway disease is an early and integral component of disease pathogenesis. Asthma is the most common misdiagnosis identified in clinical studies (1). This is understandable owing to the high frequency of cough, sputum production, and wheezing seen in large clinical cohorts (2, 3). Bronchitis is common even among those with normal spirometry (4), and COPD exacerbations are frequent among those on augmentation therapy (5, 6). How these features are impacted by airway infections caused by bacteria, viruses, or mycobacteria remains unknown (7). Observation of dramatic differences in phenotypic presentation, even in siblings, suggests either strong environmental influences, including microbial infection or colonization, and/or a significant influence of modifying genes affecting the impact of the AATD (8–11).

AATD Genotypes

Severe deficiency of AAT is a rare condition with an estimated prevalence of 60,000–100,000 individuals in the United States. The most commonly recognized deficiency allele, the Z-variant, arises from a point mutation in the *SERPINA1* gene that encodes for AAT. It is characterized by substitution of a lysine for a glutamic acid at position 342 and polymerizes within the hepatocyte (12), lowering serum and lung levels of AAT. Homozygous protease inhibitor Z genetic mutations (PiZZ) make up the severe AATD cohort in the present study. Individuals with PiZZ AATD have 10–15% of the normal baseline circulating and lung levels of AAT, but clinical presentation is highly heterogeneous and some individuals may never develop disease. Environmental exposures, especially cigarette smoke, greatly increase the risk of lung disease in those with AATD; however, it is unknown whether airway microbial factors contribute to this heterogeneity.

The decision to prescribe or receive augmentation therapy for PiZZ AATD is not made lightly. Therapy is expensive, and the burden of intravenous administration once weekly for life is not easily dismissed. Although therapy is associated with slowing of emphysema progression (13, 14), augmentation therapy has not been clearly shown to lessen the frequency or number of COPD exacerbations (13). In addition, augmentation therapy is not indicated in individuals without emphysema despite PiZZ deficiency.

PiMZ carriers of one deficiency Z and one normal M allele also appear to more frequently have COPD when the protease burden is high. The best characterized protease burden arises from cigarette smoking. In a recent study in which researchers carefully characterized PiMZ populations who had not been tested for AATD because of symptoms, COPD prevalence was higher in PiMZ compared with PiMM siblings when cigarette smoking was present (15). Other case control studies have confirmed the increased prevalence of Z alleles in COPD cohorts (16). Importantly, the PiMZ genotype is present in 1–2% of the U.S. population, up to 8% of the entire COPD cohort (17), and affects 3–6 million individuals in the United States.

There is some controversy whether bronchiectasis is a frequent clinical phenotype in AATD. Population-based bronchiectasis registries have not shown large differences in AAT allele frequencies compared with control populations (18). However, studies within AATD cohorts have shown frequent bronchiectasis. The best study was performed in 2007 on 74 subjects with PiZZ by Parr and colleagues (19), who showed computed tomography (CT)-confirmed bronchiectasis in 70 individuals (95%). Clinically significant disease, as manifested by four or more segments of airway abnormality and chronic sputum production, was seen in 20 individuals (28%). The researchers in the National Heart, Lung, and Blood Institute (NHLBI) Study of Severe Deficiency of Alpha-1 Antitrypsin recovered 42 autopsy specimens from the 204 decedents in this observational cohort. Whereas large airway bronchiectasis was present in only six (14%), dilation of membranous bronchioles was frequent (20). Further understanding of the organisms involved in bronchiectasis could impact future therapy for this disease and may affect the larger population with typical COPD.

Lung Microbiome

The lung is among the most difficult organs in which to study the microbiome, in large part because a bronchoscope used to sample the lower airways must traverse the microbe-rich upper respiratory tract. Nevertheless, the lung is open to the environment and has direct communication with the upper airway, and its microbial content is likely impacted by innumerable diseases and disease phenotypes. Although the lower airways and alveoli were once felt to be sterile, data suggest that the microbiome of the lung closely resembles that of the oropharynx, but at markedly lower density (21, 22). Microaspiration occurs in healthy individuals and may be responsible for this similarity (23). Investigators in several studies have also detected the presence of unique organisms such as *Tropheryma whipplei*, the agent of Whipple's disease, in the lung (21, 22, 24).

In typical smokers' COPD, acquisition of new strains of bacteria was once considered causative in the pathogenesis of acute exacerbations (25, 26). Data now

suggest that these pathogens are present before the exacerbation and that factors which encourage "blooms" of specific microbes are important. One reason to study AATD is that the protease inhibitor deficiency may amplify the effects of the microbial and native airway cellular interactions. The microbiome is known to influence lymphocyte, neutrophil, and macrophage populations in the lung in both healthy and pathologic conditions (27). Downstream effects on tumor necrosis factor α and matrix metalloproteinases vary in pneumonia and bronchiectasis associated with microbiota composition (28, 29). Colonization or low-grade chronic infections may drive an ongoing spiral of disease progression (30).

Although *Haemophilus influenzae*, *Chlamydia* species, *Pneumocystis jirovecii*, and other bacteria and viruses have been implicated in chronic progression of disease (31–34), some microbial species may decrease lung inflammation, as has been shown for *Lactobacillus* species (35). The lung microbiota composition in COPD has been shown to be influenced by age and inhaled corticosteroids (36). Such chronic processes in AATD are more poorly defined than in typical COPD and thus are a focus of the Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis (GRADS) Study.

AATD also appears to increase the risk of invasive pulmonary nontuberculous mycobacterial infections, with significant overrepresentation of abnormal AAT genotypes in this population (37). There is also interest in the observation that AATD modulates permissiveness for growth of HIV Type 1 (38), raising the question whether it could impact components of the viral microbiome. To investigate the interactions between AATD, the microbiome, and COPD phenotypes, we designed this study to enhance the understanding of typical COPD by illuminating the disease that generated the protease-antiprotease theory of the pathogenesis of COPD.

Relevant Pathobiology
Polymorphonuclear leukocyte (PMN, neutrophil)–mediated airways inflammation is a hallmark of AATD and typical COPD. However, few studies have evaluated whether the microbiome may be causative in PMN translocation to the airways or distal airspaces of the lung.

Noninfectious causes of neutrophil influx into the AATD lung have been more consistently studied. Human neutrophil elastase (HNE) stimulates alveolar macrophages to release LTB4, a potent PMN chemoattractant (39). Polymers of AAT, found often in the circulation of individuals with PiZZ (severe AATD) (40), stimulate the influx of lung neutrophils through an IL-8–dependent mechanism (41–43). Other sources of PMN influx include defensins (44), elastin fragments (45), and possibly the activation of cells involved in immune modulation within the lung through the unfolded protein response (46). Specifically, microbial colonization and infection have not been specifically studied as a mechanism for lung leukocytosis in AATD.

Considerable work has been done to determine the interplay between bacterial species and their associated elastases, PMN influx into the airway, and airway protease defenses in COPD. Common findings in progressive disease include persistence of neutrophilic inflammation despite optimal therapy, more rapid progression in patients colonized with some bacterial species (particularly *Pseudomonas* and its associated elastase) (47), and excess amounts of antiproteases, particularly in oxidized or fragmented form. Data have suggested that components of the sputum sol matrix such as heparan sulfate and syndecan 1 are responsible for compartmentalizing neutrophil elastase, making it inaccessible to exogenous antielastase compounds (48, 49).

In addition to acute neutrophil-associated lung destruction in AATD, recent evidence supports a chronic immune activation that resembles the lymphoid follicular response seen in moderate to advanced COPD (50). Such a response may be related to loss of immune tolerance associated with the early tissue damage resulting from the primary pathogenic processes, but other possible explanations include viral and other microbial processes that result in ongoing immune activation as occurs in typical COPD (33, 51).

Some work has been performed on biomarkers of disease progression in AATD. Bronchoalveolar lavage (BAL) has been used to characterize the elastase inhibitory capacity of the lower airway (52), which improves with AAT augmentation therapy (53). Acute bacterial pneumonia prompts an increase in measured AAT in BAL that

is functionally complexed to HNE, resulting in lower elastase inhibitory activity (54). Oxidative injury of AAT can have a variety of sources (55), including cigarette smoke. Therefore, it is reasonable to hypothesize that a complex interplay exists between immunoreactive AAT, HNE, elastase inhibitory activity, complexed AAT–HNE, and lower airway PMN numbers that depends in part on the microbiome.

Bronchiolitis is a major component of COPD. It is associated with a large excess of neutrophils in the airway (56) and with bronchial hyperreactivity, a COPD phenotype marked by frequent exacerbations, and a more rapid decline in FEV_1 compared with individuals without bronchiolitis. Elastase in the airway contributes to antigen-induced mucociliary dysfunction in animal (57) and human airways. Although a correlation between an abnormal microbiome and a bronchiolitis phenotype is likely, preliminary data are insufficient to define expected changes.

Present Study
Because AAT may function over a lifetime in a variety of novel interactions with the environment, a better understanding of the interaction between AAT and the microbial environment is the goal of the GRADS Alpha-1 Study protocol. Clinical COPD phenotypes of bronchodilator-responsive individuals, those with clinical bronchiectasis, and those with normal lung function without symptoms are all of interest in this pilot study. Companion requests for applications (RFAs) for a Genomics and Informatics Center (GIC) and for clinical centers were released by the NHLBI of the National Institutes of Health as RFA-HL-12-013 and RFA-HL-12-014, both with a planned start date of April 1, 2012.

Methods

Study Objectives
The GRADS Alpha-1 Study protocol is designed to investigate the overarching hypothesis that AAT impacts the diversity and content of the lower airway microbiota, resulting in a less inflammatory airway. This occurs whether the AAT is given by augmentation or is naturally occurring owing to the presence of one or two copies of

the normal PiM *SERPINA1* allele. The specific aims of the study are
1. To compare the lower respiratory tract microbiome and virome population diversity and content in age and stage-matched individuals with PiZZ (based on Global Initiative for Chronic Obstructive Lund Disease [GOLD] guidelines [58]) not receiving augmentation therapy, individuals with PiZZ receiving augmentation therapy, PiMZ individuals not receiving augmentation therapy, and (normal nondeficient genotype) individuals with PiMM who have COPD
2. To determine correlations between BAL and peripheral blood gene expression patterns and patterns in lung microbial and viral populations across all cohorts
3. To correlate the presence or absence of CT-confirmed bronchiectasis and bronchiolectasis with patterns in the microbiome population diversity and content
4. To identify and define novel molecular phenotypes of AATD based on computational integration of clinical, transcriptomic, and microbiomic data

Study Design
Four separate cohorts are defined for this pilot study: individuals with PiZZ not

Table 1. Inclusion and exclusion criteria

Inclusion criteria
1. Age between 35 and 80 yr (inclusive) at baseline visit
2. Alpha-1 antitrypsin genotype PiZZ or PiMZ
3. Able to tolerate and willing to undergo study procedures
4. Signed informed consent

Exclusion criteria
1. History of comorbid condition severe enough to significantly increase risks based on investigator discretion
2. Diagnosis of unstable cardiovascular disease, including myocardial infarction, in the past 6 wk; uncontrolled congestive heart failure; or uncontrolled arrhythmia
3. Pa_{O_2} on room air at rest <50 mm Hg or Sa_{O_2} on room air at rest <85%
4. Postbronchodilator FEV_1 <30% predicted
5. Use of anticoagulation (Patients taking warfarin or clopidogrel will be excluded; patients taking aspirin alone can be studied even with concurrent use.)
6. Dementia or other cognitive dysfunction that, in the opinion of the investigator, would prevent the participant from consenting to the study or completing study procedures
7. Active pulmonary infection with tuberculosis
8. History of pulmonary embolism in the past 2 yr
9. Non-COPD obstructive disease (various bronchiolitides, sarcoidosis, lymphangioleiomyomatosis, Langerhans cell histiocytosis) or parenchymal lung disease, pulmonary vascular disease, pleural disease, severe kyphoscoliosis, neuromuscular weakness, or other cardiovascular and/or pulmonary disease that, in the opinion of the investigator, limits the interpretability of the pulmonary function measures
10. Prior significant difficulties with pulmonary function testing
11. Hypersensitivity to or intolerance of albuterol sulfate or ipratropium bromide or propellants or excipients of the inhalers
12. Hypersensitivity to or intolerance of all drugs required for sedation during conscious sedation bronchoscopy
13. History of lung volume reduction surgery, lung resection, or bronchoscopic lung volume reduction in any form
14. History of lung or other organ transplant
15. History of large thoracic metal implants (e.g., AICD and/or pacemaker) that, in the opinion of the investigator, limits the interpretability of computed tomographic scans
16. Currently taking ⩾10 mg/day or 20 mg every other day of prednisone or equivalent systemic corticosteroid
17. Currently taking any immunosuppressive agent, excepting systemic corticosteroids
18. History of lung cancer or any cancer that spread to multiple locations in the body
19. Current illicit substance abuse, excluding marijuana
20. Known HIV/AIDS infection
21. History of or current exposure to chemotherapy or radiation treatments that, in the opinion of the investigator, limits the interpretability of the pulmonary function measures
22. BMI >40 kg/m^2 at baseline examination
23. Current or planned pregnancy within the study course
24. Currently institutionalized (e.g., prisons, long-term care facilities)
25. Possession of a genotype of PiMZ and ever having received intravenous or inhaled alpha-1 antitrypsin augmentation therapy (alpha-1 proteinase inhibitor)

Conditional exclusions
1. Participants who present with an upper respiratory infection or pulmonary exacerbation, either solely participant-identified or that has been clinically treated, in the preceding 6 wk can be rescreened for the study once the 6-wk window has passed.
2. Participants who present with current use of acute antibiotics or steroids can be rescreened for the study ⩾30 days after discontinuing acute antibiotics and/or steroids. This does not apply to participants who are on chronic prednisone therapy of <10 mg/day or <20 mg every other day.
3. Participants who present with a myocardial infarction or eye, chest, or abdominal surgery within 6 wk can be rescreened after the 6-wk window has passed. Study coordinators should consult with the site's principal investigator before rescreening these participants.
4. Female participants who present <3 mo after giving birth will be asked to reschedule their visit until 3 mo have passed since the birth.
5. Individuals with PiZZ who are receiving alpha-1 antitrypsin augmentation therapy (alpha-1 proteinase Inhibitor) must be off augmentation therapy for >6 mo to qualify for stratified enrollment in the PiZZ group not receiving augmentation therapy.

Definition of abbreviations: AICD = automated implantable cardiac defibrillator; BMI = body mass index; COPD = chronic obstructive pulmonary disease; PiMZ = protease inhibitor Z carrier state; PiZZ = homozygous protease inhibitor Z genetic mutations.

receiving augmentation therapy, individuals with PiZZ receiving augmentation therapy, individuals with PiMZ not receiving augmentation therapy, and individuals with PiMM. The optimal matching strategy would control for age, sex, $FEV_1\%$ predicted, smoking intensity, and bronchiectasis present on CT scans.

A control population of individuals with PiMM was felt necessary for this study and was facilitated through the Subpopulations and Intermediate Outcome Measures in COPD Study (SPIROMICS) (59). SPIROMICS BAL samples collected using a similar methodology as that used in the GRADS Study are being shared with the GRADS Alpha-1 program (59).

The study design approved by the GRADS Observational Safety Monitoring Board sought matching between the four cohorts on age within 5 years and $FEV_1\%$ predicted within one 2011 GOLD stage (58). A key feature of the study design is to find comparable patients because individuals with PiZZ are, on average, 10 years younger than individuals with PiMM with similarly severe COPD. An additional demographic difference is related to the fact that individuals choosing not to use augmentation therapy are typically less ill and have better lung function than most individuals on AAT augmentation therapy. PiMM samples are selected from the SPIROMICS cohort by matching age and FEV_1 to the PiZZ not receiving augmentation cohort.

Recruitment

Recruitment into this study is heavily dependent on contact via mail, e-mail, and published invitations to individuals affected by AATD. Recruitment is administered by the Alpha-1 Foundation Research Registry at the Medical University of South Carolina (MUSC). This registry has been in existence since 1996 and has contact information and genotypes on more than 5,000 individuals with the severe deficiency or carrier state. Additional recruitment came from the Alpha Coded Testing Study, a home genetic testing cohort at MUSC (60), Alpha-1 Foundation Education days, and Alpha-1 Foundation publications.

The study inclusion and exclusion criteria are presented in Table 1. Importantly, participants could be smoking, but they must not have used antibiotics for 4 weeks and could not be using any immunosuppressant or prednisone greater than 10 mg daily or an equivalent. Written informed consent is obtained from all participants.

Measurements

Questionnaires are collected to integrate with biosamples in future studies (Table 2).

Pulmonary function testing is performed in dedicated spirometry laboratories and includes spirometry before and after bronchodilators, measurement of plethysmographic lung volumes, and diffusing capacity of carbon monoxide (D_{LCO}) assessed using American Thoracic Society (ATS) criteria. A 6-minute-walk test is performed using ATS criteria.

Chest CT is performed with participants at TLC and residual volume by a rigid quality-controlled program following bronchodilator administration. Phantoms with a range of attenuation values are used at each center to approve scanners for use in the GRADS Study before patient scanning. Radiation exposure is determined by body mass index category and performed by helical protocol with participants in the supine position (Table 3). Scans are assessed for quality at the University of Pittsburgh Radiology Center. Structural changes in the parenchyma and airways are assessed both visually and quantitatively. A visual quantitative analysis of bronchiectasis is performed using bronchial dilatation, peribronchial wall thickening, and number of bronchiectasis segments as variables.

Quantitative analysis measures include lung volumes computed on the basis of CT (both lung and air volumes), density

Table 2. Questionnaires used for data collection

Questionnaire	Goal
Gastroesophageal Reflux Disease Questionnaire (GerdQ)	The GerdQ is a validated test for the correlate of anatomic reflux. This test determines if the lung microbiome is related to gastroesophageal reflux.
Past lung function tests and FEV_1 decline	Spirometry, TLC, RV, and D_{LCO} evaluations are collected from up to 5 yr of past tests to define an estimate of the mean yearly FEV_1 decline.
Charlson comorbidity index	This index is used to assess the comorbidities associated with COPD that might be impacted by microbiome findings.
St. George's Respiratory Questionnaire (SGRQ)	This 51-item respiratory system–specific quality of life questionnaire quantifies the 3 domains of symptoms, activity, and impact, as well as total score.
Modified Medical Research Council (mMRC) Dyspnea Scale	The mMRC quantifies activity associated dyspnea, which is an important biomarker of COPD severity and risk of death.
COPD Assessment Test (CAT)	The CAT is an 8-item questionnaire that quantifies symptom severity.
Questionnaire for Ease of Cough and Sputum Clearance	This 5-item questionnaire queries the impact of cough and sputum, and the results will be compared with those in the SPIROMICS cohort.
AlphaNet Exacerbation Questionnaire	This 10-item questionnaire addresses exacerbation frequency and duration and has been collected monthly on >2,000 individuals with severe deficiency of AAT. The results will assure the generalizability of the study cohort.
PROMIS Fatigue Instrument	This instrument queries fatigue in the affected population.
Hospital Anxiety and Depression Scale (HADS)	HADS is a 14-item questionnaire that assesses anxiety and depression, and it was also used in the SPIROMICS cohort.
MOT-Short Form 12	This is a 12-item general quality of life questionnaire.

Definition of abbreviations: AAT = alpha-1 antitrypsin; COPD = chronic obstructive pulmonary disease; D_{LCO} = diffusing capacity of carbon monoxide; MOT = Medical Outcomes Trust; PROMIS = Patient Reported Outcomes Measurement Information System; RV = residual volume; SPIROMICS = Subpopulations and Intermediate Outcome Measures in COPD Study.

Table 3. Chest computed tomography scanner protocols

	Siemens Healthcare			GE Healthcare	Philips Healthcare
	Sensation 64	SOMATOM Definition	SOMATOM Definition AS+ Definition Flash	VCT CT750 HD	Brilliance 64 iCT 128
Acquisition (tube current modulation off: CareDose, Auto mA, DoseRight, ACS)					
Acquisition mode	Spiral	Spiral	Spiral	Helical	Spiral Helix
Detector collimation (mm)	0.6	0.6	0.6	0.625	0.625
Energy (kVp)	120	120	120	120	120
Pitch	1.00	1.00	1.00	0.984	0.920
Gantry rotation time (s)	0.5	0.5	0.5	0.5	0.5
Exposure	Effective mAs	Effective mAs	Effective mAs	Tube Current (mA)	Effective mAs
Inspiration scan					
BMI <20 kg/m^2	80	85	90	145	105
BMI 20–30 kg/m^2	100	105	110	180	130
BMI >30 kg/m^2	145	150	165	270	190
Expiration scan					
BMI <20 kg/m^2	55	60	65	100	70
BMI 20–30 kg/m^2	55	60	65	100	70
BMI >30 kg/m^2	80	85	90	145	105
Reconstructions (no iterative reconstruction algorithms: iDOSE, ASIR, IRIS, IQ Enhance)					
Reconstruction 1					
Kernel	B35f	B35f	B35f	Standard	B
Thickness (mm)	0.750	0.750	0.750	0.625	0.67
Interval (mm)	0.500	0.500	0.500	0.500	0.50
Reconstruction 2					
Kernel	B46f	B46f	B46f	Bone	D
Thickness (mm)	0.750	0.750	0.750	0.625	0.67
Interval (mm)	0.500	0.500	0.500	0.500	0.50
Reconstruction 3					
Kernel	B60f	B60f	B60f	Lung	E
Thickness (mm)	2.000	2.000	2.000	2.500	2.500
Interval (mm)	2.000	2.000	2.000	2.500	2.500

Definition of abbreviations: ACS = automatic current selection; ASIR = adaptive statistical iterative reconstruction; BMI = body mass index; IRIS = iterative reconstruction in image space; kVp = kilovolt peak; mAs = milliampere seconds.
Reconstruction matrix 512 × 512 for all images.

mask analysis (percentage of voxels minus a predefined Hounsfield unit [HU] value threshold, such as −950 HU, −910 HU, or −856 HU), count of all automatically detected airway sections, lumen area, lumen perimeter, wall area, wall thickness, and wall area as a percentage of the total airway area.

Bronchoscopy is performed under conscious sedation on Visit 2 of the protocol. Oral samples are taken before lidocaine and include both saline rinses and tongue scrapings. The bronchoscope is instilled with saline collected for microbiome analysis before insertion into the patient, then passed orally without suction to the right middle lobe or lingula into a wedged position. A bronchial wash (20 ml) and BAL aliquots to total 360 ml of saline are infused into two adjacent segments. Bronchial brushings and biopsies are allowed by local sample acquisition protocols. A stool sample is collected for microbiome analysis to compare the lung and stool microbiomes.

Biosamples are collected for current and future use by following a quality-controlled protocol with sample processing done on the day of collection (Table 4). To maximize recovery of nucleic acids, BAL, sputum, and lung tissue specimens are processed according to a combination of procedures recently described (61, 62). Cellular material is pelleted, after which the supernatant is used for viral isolation and the pellet is used for bacterial DNA isolation.

For bacterial microbiome analysis, an approach is used that relies on sequence tags that enable taxonomic identification. DNA is extracted and subjected to polymerase chain reaction (PCR) amplification using primers directed at the bacterial ribosomal RNA gene (*16S* rRNA) that all bacteria possess. This gene has highly conserved regions and highly variable (V) regions (which can be used for taxonomic identification). The V1–V3 region of the *16S* rRNA gene will be amplified. PCR amplicon libraries are gel purified,

quantified, pooled in equal ratios by mass, and subjected to deep sequencing using the Illumina platform (Illumina, San Diego, CA). Sequences are grouped together into "taxons" based on sharing 97% identity, and these taxons are aligned with reference databases to assign each to a particular bacterial group (family, genus, or species level, depending on the level of precision possible). Bacterial numbers are generated by quantitative PCR of the *16S* gene.

To identify the virome, supernatants are passed through 0.45-mm and 0.22-mm filters. Viral DNA and RNA are isolated according to protocols recently published (63). Because there are no sequences shared by all viruses that could be used for sequence tag identification as with bacteria, viruses are identified by massive sequencing of all nucleic acids present (following physical separation from bacterial and host cells), termed *shotgun metagenomics*. The DNA and RNA from viral preparations are converted into bar-coded DNA and cDNA

Table 4. Study procedures and samples collected

Procedure	V1	V2
Informed consent	X	
Vital signs	X	
Pulmonary function testing		
Spirometry before and after bronchodilators	X	
Slow vital capacity	X	
Plethysmographic lung volumes	X	
Carbon Monoxide Diffusing Capacity	X	
Exercise: 6-minute walk test with oximetry and Borg Dyspnea Scale	XX	
Chest computed tomography with inspiration and expiration images	X	
Blood		
Serum	X	
EDTA plasma	X	
Citrate plasma	X	
P100 plasma	X	
Cell preparation tubes (two; centrifuged at the site)	X	
RNA (one with PAXgene kit [PreAnalytiX, Hombrechtikon, Switzerland])	X	
Urine		
Cotinine	X	
Stool		X
Bronchoscopy		
Mouth rinse specimen		X
Bronchoalveolar lavage 20-ml proximal aliquot		X
Bronchoalveolar lavage distal collection		X
Bronchoalveolar lavage cell count and differential		X
Mycobacterial culture		X

Definition of abbreviations: EDTA = ethylenediaminetetraacetic acid; V1 = Visit 1; V2 = Visit 2 (usually next day, but must be <30 days).

and deep sequenced on the Illumina HiSeq2000 platform. Each HiSeq run can generate up to 250 GB of data, allowing GRADS Study researchers to sample the virome at a very high depth of coverage.

Virome sequencing is accomplished by using a paired-end read strategy. The individual sequences are screened for human and bacterial sequence, trimmed for quality, then assembled using several approaches. The resulting coding sequence is then searched against several databases, including collections of mobile genetic elements (e.g., ACLAME database: http://aclame.ulb.ac.be/) and other sequenced metagenomes, to augment viral discovery and annotation.

Data Management and Analysis Plan
This cross-sectional study has a primary analysis goal of determining whether individuals with PiZZ on augmentation therapy have a difference in lower respiratory tract microbes compared with matched individuals with PiZZ who are not on augmentation therapy. Mixed models analysis will be used to adjust for FEV_1, age, sex, oral or inhaled corticosteroid use, bronchiectasis presence on CT examination

and its severity, historical exacerbation frequency, preceding rate of FEV_1 decline, serum C-reactive protein, BAL neutrophil percentage, BAL neutrophil elastase, and serum Z polymer concentration. Interaction effects will be characterized.

Sample size calculations for analysis of variance (ANOVA) suggested that a minimum of 35 samples per group would be required. A sample size of 200 patients overall for this protocol, 50 in each group, is optimal, including close to 40 with no detectable emphysema on CT scans and evenly split among individuals with no obstruction or mild, moderate, or severe obstruction.

For the primary analysis, matched cohorts of similar patients allow for paired t tests to compare the microbial content as measured by the number, richness, and diversity of microbes. McNemar's test is used to compare the lower respiratory tract microbiome and virome, assessed by the presence or absence of specific organisms. Conditional logistic regression is used to determine if there is an independent association with the use of augmentation therapy after controlling for presenting characteristics that differ between the two

populations. In secondary comparisons between all patients with PiZZ, we will compare the content and diversity of the microbiome in two cohorts of individuals stratified by time on augmentation therapy.

The remainder of the specific aims will be met using identical methods. *P* values less than 0.05 are considered significant following appropriate adjustment for multiple comparisons.

Integrated Genomics
State-of-the-art methods are applied for transcript profiling of mRNA, microRNA, and large noncoding RNA, including microarrays and sequencing techniques. The GIC uses state-of-the-art analytic approaches as well as new algorithms (mirConnX) that can be used to coanalyze mRNA and microRNA expression data from time-course or case–control studies to identify regulatory network modules relevant to the particular phenotype or disease (64). The GIC uses a combination of methods to generate significant testable hypotheses at the regulatory and protein interaction level. For global pathway analysis, we will use Ingenuity Pathway Analysis (Qiagen, Redwood City, CA) and MetaCore (GeneGo/Thomson Reuters, http://thomsonreuters.com/metacore/), and gene set analysis will be performed to identify specific signatures potentially relevant to infection. R packages for genomic meta-analysis are integrated: MetaDiagnosis for assessing study quality, MetaDE for differentially expressed gene detection, MetaPath for pathway analysis, MetaClass for classification analysis, MetaDimR for dimension reduction, MetaClust for gene clustering, and MetaNetwork for regulatory network construction.

We will use the taxonomic and operational taxonomic unit (OTU)-based profiles in a series of ordination, clustering, and community structure analyses designed to compare and identify significant shifts in 16S profiles using computational algorithms tuned for high-throughput next-generation sequencing. This includes the application of refined statistical approaches to identify core membership in the microbiome between groups of individuals, including analyses and comparisons of very low-abundance entities (using a suite of computational tools and visual analyses known as the Corbata tools) (65). Of particular importance in this study are to examine the derivation of OTUs from
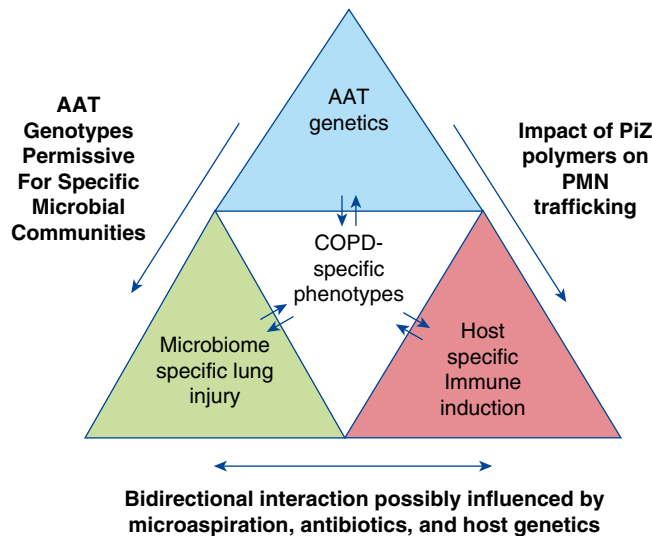
Figure 1. Alpha-1 Antitrypsin (AAT) deficiency, with its associated intermediate deficiency genotypes, can inform the study of chronic obstructive pulmonary disease (COPD) pathogenesis related to protease–antiprotease balance related to microbial communities and their immune impact. The resulting COPD clinical phenotypes may be related to a complex interaction between AAT genetics, microbial diversity or specific microbes, and immune phenotypes. A systems biology approach to this model will be applied in the Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis Alpha-1 Study. PiZ = protease inhibitor Z genotypes; PMN = polymorphonuclear leukocytes.

exogenous sources (e.g., patient sampling and laboratory procedures) versus the actual patient and, further, to better discriminate between legitimate biological mixing that can occur between the oral cavity and lower lung. To address this issue, we will apply a probabilistic approach to examining the microbial composition based on the consistency (i.e., variance) of the relative distribution of the taxonomic assignments or OTUs across the entire set of samples (i.e., laboratory controls, BAL controls, and experimental samples).

Further, we will integrate the availability of associated clinical patient metadata using various linear regression analyses to identify the factors that may be affecting the microbiome compositions and to define significant associations of groups of samples related by their 16S profiles identified within the cohort with clinical patient metadata (e.g., FEV_1, $D_{L_{CO}}$) using multivariate analyses. To identify the effect of factors on individual taxa, the taxonomic abundances in the profiles will first be analyzed using additive log ratio transformation and used as the response variable for a multivariate regression. To identify the effect of factors affecting the differences between samples, PERMANOVA will be used. PERMANOVA is a nonparametric permutation-based

algorithm for performing ANOVA between groups of samples.

Potential Outcomes and Conclusions

By characterizing the microbiome in AATD, we hope to define new phenotypes of COPD that explain some of the diversity of clinical presentations (Figure 1). This unique genetic cause of COPD has the potential to add to knowledge of the disease with implications for typical COPD pathogenesis, as well as the promise to increase understanding of the complex interplay between environment and genetics.

In addition, the GRADS Study biosamples allow us to use an integrative systems biology strategy designed to identify the functional and regulatory pathways that play roles in COPD pathophysiology. This approach relies on global genome, transcriptome, proteome, metabolome, and microbiome data sets collected in these cross-sectional patient cohorts. Although the biological approaches are hypothesis-generating, the results have the potential to change future hypotheses, lead to development of novel biomarkers, increase understanding of COPD phenotypes, and allow novel diagnostic and therapeutic trials

in AATD and COPD that can be validated in prospective clinical trials. ∎

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