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Authors

Moller, David R
Koth, Laura L
Maier, Lisa A
et al.

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Rationale and Design of the Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis (GRADS) Study

Sarcoidosis Protocol

David R. Moller[1], Laura L. Koth[2], Lisa A. Maier[3], Alison Morris[4], Wonder Drake[5], Milton Rossman[6], Joseph K. Leader[4], Ronald G. Collman[6], Nabeel Hamzeh[3], Nadera J. Sweiss[7], Yingze Zhang[4], Scott O'Neal[9], Robert M. Senior[10], Michael Becich[4], Harry S. Hochheiser[4], Naftali Kaminski[8], Stephen R. Wisniewski[9], and Kevin F. Gibson[4]; for the GRADS Sarcoidosis Study Group*

[1]Johns Hopkins University School of Medicine, Baltimore, Maryland; [2]Department of Medicine, University of California, San Francisco, San Francisco, California; [3]National Jewish Health, School of Medicine, Denver, Colorado; University of Colorado, Aurora, Colorado; [4]School of Medicine and [9]Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania; [5]Vanderbilt University School of Medicine, Nashville, Tennessee; [6]University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania; [7]University of Illinois Hospital and Health Sciences System, Chicago, Illinois; [8]Yale School of Medicine, New Haven, Connecticut; and [10]Barnes-Jewish Hospital/Washington University, St. Louis, Missouri

Abstract

Sarcoidosis is a systemic disease characterized by noncaseating granulomatous inflammation with tremendous clinical heterogeneity and uncertain pathobiology and lacking in clinically useful biomarkers. The Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis (GRADS) study is an observational cohort study designed to explore the role of the lung microbiome and genome in these two diseases. This article describes the design and rationale for the GRADS study sarcoidosis protocol. The study addresses the hypothesis that distinct patterns in the lung microbiome are characteristic of sarcoidosis phenotypes and are reflected in changes in systemic inflammatory responses as measured by peripheral blood changes in gene transcription. The goal is to enroll 400 participants, with a minimum of 35 in each of 9 clinical phenotype subgroups prioritized by their clinical relevance to understanding of the pathobiology and clinical heterogeneity of sarcoidosis. Participants with a confirmed diagnosis of sarcoidosis undergo a baseline visit with self-administered questionnaires, chest computed tomography, pulmonary function tests, and blood and urine testing. A research or clinical bronchoscopy with a research bronchoalveolar lavage will be performed to obtain samples for genomic and microbiome analyses. Comparisons will be made by blood genomic analysis and with clinical phenotypic variables. A 6-month follow-up visit is planned to assess each participant's clinical course. By the use of an integrative approach to the analysis of the microbiome and genome in selected clinical phenotypes, the GRADS study is powerfully positioned to inform and direct studies on the pathobiology of sarcoidosis, identify diagnostic or prognostic biomarkers, and provide novel molecular phenotypes that could lead to improved personalized approaches to therapy for sarcoidosis.

Keywords: bronchoalveolar lavage; lung; microbiome; phenotype; transcriptome

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*A complete list of members may be found before the beginning of the References.

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Correspondence and requests for reprints should be addressed to David R. Moller, M.D., Division of Pulmonary and Critical Care Medicine, Johns Hopkins University School of Medicine, 5501 Hopkins Bayview Circle, Baltimore, MD 21224. E-mail: dmoller@jhmi.edu

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Sarcoidosis is a multisystem inflammatory disease characterized by noncaseating granulomas at sites of inflammation (1). The lungs or intrathoracic lymph nodes are involved in over 90% of cases, with variable involvement of other organs and tissues. There is tremendous heterogeneity in sarcoidosis clinical manifestations, severity, and clinical course that vary among different ethnic and racial groups. Despite progress in the understanding of the genetic and immunologic basis of sarcoidosis, the determinants of its disease manifestations and clinical course remain unclear, as does its etiology. There are currently no validated biomarkers that provide for global organ assessment, prediction of disease course, or response to therapy.

The human microbiome is a critical determinant of health and disease (2). As the lung microbiome is likely to have a critical role in the pathobiology of immunologic lung diseases such as sarcoidosis, the National Heart, Lung, and Blood Institute solicited applications for the Genomic Research in Alpha-1 Antitrypsin Deficiency and Sarcoidosis (GRADS) study. This multicenter study involves a collaborative effort of a Genomics Informatics Center (GIC) and seven clinical centers.

The purpose of this article is to describe the design of and rationale for the sarcoidosis protocol of the GRADS study. This protocol was derived by the Sarcoidosis Protocol Development Committee from the initial hypothesis of the GIC that distinct patterns in the lung microbiome are characteristic of sarcoidosis phenotypes and are reflected in changes in systemic inflammatory responses as measured by peripheral blood changes in gene transcription.

Overview of Sarcoidosis

Clinical Heterogeneity

The clinical manifestations and disease course of sarcoidosis are both variable and largely unpredictable, representing a challenge to both the patient and clinician. Manifestations range from patients with acute sarcoidosis (or Löfgren syndrome) to those with chronic severe disease, including advanced lung disease, pulmonary hypertension, and cardiomyopathy (3). A majority of patients experience remission, typically within the first 2–3 years. However, 30–50% of patients have unremitting chronic disease that can lead to significant organ dysfunction requiring long-term corticosteroid or immunosuppressive therapy. Mortality may reach 12% among those with advanced disease (4).

The traditional method for characterizing sarcoidosis lung disease is to use a chest radiograph–based classification scheme referred to as the *Scadding stage staging system* (Table 1) (5). There is epidemiologic evidence that the Scadding stage offers some predictive value in estimating the likelihood of spontaneous remission (high in Scadding stage 0 or I but low in Scadding Stages III and IV). Unfortunately, evidence that this staging system or other biomarkers can accurately predict disease course is lacking.

Pathobiology

The pathologic hallmark of sarcoidosis is epithelioid granulomas consisting mainly of monocytes, macrophages, epithelioid and multinucleated giant cells, and T lymphocytes. Highlights of some of the central features of the pathobiology of sarcoidosis and findings that currently lack consensus are summarized in Table 2 (6). In the lung, the sarcoidosis inflammatory cascade displays a polarized T helper Type 1 (Th1) lymphocyte response, with increased production of IFN-γ, IL-2, IL-12, and IL-18 (7). Chemotactic and proinflammatory effector and regulatory cytokine signals lead to recruitment of additional monocytes and lymphocytes to the lung, macrophage differentiation into epithelioid and multinucleated giant cells, and eventually granuloma formation. More recently, studies have suggested that Th17 effector T-cell responses may contribute to sarcoidosis pathobiology, but whether these responses are critical to disease pathogenesis or play a role in shaping clinical phenotype remains uncertain (8, 9).

Both genetic susceptibility and environmental triggers are thought to cause sarcoidosis. Independent molecular and immunologic investigations have suggested the association of pathogenic mycobacteria with some cases of sarcoidosis (10). GRADS study investigators have contributed molecular evidence of mycobacterial RNA, DNA, and proteins in sarcoidosis tissues that are not found in control nonsarcoidosis tissues (11–13). Several research groups have reported enhanced Th1 immune responses to specific mycobacterial proteins in subjects with sarcoidosis compared with control subjects (14–18). Several studies show significant overlap between the transcriptome of patients with sarcoidosis and patients with tuberculosis (19–21). Japanese investigators have reported a strong linkage of propionibacteria and sarcoidosis among Japanese subjects (22, 23). However, there is no consensus on the role of microbial infection in sarcoidosis. Whereas some research groups promote the idea that chronic sarcoidosis results from an active, replicating mycobacterial or propionibacterial infection that may respond to antimicrobial therapies, others posit that sarcoidosis results from a hyperimmune Th1 response to tissue antigens (including those from remnant microbial antigens) that does not involve an active, ongoing infection (6, 24).The latter possibility may be associated with an aberrant innate response involving aggregation of serum amyloid A within granulomas (6, 25). These studies highlight the potential importance of interactions between the lung microbiome and genomic regulation in determining clinical phenotype and course in sarcoidosis, both of which are areas of focus in the GRADS study.

Molecular Signatures of Sarcoidosis Phenotypes

In recent studies, researchers have used genome-wide transcriptome profiling to better define sarcoidosis pathogenesis.

Table 1. Chest radiograph sarcoidosis Scadding staging system

Stage	Finding	Approximate Remission Rate
0	Normal chest radiography	Variable
I	Bilateral hilar lymphadenopathy (BHL)	60–70%
II	BHL plus pulmonary infiltrations	50%
III	Pulmonary infiltrations (without BHL)	20%
IV	Pulmonary fibrosis	<5%

Table 2. Central features and questions in the pathobiology of sarcoidosis

Pathobiologic features of sarcoidosis
• Highly polarized Th1 immunity at sites of disease
• Oligoclonal T-cell expansions consistent with T-cell antigen-driven inflammation
• Regulatory T-cell functional deficiency
• Genetic susceptibility predominantly involving HLA genes within the MHC locus
• Microbial triggers implicated in the etiology of a subset of sarcoidosis
• Innate immune pathway dysregulation
Pathobiologic questions in sarcoidosis
• Role of specific microorganisms in sarcoidosis etiology and pathogenesis (e.g., mycobacteria, propionibacteria, others)
• Role of active infection in sarcoidosis
• Role of serum amyloid A aggregation in promoting chronic disease
• Role of Th17 immunity in clinical phenotype
• Role of Th1, Th17, and Th2 immune transition in fibrotic sarcoidosis

Definition of abbreviations: HLA = human leukocyte antigen; MHC = major histocompatibility complex; Th = T helper cell.

These studies have confirmed an association with Th1 genes and pathways known to be relevant in sarcoidosis, including T-cell receptor pathways; cell signaling and proliferation; and cytokine genes, including IFN-γ (26, 27). GRADS study investigators evaluating peripheral blood gene expression in sarcoidosis found patterns of gene expression that are associated with disease severity (26, 27) and disease course (26). Together, these studies support the promise of the GRADS study to discover new molecular signatures to define sarcoidosis phenotypes and outcomes.

Overview of the Lung Microbiome

Human Microbiome

The *microbiome* refers to the set of microbial agents and their genes contained in an environment (2), and the term *metagenomics* refers to the analysis of genetic material recovered directly from an environment. Standard microbiologic and virologic methods can detect only a small proportion of the bacteria and viruses present in various body sites because the great majority of these organisms are uncharacterized or uncultivable. Thus, in practice, microbiomic studies rely on the use of metagenomic approaches—that is, sequence-based rather than culture-based approaches—to investigate microbial communities. Microbiomic studies may focus on fully enumerating all microbial agents in a body habitat through use of sequence tags that are shared between organisms within a group, such as the 16S ribosomal rRNA gene shared by all bacteria and archaea, or can define all of the genes within a community through "shotgun" metagenomic sequencing of all genetic material present (28–31).

Metagenomics has provided insight into the complex composition of the microbiome of several body sites, and this information has allowed us to draw tentative conclusions about the relationship between specific microbiomes and health (32–36). Understanding of the overlap and degree of communication between these microbiome niches is rudimentary at best. Perhaps the most extensively studied human microbiome is the human gut microbiome (37), where the interaction of the gut microflora, independently or through interaction with the genetic makeup of the host, plays a role in obesity, Crohn disease and ulcerative colitis (38–41).

Lung Microbiome in Health and Disease

In contrast to the extensive literature about the gut, much less is known about the composition of the microbial population of the lower respiratory tract in health or disease. It is likely that there are undetected organisms as well as complex relationships between the multiple pathogens seen in patients with chronic lung diseases. Although the healthy lung has traditionally been thought to be sterile, emerging data suggest that the microbial DNA present in the healthy lung closely resembles that of the oropharynx, but in markedly lesser amounts, perhaps resulting from microaspiration that occurs even in healthy individuals (42, 43). Researchers have also detected the presence of unique organisms such as *Tropheryma whipplei*, the agent of Whipple disease, in the lungs of some healthy people (42, 43) Studies of the lung microbiome are challenging, given the difficulty in distinguishing true residents of the lung versus those organisms that are carried over from the mouth by the bronchoscope, as well as difficulties of environmental contamination, given the low biomass of bronchoalveolar lavage (BAL) samples.

Role of Human Microbiome in Sarcoidosis

Recent studies support the emerging concept that the lung microbiome is altered in lung diseases such as chronic obstructive pulmonary disease, asthma, and cystic fibrosis (44–49), but little is known about the microbiome in other lung diseases, such as sarcoidosis. The presence of a chronic granulomatous inflammatory response in the lung in sarcoidosis has long suggested that infectious agents are important in sarcoidosis; to date, however, no culture-based studies have directly proven an infectious pathogenesis of the disease. In contrast to the limitations of traditional microbial cultures, culture-independent techniques have the capacity to enhance understanding of the identity and complexity of the microbial community within the lung in patients with sarcoidosis. Use of unbiased 16S sequencing in sarcoidosis has been examined in only one study (50). The investigators in that study did not find dramatic changes in the lung microbiome, but they examined only seven patients with sarcoidosis. Larger studies that encompass the multiple phenotypes of sarcoidosis are needed to determine the role of the microbiome in this disease.

Rationale for the GRADS Study

As indicated above, published studies suggest that the lung microbiome and genomic network interactions are likely to play critical roles in disease pathogenesis and determining clinical phenotypes in sarcoidosis. However, the current state of knowledge in these areas is rudimentary. The goal of the GRADS study is to perform a comprehensive analysis of the microbiome and genomic patterns and their interactions

in well-defined sarcoidosis phenotypes. Among the questions to be addressed are the following: How does the lung microbiome contribute to the phenotypic landscape? What are the critical interactions between the lung microbiome and host immune response networks that contribute to clinical phenotype and, particularly, clinical outcome? Are there genomic or microbiome signatures associated with clinical phenotype or outcome?

Study Objectives

To begin to answer these questions, the study investigators outlined the following aims: (*1*) to identify peripheral blood mononuclear cell (PBMC) gene expression patterns that characterize distinct sarcoidosis phenotypes; (*2*) to determine whether patterns in the lung microbiome are associated with sarcoidosis severity and disease phenotypes; (*3*) to correlate mRNA and microRNA expression patterns in sarcoidosis-affected organs with changes in microbiome, clinical parameters, and PBMC gene expression patterns; and (*4*) to integrate clinical, transcriptomic and microbiome data to identify novel molecular phenotypes in sarcoidosis.

Clinical Phenotypes

The aims of the GRADS study are to understand how genomic and microbiomic relationships associate with clinical phenotype, so it was necessary to define specific phenotypes important to the study. Not all manifestations could be included, owing to disease heterogeneity and the limited sample size of the study. The Sarcoidosis Protocol Development Committee prioritized nine phenotypes (Table 3) on the basis of considerations described in the paragraphs that follow.

First, more than 90% of patients with sarcoidosis have involvement of the lung, which is a well-studied site of disease pathogenesis and immune activity; thus, patients with this manifestation dominate our groupings (1, 51). Second, perhaps the most critical phenotypic distinction of biologic importance is the separation of remitting versus chronic sarcoidosis, the latter being disproportionate in its disease impact. Given the limitations of the study that precluded a lengthy follow-up period sufficient to confidently determine a chronic versus remitting outcome, phenotypes with known outcome expectations were chosen. For pulmonary sarcoidosis, we chose to distinguish pulmonary phenotypes on the basis of the Scadding chest radiograph staging system because different stages are associated with differences in outcome (Table 1). We theorize that the prognostic information reflects changing pathobiologic processes underlying the different radiographic stages. Third, both advanced lung disease with pulmonary fibrosis and cardiac sarcoidosis represent phenotypes that portend the highest mortality associated with sarcoidosis and thus deserve specific attention. Fourth, the effects of treatment were judged to be critical to interpretations of genomic and microbiome data.

Limitations in any phenotypic grouping of sarcoidosis are readily apparent. The instruments and definitions used to define organ involvement (and thus phenotype) are based on previously published studies (51, 52) but have not been validated in large clinical trials (*see* online supplement). In part this is due to the fact that instruments and definitions remain a "moving target" because advances in imaging continue to improve the sensitivity in detecting inflammatory sites in sarcoidosis. However, not all of these imaging changes have proven clinical, functional, or phenotypic significance. For example, the prognoses associated with the Scadding chest radiograph staging system were first used by international convention in the 1950s and were validated in multiple international studies in the subsequent decades. There is no similar scope of studies in which chest computed tomography (CT) correlates were used for prognostic staging. Despite these drawbacks, we selected the following nine phenotypes based on data from prior published clinical studies, such as A Case Control Etiologic Study of Sarcoidosis (ACCESS) (51) and clinicians' experiences:

- *Phenotype 1: Multiorgan sarcoidosis.* Patients with sarcoidosis with clinically significant inflammation in many organs may represent one of the purest phenotypes in terms of outcome because they invariably have chronic, unremitting disease. These patients have widespread inflammatory changes, often with the presence of abdominal sarcoidosis (typically with liver, spleen, bone, or bone marrow involvement and subdiaphragmatic lymphadenopathy) along with pulmonary or other extrapulmonary organ involvement. Although the number of organs that define this phenotype will vary with the instruments and definitions used, our intent was to define a group having a high likelihood of representing chronic sarcoidosis. To this end, we settled on five or more organs to meet the criteria for this phenotype, based more on clinical experience than on any specific data.
- *Phenotype 2: Stage I pulmonary sarcoidosis, untreated.* This fundamental phenotype represents a group with active sarcoidosis based on intrathoracic lymph node granulomatous inflammation, but

Table 3. Clinical phenotype study groups

Clinical Phenotype Focus Group	Presentation	Scadding stage	Treatment >3 Mo	Multiorgan	Clinical Course	Cardiac
Group 1: Multiorgan	N	Any	Any	Yes	C, U	Any
Group 2: Nonacute, Stage I, untreated	N	I	No	No	C, U	No
Group 3: Stage II-III, treated	N	II, III	Yes	No	C, U	No
Group 4: Stage II-III, untreated	N	II, III	No	No	C, U	No
Group 5: Stage IV, treated	N	IV	Yes	No	C, U	No
Group 6: Stage IV, untreated	N	IV	No	No	C, U	No
Group 7: Acute sarcoidosis	A	I, II, III	No	Any	C, U	No
Group 8: Remitting, untreated	Any	Any	No	Any	R	Any
Group 9: Cardiac defining therapy	Any	Any	Any	No	C, U	Yes

Definition of abbreviations: A = acute; C = chronic; N = nonacute; R = remitting; U = uncertain.

without radiographic evidence of lung inflammation. Although the patients with this phenotype overall have been found to undergo remission 70–80% of the time, there is heterogeneity within this subgroup as this stage is frequently seen in patients with extrapulmonary sarcoidosis with uncertain prognoses. Our planned study analysis will test whether biologic signatures in this group are associated with extrapulmonary organ involvement (or with those who require treatment) compared with those who are untreated and likely to experience remission.

- *Phenotypes 3 and 4: Stages II and III pulmonary sarcoidosis, untreated and treated.* The inclusion of these phenotypes is obvious because they represent subjects with known lung inflammation. The prognosis is uncertain, with remission occurring in approximately 50% of those with stage II and about 20% of those with Stage III pulmonary sarcoidosis when assessed at initial presentation. The Stage II and Stage III groups were combined because of the difficulty in reliably distinguishing a Stage II from a Stage III chest radiograph (51). Because therapy for sarcoidosis has the potential to change not only the immune response but also the lung microbiome, the GRADS Protocol Committee decided to separate treated and untreated pulmonary sarcoidosis phenotypes.
- *Phenotypes 5 and 6: Stage IV pulmonary sarcoidosis, untreated and treated.* These important phenotypes are characterized by pulmonary fibrosis that is associated with chronic, unremitting disease in more than 95% of patients. The characteristics of the changes in the inflammatory trajectory that preferentially lead to fibrosis in this group remain poorly understood.
- *Phenotype 7: Acute sarcoidosis, untreated.* Acute sarcoidosis, also known as Löfgren syndrome, is characterized by acute arthritis, bilateral hilar lymphadenopathy, and typically erythema nodosum and uveitis. This phenotype is unique in having a defined onset marked by arthritis or erythema nodosum, distinct genetic and immunologic signatures in Scandinavian patients (53), and a good prognosis, with over 70% of patients experiencing remission in subgroups with specific major histocompatibility complex haplotypes. This phenotype is much less common in black populations than in white populations in the United States and Europe, and its biologic determinants are not well defined.
- *Phenotype 8: Remitting sarcoidosis.* This phenotype is typically defined by patients who have had no evidence of active clinical disease for more than 1 year. It is not clear whether the biologic correlates of this phenotype will resemble those of healthy control subjects or will have remnants of biologic signatures of active disease that persist (but, e.g., at lower levels). Identifying those specific pathways may help focus research efforts on the relevant mechanisms underlying this transition.
- *Phenotype 9: Cardiac sarcoidosis.* Cardiac sarcoidosis is a major cause of sarcoidosis mortality and morbidity. Recent anecdotal experiences at sarcoidosis centers suggest that there has been a surge in the number of diagnosed cases in the past decade in the United States. We required this group to have cardiac manifestations sufficient to drive antiinflammatory intervention (rather than pulmonary or other manifestations) to maximize the likelihood of detecting candidate biological signatures that uniquely define this subgroup.

Study Design

We are conducting an observational cohort study to explore the role of microbiome and genomic regulation as they relate to specific sarcoidosis clinical phenotypes. The study was not designed as a case–control study to study the microbiome in sarcoidosis etiology. To achieve the goals of the study, clinical centers are recruiting subjects who meet the initial entry criteria (detailed below and in Table 4), including American Thoracic Society/European Respiratory Society criteria (1), for a diagnosis of sarcoidosis. Participants are either (*1*) classified within the targeted clinical phenotypes defined within this protocol upon review of their clinical information gathered from the initial study visit or (*2*) excluded from additional study participation. Eligible participants undergo self-administered questionnaires, physical examinations, research chest CT examinations, pulmonary function tests, and blood and urine tests (Table 5). Most subjects either undergo research BAL as part of an otherwise clinically indicated bronchoscopy for suspected pulmonary sarcoidosis or are recruited to undergo a research-only bronchoscopy with BAL. In the former group, if a diagnosis of sarcoidosis is confirmed by biopsy, the patient is then entered into the database of participants undergoing full testing and sample collection. If an alternative diagnosis is made for these recruits, they will not undergo any further testing. Participants can elect to undergo bronchial brushings and, if appropriate, skin biopsy of a suspected sarcoidosis skin lesion. Those subjects in the cardiac phenotype group and a subset of Stage IV pulmonary sarcoidosis participants will not undergo bronchoscopy, owing to a higher theoretical risk of bronchoscopic complications.

The rationale for collecting BAL, bronchial brushing, and blood samples is clear. High-throughput unbiased analyses of the lung microbiome can potentially identify patterns in the lung microbiome that determine disease activity and persistence as well as response to therapy. Sampling by BAL, bronchial brushing, and oral washing offer the opportunity to compare regional lung microbiome environments in the alveolar compartment with more proximal bronchial wall and oral environments. Focusing on accessible blood cells for genomic analyses enables GRADS study researchers to identify markers for disease phenotype, severity, and outcome that are easily transferable to the clinical arena. In addition, such studies may indicate whether blood sampling can be a surrogate for BAL sample analysis. Analysis of the transcriptomes in sarcoidosis tissues (BAL cells and peripheral blood), the microbiome in BAL fluid, and biochemical signatures in BAL, serum, plasma, and urine provides an opportunity to identify new molecular phenotypes that correlate with disease phenotype and organ system involvement.

Recruitment Goals

To achieve the aims of the study, we plan a recruitment goal of 400 participants with at least 35 participants in each phenotypic group based on sample size analysis (*see below*). Recruitment of participants with defined clinical phenotypes is monitored. To obtain information regarding clinical course, participants are asked to return for

Table 4. Inclusion and exclusion criteria

Inclusion criteria
1. Age between 18 and 85 yr
2. (a) Have a diagnosis of sarcoidosis established by consensus criteria (ATS/ERS) (1) and confirmed by either biopsy or by manifestations consistent with acute sarcoidosis (Löfgren syndrome) in absence of other known diagnosis; or (b) have a suspected diagnosis of sarcoidosis and scheduled to undergo biopsy procedure to confirm diagnosis of sarcoidosis using same consensus criteria (ATS/ERS) (1)
3. Able to tolerate and willing to undergo study procedures
4. Be capable of understanding study forms
5. Provide signed informed consent
Exclusion criteria
1. History of comorbid condition severe enough to significantly increase risks based on investigator discretion
2. Currently an active smoker
3. Undergoing bronchoscopy (clinical or research) with any one of the following:
a. Severe pulmonary impairment (<50% predicted FVC, <1 L FEV_1, D_{LCO} <40% predicted, resting hypoxemia <92% with or without supplemental oxygen)
b. Other comorbid disease that would preclude bronchoscopy
c. Hypersensitivity to or intolerance of any of the drugs required for sedation during conscious sedation bronchoscopy
4. Known systemic autoimmune disease such as rheumatoid arthritis, lupus, scleroderma, Sjögren syndrome
5. Found to have an alternative interstitial lung disease during evaluation and/or screening
6. Diagnosis of unstable cardiovascular disease, including myocardial infarction, in the past 6 wk; uncontrolled congestive heart failure; or uncontrolled arrhythmia
7. Use of anticoagulants (Patients taking warfarin or clopidogrel will be excluded; patients on aspirin alone can be studied even with concurrent use.)
8. Dementia or other cognitive dysfunction that, in the opinion of the investigator, would prevent the participant from consenting to or completing study procedures
9. Nonsarcoidosis pulmonary disease (e.g., rheumatoid arthritis, lupus, scleroderma) that, in the opinion of the investigator, limits the interpretability of the analysis of sarcoidosis pulmonary disease
10. Primary biliary cirrhosis or autoimmune hepatitis
11. Crohn disease
12. Chronic beryllium disease
13. Have an active bacterial or viral infection at time of screening
14. Have an active or ongoing serious infection, including HIV, HBV, and HCV
15. Active tuberculosis or are taking any medication for tuberculosis
16. Have a history of demyelinating diseases, lymphoproliferative diseases, or other malignancies other than presumed cured nonmetastatic skin cancer
17. Have evidence of a likely malignancy on chest X-ray
18. Are currently pregnant at time of screening
19. Currently institutionalized (e.g., prisons, long-term care facilities)
20. Hypersensitivity to or intolerance of albuterol sulfate or propellants or excipients of the inhalers
21. History of lung volume reduction surgery, lung resection, or bronchoscopic lung volume reduction in any form
22. History of lung or other organ transplant
23. Unable to comprehend consent document and/or questionnaires
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 4 wk can be rescreened for the study once the 4-wk window has closed.
2. Participants who present with current use of acute antibiotics or have taken acute antibiotics within the preceding 4 wk can be rescreened for the study ⩾28 d after discontinuing acute antibiotics.
3. Female participants who present <3 mo after giving birth will be asked to reschedule their visit until 3 mo have passed since the birth.
4. Former smokers who quit <3 mo before enrollment.

Definition of abbreviations: ATS = American Thoracic Society; ERS = European Respiratory Society; D_{LCO} = diffusing capacity of carbon monoxide; HBV = hepatitis B virus; HCV = hepatitis C virus.

clinical follow-up at 6 months if within the study timeline.

Clinical Outcome Measures

Questionnaires

Self-administered questionnaires are collected to assess dyspnea, fatigue, and quality of life using published instruments (Table 6 and online supplement).

Information on personal and medical risk factors, including demographic variables, current and past medical history, reflux history, and current and past medication use, is obtained. Detailed information on occupational, recreational, environmental, and residential exposures is assessed using modifications of the ACCESS occupational and environmental questionnaires (54). These variables will be correlated with phenotype-specific associations and with microbiome and genome expression patterns.

Physiology

Pulmonary manifestations of sarcoidosis are variable, with different subgroups demonstrating dominant restrictive, obstructive, or reduced diffusing capacity impairment or a mixture of these physiologic impairments. Pre- and post-bronchodilator spirometry is performed to assess the degree of bronchodilator responsiveness, which has not been systematically studied in sarcoidosis. To confirm restrictive ventilatory impairment,

Table 5. Study timeline

Data and Sample Collection	Screening Visit	Initial Visit, 0–21 d	6-Mo Visit
Informed consent	X		
History and physical examination		X	X
Blood, urine, and stool sample collection		X	X
Spirometry and D_{LCO}, and lung volumes if FVC <80%		X	
Subject self-administered questionnaires		X	X
Organ assessment instrument modified from the ACCESS instrument		X	
Research or clinical bronchoscopy		X	
Radiology (chest radiograph and chest CT)		X	
Skin biopsy (if deemed appropriate)		X	

Definition of abbreviations: ACCESS = A Case Control Etiologic Study of Sarcoidosis; CT = computed tomography; D_{LCO} = diffusing capacity of carbon monoxide.

lung volumes are obtained on participants whose slow vital capacity is less than 80% of the predicted value. American Thoracic Society/European Respiratory Society guidelines (55) serve as the primary guidance for the conduct and interpretation of spirometric (56) and lung volume (57) measurements and measurements of the single-breath carbon monoxide diffusing capacity (58).

Radiology

Radiologic studies include a standard chest radiograph to classify participants according to the Scadding staging system and a research chest CT scan (59). The chest CT protocol was developed to account for potential scanner variation across the clinical centers and body habitus (Table E1 in the online supplement) (60, 61). The helical (spiral) chest CT data are acquired with the participants in the supine position during breath holding at end inspiration (or total lung capacity) and without radiopaque contrast dye. Radiation exposure is based on three body mass index categories.

Study Procedures and Sample Collection

Recruitment

Potential study participants having either an established diagnosis of sarcoidosis or who are highly suspected of having sarcoidosis are recruited at the clinical centers through flyers, websites, mailings, and physician referrals. The determination of eligibility for study participation based on the inclusion and exclusion criteria listed in Table 4. Eligible participants with a confirmed sarcoidosis diagnosis are approached to request consent to undergo the study procedures using one of three consent forms, depending on whether a research bronchoscopy, clinical bronchoscopy, or no bronchoscopy is planned. All recruitment and consenting procedures are compliant with current Health Insurance Portability and Accountability Act regulations and were approved by the local institutional review board (IRB) as well as the IRB of the GIC.

A phenotype based on the categories noted above is determined with the use of a modified organ assessment instrument initially developed in the ACCESS study (52, 62). Eligible participants without a confirmed clinical diagnosis are consented before their clinical bronchoscopy; if a diagnosis is confirmed by tissue biopsy, the participant is enrolled in the study database and completes testing. Participants are asked to consent to a skin biopsy if cutaneous involvement is deemed consistent with sarcoidosis and a biopsy is planned for clinical purposes.

Bronchoscopy

Bronchoscopy is performed with the goal of minimizing bias in the collection of samples for microbiome analysis as previously described by GRADS study investigators (43). Briefly, before anesthesia is induced, the bronchoscopy is preceded by a tongue scraping and an oral rinse to collect microbial communities on the tongue and oral mouth and upper airway. The participant is then asked to gargle with an antiseptic wash for oral decontamination. A sample of the working channel of the bronchoscope is also collected to assess preexisting levels of microbial contamination. BAL is performed using a predesigned algorithm guided by radiographic sites of involvement in the chest radiograph or CT scan, with preferential locations being the right middle lobe and lingula. For those participants who agree to undergo

Table 6. Outcome questionnaires

1. Demographics, including race, age, sex, and SES; and past and current medical history, including infections, other medical conditions, and drugs (based on the ACCESS and Lung Microbiome Questionnaires) (67)
2. Gastroesophageal Reflux Disease Questionnaire (68)
3. An occupational, recreational, and environmental exposures questionnaire based on the ACCESS Research Group questionnaire
4. University of California San Diego Shortness of Breath Questionnaire
5. Fatigue Assessment Scale
6. Patient-Reported Outcomes Measurement Information System fatigue profile
7. Cognitive Failure Questionnaire
8. Medical Outcomes Study 12-item Short Form Health Survey

Definition of abbreviations: ACCESS = A Case Control Etiologic Study of Sarcoidosis; SES = socioeconomic status.

bronchial brushing, this procedure is performed following BAL.

Sample Collection and Processing

Samples collected during the study are detailed in Table E2. Bronchoscopic samples are processed to maximize sample integrity for RNA, microRNA, DNA, and proteins as recently described (63, 64). A portion of the BAL fluid is centrifuged to separate it into a cell-free BAL fluid supernatant and a BAL cell pellet for microbiome, transcriptomic, and biochemical analyses. For subjects undergoing a clinical bronchoscopy, a portion of the BAL sample is sent for routine clinical tests at the discretion of the bronchoscopist, and the remainder is collected for research purposes. Bronchial brushings are placed in a solution to preserve RNA integrity.

Blood is collected by phlebotomy, with aliquots sent to the clinical laboratory for tests typically obtained in assessing patients with sarcoidosis patients (Table E2). PBMCs are separated using a single-tube separation system, and serum and plasma are stored frozen. Aliquots of whole blood are placed in solutions designed to preserve RNA and DNA integrity. A urine sample is obtained and assayed for cotinine at the clinical site for an objective test of recent smoking. Stool samples are requested from participants who are undergoing research BAL collection for future gut microbiome comparison with the lung microbiome. Samples are shipped to the GIC, where they are stored in a biorepository.

Data Handling and Biorepository

Data are entered at the clinical sites using an encrypted, password protected, web-based system. Clinician-rated data are entered into this system from paper forms, and participant-rated data are entered directly into the system via a tablet computer. Radiographic data are transferred electronically to the GIC in a deidentified format compliant with the Digital Imaging and Communications in Medicine standard.

Data sharing is an important feature of the GRADS study. The GIC has developed a web-based data portal that provides dynamic exploratory cohort selection tools which allow data exploration across a large number of clinical variables. Microbiome and genomic data will be deposited in the appropriate National Center for Biotechnology Information repositories, such as the Database of Genotypes and Phenotypes, known as "dbGaP," in accordance with National Institutes of Health policies.

Study Analyses

Integrated Microbiome Analysis

For the identification of bacterial species within an environment, the amplification of 16S rRNA genes (or 16S rDNA) is useful because this genetic locus is present in all bacterial species. Polymerase chain reaction primers target conserved regions of the gene that are shared by all bacteria but flank hypervariable regions that differ. The nine hypervariable regions of the 16S rDNA can be used for bacterial species identification, with some regions having better discriminatory value than others. In contrast, there are no conserved genes that can be targeted for identification of all viruses; thus, whole shotgun sequencing of a sample enriched for viruses (such as by filtering) can lead to an effective characterization of viral communities. The GRADS study researchers will perform bacterial and viral analyses of oral washes, BAL samples, and environmental control samples to determine differences in microbial community structure in relation to sarcoidosis phenotype and clinical course.

Integrated Genomics Analysis

Few studies have incorporated host gene expression and microbiomic data to determine the interactions that occur between host and organisms. By integrating clinical, transcriptomic, and microbiome data, the GRADS study is positioned to identify novel molecular phenotypes as well as genomic correlates of known clinical phenotypes that will form the basis for molecular fingerprints to predict both disease severity and response to therapy as well as patient stratification for clinical trials.

Sample Size Analysis

Normalized gene expression levels will be related to established phenotypes and cross-phenotype characteristics using linear models (i.e., analysis of variance or linear regression tools). Sample size calculations for the analysis of variance are based on prior data showing that a minimum of 35 samples per group is required for detection with power exceeding 95% for changes of 1.25 or more, allowing for a single false positive and assuming 1,500 genes are truly differentially expressed (65, 66). Thus, an overall sample size of 400 participants, with a goal of 44 or 45 subjects per phenotype, is planned to allow for incomplete data collection.

Study Potential

Fundamentally, the GRADS study is a study of interactions. It integrates state-of-the-art high-throughput microbiome and genomic analyses in well-defined clinical phenotypes with measures of quality of life and environmental variables in a disease with tremendous unexplained clinical heterogeneity. This information is likely to provide a wealth of information on potential mechanistic pathways and interactions that play critical roles in disease pathogenesis and determine clinical manifestations, clinical course, and response to therapy. Future studies based on the data foundation provided by the GRADS study might include the multiple potential avenues of investigation shown in Table 7.

Table 7. GRADS study as a foundation for future studies of sarcoidosis

Associations of microbiomic and genomic signatures with clinical phenotypes provide for focused mechanistic studies on disease pathobiology
Genomic analyses from lung, epithelial brushings, and blood direct studies of local and systemic inflammatory pathways in lung and extrapulmonary disease
Validation of novel and previously discovered genomic biomarkers
Discovery of molecular phenotypes to inform personalized therapies based on relevant pathways
Molecular signatures of remitting vs. chronic sarcoidosis direct studies on manipulating relevant pathways and microflora for disease cure

Conclusions

The GRADS study is a multicenter collaboration tasked with investigating the role of the lung microbiome and system-wide genome expression in two orphan

diseases: α_1-antitrypsin deficiency and sarcoidosis. The sarcoidosis protocol is designed to explore how the lung microbiome and peripheral and lung genomes may shape different clinical phenotypes and outcomes in sarcoidosis, a disease with tremendous clinical heterogeneity and uncertain pathobiology and lacking in clinically useful biomarkers. The GRADS study researchers spearhead a singular effort to integrate microbiome and genomic analyses with rigorous clinical phenotyping of sufficient scope that should inform and direct future studies on the pathobiology and management of sarcoidosis for the foreseeable future. ■

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The GRADS Study Group

Study Chair

Barnes-Jewish Hospital/Washington University: Robert M. Senior, M.D.

Clinical Centers

Arizona Health Sciences Center: Cristine Berry, M.D., MHS; Nancy Casanova, M.D.; Joe Garcia, M.D.; James Knepler, Jr., M.D.; Kenneth Knox, M.D.; Jorge Navarrete, M.D.; and Isabel Oliva, M.D.

Johns Hopkins University: Linda Breslin, CCRC, CPFT; Edward Chen, M.D.; David Moller, M. D.; Rebecca Robinson, RN; and Zhimin Song, M.D.

Medical University of South Carolina: Rahul Argula, M.D.; Tatsiana Beiko, M.D.; Mary Brooks; Kimberly Brown, M.S.; Yaser Dawod, M. D.; Shalini Kanukala, BDS, MSCR; Luca Paoletti, M.D.; Richard Rissmiller, M.D.; Lynn Schnapp, M.D.; Charlie Strange, M.D.; Danielle Woodford, M.S.; and Deirdre Walker

National Jewish Health: Gina Atnes, B.A.; Briana Barkes, MPH; Russell Bowler, M.D., Ph.D.; Jill Elliott, M.S.; May Gillespie, B.A.; Nabeel Hamzeh, M.D.; Li Li, M.D., Ph.D.; Kristyn MacPhail, B.S.; Lisa Maier, M.D.; Peggy Mroz, MSPH; Robert Sandhaus, M.D.; and Lori Silveira, Ph.D.

University of California, San Francisco: Nirav Bhakta, M.D., Ph.D.; Melissa Ho, B.S.; Laura L. Koth, M.D.; Joris Ramstein, B.A.; Sara Sun, B.S.; and Prescott Woodruff, M.D., MPH

University of Illinois, Chicago: Nadera Sweiss, M.D.

University of Pennsylvania: Deborah Arnold; Eduardo Barbosa, M.D.; Frederic Bushman, Ph. D.; Ronald Collman, M.D.; Ayannah Fitzgerald, BSN, RN; Lisa Gardo, RN; Ize Imai; Maryl Kreider, M.D.; Karen Patterson, M.D.; Tamyra Riggs; and Milton Rossman, M.D.

University of Pittsburgh: Stephen Bruno, B.A.; Kevin Gibson, M.D.; and Yingze Zhang, Ph.D.

Vanderbilt University: Wonder Drake, M.D.

Yale School of Medicine: Percy Adonteng-Boateng, MB CHB, MPH; Donna Carrano, BSN; Mridu Gulati; and Erica Herzog, M.D., Ph.D.

Genomics and Informatics Centers

New York University: Elodie Ghedin, Ph.D.

J. Craig Venter Institute: Barbara Methe, Ph.D.

University of Pittsburgh: G. K. Balasubramani, Ph.D.; Michael Becich, M.D., Ph.D.; Rebecca Boes, B.S.; Kevin Gibson, M.D.; Jennifer Haverty, M.D.; Harry Hochheiser, Ph.D.; Naftali Kaminski, M.D.; Joseph K. Leader, Ph.D.; Melody Macey-Kalcevic, M.Ed.; Jeff Martin, MBA; Mary Martinez, M.S.; Alison Morris, M.D.; Scott O'Neal, M.A.; Nancy Petro, B.S.; Darina Protivnak, MSIS; Frank Sciurba, M.D.; Bill Shirey, M.S.; Laurie Silfies, B.S.; Mary Tranchine, B.S.; Stephen Wisniewski, Ph.D.; and Yingze Zhang, Ph.D.

Medical University of South Carolina

Charlie Strange, M.D.

Vanderbilt University

Wonder Drake, M.D.

Yale University

Heather Lynn, B.S., Naftali Kaminski, M.D.

NHLBI: Kevin J. Heath; Timothy M. Moore, M.D., Ph.D. (Observational Study Monitoring Board Executive Secretary); Antonello Punturieri, M.D., Ph.D.; and Lisa M. Viviano, BSN

Observational Study Monitoring Board

Jerome S. Brody, M.D.; Alicia Gerke, M.D., MBA; Stephen R. Gill, Ph.D.; Richard Holubkov, Ph.D.; and James Stoller, M.D. (Chair)

References

1 Statement on sarcoidosis: Joint Statement of the American Thoracic Society (ATS), the European Respiratory Society (ERS) and the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) adopted by the ATS Board of Directors and by the ERS Executive Committee, February 1999. *Am J Respir Crit Care Med* 1999;160:736–755.

2 Ley RE, Lozupone CA, Hamady M, Knight R, Gordon JI. Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat Rev* 2008;6: 776–788.

3 Iannuzzi MC, Rybicki BA, Teirstein AS. Sarcoidosis. *N Engl J Med* 2007; 357:2153–2165.

4 Swigris JJ, Olson AL, Huie TJ, Fernandez-Perez ER, Solomon J, Sprunger D, Brown KK. Sarcoidosis-related mortality in the United States from 1988 to 2007. *Am J Respir Crit Care Med* 2011;183: 1524–1530.

5 Scadding JG. Prognosis of intrathoracic sarcoidosis in England: a review of 136 cases after five years' observation. *BMJ* 1961;2: 1165–1172.

6 Chen ES, Moller DR. Sarcoidosis—scientific progress and clinical challenges. *Nat Rev Rheumatol* 2011;7:457–467.

7 Müller-Quernheim J, Prasse A, Zissel G. Pathogenesis of sarcoidosis. *Presse Med* 2012;41:e275–e287.

8 Richmond BW, Ploetze K, Isom J, Chambers-Harris I, Braun NA, Taylor T, Abraham S, Mageto Y, Culver DA, Oswald-Richter KA, *et al*. Sarcoidosis Th17 cells are ESAT-6 antigen specific but demonstrate reduced IFN-γ expression. *J Clin Immunol* 2013;33:446–455.

9 Facco M, Cabrelle A, Teramo A, Olivieri V, Gnoato M, Teolato S, Ave E, Gattazzo C, Fadini GP, Calabrese F, *et al*. Sarcoidosis is a Th1/Th17 multisystem disorder. *Thorax* 2011;66:144–150.

10 Gupta D, Agarwal R, Aggarwal AN, Jindal SK. Molecular evidence for the role of mycobacteria in sarcoidosis: a meta-analysis. *Eur Respir J* 2007;30:508–516.

11 Song Z, Marzilli L, Greenlee BM, Chen ES, Silver RF, Askin FB, Teirstein AS, Zhang Y, Cotter RJ, Moller DR. Mycobacterial catalase-peroxidase is a tissue antigen and target of the adaptive immune response in systemic sarcoidosis. *J Exp Med* 2005;201:755–767. [Published erratum appears in J Exp Med 2005; 202:721.]

12 Drake WP, Pei Z, Pride DT, Collins RD, Cover TL, Blaser MJ. Molecular analysis of sarcoidosis tissues for mycobacterium species DNA. *Emerg Infect Dis* 2002;8:1334–1341.

13 Oswald-Richter KA, Beachboard DC, Seeley EH, Abraham S, Shepherd BE, Jenkins CA, Culver DA, Caprioli RM, Drake WP. Dual analysis for mycobacteria and propionibacteria in sarcoidosis BAL. *J Clin Immunol* 2012;32:1129–1140.

14 Chen ES, Wahlström J, Song Z, Willett MH, Wikén M, Yung RC, West EE, McDyer JF, Zhang Y, Eklund A, *et al*. T cell responses to mycobacterial catalase-peroxidase profile a pathogenic antigen in systemic sarcoidosis. *J Immunol* 2008;181: 8784–8796.

15 Oswald-Richter KA, Culver DA, Hawkins C, Hajizadeh R, Abraham S, Shepherd BE, Jenkins CA, Judson MA, Drake WP. Cellular responses to mycobacterial antigens are present in bronchoalveolar lavage fluid used in the diagnosis of sarcoidosis. *Infect Immun* 2009; 77:3740–3748.
16 Oswald-Richter KA, Beachboard DC, Zhan X, Gaskill CF, Abraham S, Jenkins C, Culver DA, Drake W. Multiple mycobacterial antigens are targets of the adaptive immune response in pulmonary sarcoidosis. *Respir Res* 2010;11:161.
17 Oswald-Richter K, Sato H, Hajizadeh R, Shepherd BE, Sidney J, Sette A, Newman LS, Drake WP. Mycobacterial ESAT-6 and katG are recognized by sarcoidosis CD4+ T cells when presented by the American sarcoidosis susceptibility allele, DRB1*1101. *J Clin Immunol* 2010;30:157–166.
18 Dubaniewicz A, Trzonkowski P, Dubaniewicz-Wybieralska M, Dubaniewicz A, Singh M, Myśliwski A. Mycobacterial heat shock protein-induced blood T lymphocytes subsets and cytokine pattern: comparison of sarcoidosis with tuberculosis and healthy controls. *Respirology* 2007;12:346–354.
19 Koth LL, Solberg OD, Peng JC, Bhakta NR, Nguyen CP, Woodruff PG. Sarcoidosis blood transcriptome reflects lung inflammation and overlaps with tuberculosis. *Am J Respir Crit Care Med* 2011;184: 1153–1163.
20 Thillai M, Eberhardt C, Lewin AM, Potiphar L, Hingley-Wilson S, Sridhar S, Macintyre J, Kon OM, Wickremasinghe M, Wells A, *et al*. Sarcoidosis and tuberculosis cytokine profiles: indistinguishable in bronchoalveolar lavage but different in blood. *PLoS One* 2012;7:e38083.
21 Maertzdorf J, Weiner J III, Mollenkopf HJ, Bauer T, Prasse A, Müller-Quernheim J, Kaufmann SH; TBornotTB Network. Common patterns and disease-related signatures in tuberculosis and sarcoidosis. *Proc Natl Acad Sci USA* 2012;109:7853–7858.
22 Zhou Y, Hu Y, Li H. Role of *Propionibacterium acnes* in sarcoidosis: a meta-analysis. *Sarcoidosis Vasc Diffuse Lung Dis* 2013;30:262–267.
23 Ebe Y, Ikushima S, Yamaguchi T, Kohno K, Azuma A, Sato K, Ishige I, Usui Y, Takemura T, Eishi Y. Proliferative response of peripheral blood mononuclear cells and levels of antibody to recombinant protein from *Propionibacterium acnes* DNA expression library in Japanese patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2000;17:256–265.
24 Oswald-Richter KA, Drake WP. The etiologic role of infectious antigens in sarcoidosis pathogenesis. *Semin Respir Crit Care Med* 2010;31: 375–379.
25 Chen ES, Song Z, Willett MH, Heine S, Yung RC, Liu MC, Groshong SD, Zhang Y, Tuder RM, Moller DR. Serum amyloid A regulates granulomatous inflammation in sarcoidosis through Toll-like receptor-2. *Am J Respir Crit Care Med* 2010;181:360–373.
26 Su R, Li MM, Bhakta NR, Solberg OD, Darnell EP, Ramstein J, Garudadri S, Ho M, Woodruff PG, Koth LL. Longitudinal analysis of sarcoidosis blood transcriptomic signatures and disease outcomes. *Eur Respir J* 2014;44:985–993.
27 Zhou T, Zhang W, Sweiss NJ, Chen ES, Moller DR, Knox KS, Ma SF, Wade MS, Noth I, Machado RF, *et al*. Peripheral blood gene expression as a novel genomic biomarker in complicated sarcoidosis. *PLoS One* 2012;7:e44818.
28 Handelsman J, Rondon MR, Brady SF, Clardy J, Goodman RM. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem Biol* 1998;5:R245–R249.
29 Smit E, Leeflang P, Gommans S, van den Broek J, van Mil S, Wernars K. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Appl Environ Microbiol* 2001;67: 2284–2291.
30 Dinsdale EA, Pantos O, Smriga S, Edwards RA, Angly F, Wegley L, Hatay M, Hall D, Brown E, Haynes M, *et al*. Microbial ecology of four coral atolls in the Northern Line Islands. *PLoS One* 2008;3:e1584.
31 Morgan XC, Huttenhower C. Chapter 12: human microbiome analysis. *PLoS Comput Biol* 2012;8:e1002808 .
32 Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. *Proc Natl Acad Sci USA* 1999;96:14547–14552.
33 Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE. Bacterial diversity in human subgingival plaque. *J Bacteriol* 2001;183:3770–3783.
34 Gao Z, Tseng CH, Pei Z, Blaser MJ. Molecular analysis of human forearm superficial skin bacterial biota. *Proc Natl Acad Sci USA* 2007;104:2927–2932.
35 Bik EM, Eckburg PB, Gill SR, Nelson KE, Purdom EA, Francois F, Perez-Perez G, Blaser MJ, Relman DA. Molecular analysis of the bacterial microbiota in the human stomach. *Proc Natl Acad Sci USA* 2006;103:732–737.
36 Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. *Science* 2006;312: 1355–1359.
37 Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, *et al*.; MetaHIT Consortium. Enterotypes of the human gut microbiome. *Nature* 2011;473:174–180.
38 Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 2006;444:1027–1031.
39 Gophna U, Sommerfeld K, Gophna S, Doolittle WF, Veldhuyzen van Zanten SJ. Differences between tissue-associated intestinal microfloras of patients with Crohn's disease and ulcerative colitis. *J Clin Microbiol* 2006;44:4136–4141.
40 Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* 2007;104:13780–13785.
41 Spor A, Koren O, Ley R. Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat Rev Microbiol* 2011;9: 279–290.
42 Charlson ES, Bittinger K, Haas AR, Fitzgerald AS, Frank I, Yadav A, Bushman FD, Collman RG. Topographical continuity of bacterial populations in the healthy human respiratory tract. *Am J Respir Crit Care Med* 2011;184:957–963.
43 Morris A, Beck JM, Schloss PD, Campbell TB, Crothers K, Curtis JL, Flores SC, Fontenot AP, Ghedin E, Huang L, *et al*.; Lung HIV Microbiome Project. Comparison of the respiratory microbiome in healthy nonsmokers and smokers. *Am J Respir Crit Care Med* 2013; 187:1067–1075.
44 Flanagan JL, Brodie EL, Weng L, Lynch SV, Garcia O, Brown R, Hugenholtz P, DeSantis TZ, Andersen GL, Wiener-Kronish JP, *et al*. Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with *Pseudomonas aeruginosa*. *J Clin Microbiol* 2007;45:1954–1962.
45 Bahrani-Mougeot FK, Paster BJ, Coleman S, Barbuto S, Brennan MT, Noll J, Kennedy T, Fox PC, Lockhart PB. Molecular analysis of oral and respiratory bacterial species associated with ventilator-associated pneumonia. *J Clin Microbiol* 2007;45:1588–1593.
46 Bittar F, Richet H, Dubus JC, Reynaud-Gaubert M, Stremler N, Sarles J, Raoult D, Rolain JM. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *PLoS One* 2008;3:e2908.
47 Kistler A, Avila PC, Rouskin S, Wang D, Ward T, Yagi S, Schnurr D, Ganem D, DeRisi JL, Boushey HA. Pan-viral screening of respiratory tract infections in adults with and without asthma reveals unexpected human coronavirus and human rhinovirus diversity. *J Infect Dis* 2007;196:817–825.
48 Hilty M, Burke C, Pedro H, Cardenas P, Bush A, Bossley C, Davies J, Ervine A, Poulter L, Pachter L, *et al*. Disordered microbial communities in asthmatic airways. *PLoS One* 2010;5:e8578.
49 Erb-Downward JR, Thompson DL, Han MK, Freeman CM, McCloskey L, Schmidt LA, Young VB, Toews GB, Curtis JL, Sundaram B, *et al*. Analysis of the lung microbiome in the "healthy" smoker and in COPD. *PLoS One* 2011;6:e16384.
50 Garzoni C, Brugger SD, Qi W, Wasmer S, Cusini A, Dumont P, Gorgievski-Hrisoho M, Mühlemann K, von Garnier C, Hilty M. Microbial communities in the respiratory tract of patients with interstitial lung disease. *Thorax* 2013;68:1150–1156.
51 Baughman RP, Teirstein AS, Judson MA, Rossman MD, Yeager H Jr, Bresnitz EA, DePalo L, Hunninghake G, Iannuzzi MC, Johns CJ, *et al*.; Case Control Etiologic Study of Sarcoidosis (ACCESS) research group. Clinical characteristics of patients in a case control study of sarcoidosis. *Am J Respir Crit Care Med* 2001;164:1885–1889.

52 Judson MA, Costabel U, Drent M, Wells A, Maier L, Koth L, Shigemitsu H, Culver DA, Gelfand J, Valeyre D, *et al*. The WASOG Sarcoidosis Organ Assessment Instrument: an update of a previous clinical tool. *Sarcoidosis Vasc Diffuse Lung Dis* 2014;31:19–27.
53 Grunewald J, Brynedal B, Darlington P, Nisell M, Cederlund K, Hillert J, Eklund A. Different HLA-DRB1 allele distributions in distinct clinical subgroups of sarcoidosis patients. *Respir Res* 2010;11:25.
54 Newman LS, Rose CS, Bresnitz EA, Rossman MD, Barnard J, Frederick M, Terrin ML, Weinberger SE, Moller DR, McLennan G, *et al*.; ACCESS Research Group. A case control etiologic study of sarcoidosis: environmental and occupational risk factors. *Am J Respir Crit Care Med* 2004;170:1324–1330.
55 Miller MR, Crapo R, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Enright P, van der Grinten CP, Gustafsson P, *et al*. General considerations for lung function testing. *Eur Respir J* 2005;26:153–161.
56 Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, *et al*. Standardisation of spirometry. *Eur Respir J* 2005;26:319–338.
57 Wanger J, Clausen JL, Coates A, Pedersen OF, Brusasco V, Burgos F, Casaburi R, Crapo R, Enright P, van der Grinten CP, *et al*. Standardisation of the measurement of lung volumes. *Eur Respir J* 2005;26:511–522.
58 Macintyre N, Crapo RO, Viegi G, Johnson DC, van der Grinten CP, Brusasco V, Burgos F, Casaburi R, Coates A, Enright P, *et al*. Standardisation of the single-breath determination of carbon monoxide uptake in the lung. *Eur Respir J* 2005;26:720–735.
59 Drent M, De Vries J, Lenters M, Lamers RJ, Rothkranz-Kos S, Wouters EF, van Dieijen-Visser MP, Verschakelen JA. Sarcoidosis: assessment of disease severity using HRCT. *Eur Radiol* 2003;13: 2462–2471.
60 Leader JK, Zheng B, Rogers RM, Sciurba FC, Perez A, Chapman BE, Patel S, Fuhrman CR, Gur D. Automated lung segmentation in X-ray computed tomography: development and evaluation of a heuristic threshold-based scheme. *Acad Radiol* 2003;10:1224–1236.
61 Pu J, Leader JK, Zheng B, Knollmann F, Fuhrman C, Sciurba FC, Gur D. A computational geometry approach to automated pulmonary fissure segmentation in CT examinations. *IEEE Trans Med Imaging* 2009;28:710–719.
62 Judson MA, Baughman RP, Teirstein AS, Terrin ML, Yeager H Jr. Defining organ involvement in sarcoidosis: the ACCESS proposed instrument. ACCESS Research Group—A Case Control Etiologic Study of Sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 1999;16: 75–86.
63 Victoria JG, Kapoor A, Dupuis K, Schnurr DP, Delwart EL. Rapid identification of known and new RNA viruses from animal tissues. *PLoS Pathog* 2008;4:e1000163.
64 Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, Gordon JI. Viruses in the faecal microbiota of monozygotic twins and their mothers. *Nature* 2010;466:334–338.
65 Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol* 1995;57:289–300.
66 Davé NB, Kaminski N. Analysis of microarray experiments for pulmonary fibrosis. *Methods Mol Med* 2005;117:333–358.
67 ACCESS Research Group. Design of a case control etiologic study of sarcoidosis (ACCESS). *J Clin Epidemiol* 1999;52:1173–1186.
68 Jones R, Junghard O, Dent J, Vakil N, Halling K, Wernersson B, Lind T. Development of the GerdQ, a tool for the diagnosis and management of gastro-oesophageal reflux disease in primary care. *Aliment Pharmacol Ther* 2009;30:1030–1038.