The AZITRAMBA trial: Azithromycin treatment for the airway microbiome in asthma

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Background

Asthma is a common, complex, heterogeneous disease with increasing prevalence and morbidity that results from interactions between genetic and environmental factors (1-4). A modern, Western lifestyle, exposures to pollutants and diesel exhaust, key viral and bacterial infections in early life, and increased contact with select allergens, all promote asthma risk and over time lead to persistent airway inflammation and remodeling (5-16). Important investigations are focused appropriately on the origins and early-life development of asthma, yet there are tens of millions of adults with chronic asthma. Associations between bacterial colonization of infant airways and subsequent development of asthma (17), presence of specific bronchial bacterial pathogens in adults with asthma (18), efficacy of macrolide antibiotic treatment in selected asthmatic subjects (19,20), and data from culture-independent studies (21-28) suggest the tantalizing concept of a dysbiotic microbiome in chronic asthma. If that is true, if we modify that dysbiotic microbiome, can we improve asthma control?

Why study macrolides as a potential therapy for asthma? One enticing idea is to modify the airway microbiota using antibiotics such as azithromycin. Macrolides have both anti-inflammatory and antibiotic activity (29-32), and have been used in the treatment of bronchiectasis, diffuse pan-bronchiolitis, cystic fibrosis and COPD, particularly to decrease exacerbation rates (33-40). Several trials have evaluated the use of macrolides as adjunct therapy in adult asthma. Two small trials suggested that macrolides alter bronchial responsiveness (19,41). The AZISAST trial from Belgium in 2013, a randomized, double-blind, placebo controlled trial of azithromycin, 250 mg daily, as add-on therapy to combined inhaled corticosteroids and long-acting beta agonists over 6 months in 110 adult subjects, demonstrated a significant reduction in exacerbations in patients with non-eosinophilic, severe asthma (20). This particular sub-group responds poorly to other anti-inflammatory therapies (42,43). However, Cameron et al suggested a lack of efficacy of azithromycin in a 3 month trial in adult asthmatic smokers (44). Two studies examined adult patients with asthma who had serological evidence of infection with 'atypical' organisms: Black et al, demonstrated no improvement after treatment with roxithromycin over 6 weeks in patients with *C pneumoniae* (45), whereas Kraft et al, demonstrated improvement in FEV1 in patients with evidence for either *C pneumoniae* or *M pneumoniae* treated with clarithromycin for 6 weeks (46). The most recent randomized clinical trial in Australia and New Zealand, the AMAZES trial published in 2017, demonstrated that azithromycin added to combination controller therapy reduces asthma exacerbations over a 48 week period (47).

Why are these studies in conflict? The several studies above noted the mixed and sometimes negative results with macrolides. However, these studies were non-selective, and there is clear evidence that for some asthma therapies (as one example, the experience with anti-IL-5 antibodies) selecting the right patient population is of paramount importance. Asthma is a heterogeneous disease with multiple phenotypes (e.g., Th2-high versus Th2-low). The recent AsthmaNet

Microbiome study (26) suggested that patients with Th2-low asthma, the same group that responded in the AZISAST study, have a higher microbial burden than patients with the Th2-high phenotype. The AMAZES trial, in contrast, did not distinguish a difference in Th2-high versus Th2-low asthma in the efficacy of azithromycin on exacerbations. Differences in bacterial biomass or composition, all of which may reflect a dysbiotic microbiome, may be one key factor: azithromycin may work in these patients. None of the above-cited studies examined the microbiome, and so a key link to understand how macrolides might benefit patients with poorly-controlled asthma is missing. Neutrophilic asthma, one key Th2-low subtype, is associated with increased bacterial load and pathogens (identified by culture) such as *Hemophilus*, *Pseudomonas*, and *Moraxella* (48). Identifying the correct patient group may be the key to resolving the conflict of whether azithromycin is useful in asthma.

Why would a new study succeed where past, non-selective studies have failed? We hypothesize that in the right asthma phenotype – neutrophil dominant, Th2-low – modifying the dysbiotic microbiome in turn improves clinical control. Our trial will focus on the relation of asthma control to changes in the microbiome induced by azithromycin and understanding the influence of Th2-based phenotypes on this response. By careful examination of the dysbiotic microbiome, careful focus on Th2-low asthmatic patients, and focusing on patients with poor control in which the potential signal is substantial, we will answer the question definitively.

Purpose

The purpose of this protocol is to perform a short-term pilot study so as to acquire data critical to the proper design of a full-scale RCT. The primary objectives of the pilot study are to:

a) Estimate variability of microbiome composition and diversity over time in control and asthmatic subjects to provide a baseline for the future RCT
b) Estimate the response of the airway microbiome to azithromycin over time, so that we can generate appropriate power calculations and sample size estimates for the future RCT
c) Refine our microbial informatics pipeline for the future RCT
d) Refine our sputum gene expression and cytokine array testing
e) Test the feasibility of our intervention in a small cohort of subjects with poorly-controlled asthma

Study Design

This pilot protocol examines the change in the airway microbiome after azithromycin over an 8 week period. 40 subjects with poorly controlled asthma and 10 control subjects with no known lung disease will undergo clinical data collection including ACT score, lung function, exhaled nitric oxide (FeNO), methacholine challenge, and induced sputum and blood collection. Participants with asthma then are randomized 1:1 to receive azithromycin, 250 mg, or placebo, daily for 8 weeks. Clinical parameters including ACT, oral wash, adherence, side-effect profile, spirometry, and biological specimens (sputum, blood, FeNO) are collected again at 4 and 8 weeks. Control subjects undergo the same measurements, but do not receive any intervention.

Schedule of Events

	Asthma (A) or Control (C)	Visit 1/Week 0 (Screening) (+/- 3 days)	Visit 2/Week 4 (+/- 3 days)	Week 6 (+/- 3 days)	Visit 3/Week 8 (+/- 3 days)	Week 10 (+/- 3 days)	Visit 4/Week 12 (+/- 3 days)	Week 14 (+/- 3 days)
Informed Consent	A,C	X						
Site Visit	A,C	X	X		X		X	
Phone Call	A			X		X		X
Confirmation of Eligibility	A,C	X	X					
Randomization	A		X					
Drug Dispensation	A	X[1,2]	X[1,2,3]		X[1,2,3]		X[1,2]	
Demographics	A,C	X						
Medical History Review	A,C	X						
Asthma History	A	X						
Smoking History	A,C	X						
Concomitant Medication Review	A,C	X	X		X		X	
Physical Exam/Vital Signs	A,C	X	X		X		X	
Asthma Control Test	A	X						
Demographics Questionnaire	A,C	X						
Family History Questionnaire	A,C	X						
Work Exposures Questionnaire	A,C	X						
Home Exposures Questionnaire	A,C	X						
Physical Activity Questionnaire	A,C	X						
Secondhand Smoke Exposure Questionaire	A,C	X						
Peak Flow Meter Readings[5]	A	X	X	X	X	X	X	X
Electronic Diary [4]	A	X	X	X	X	X	X	X
ECG	A,C	X	X		X		X	
Spirometry with Reversibility[6]	A,C	X	X		X		X	
Exhaled NO	A,C	X	X		X		X	
Urine Pregnancy Test[7]	A,C	X						
Methacholine Challenge[8]	A,C	X	X		X		X	
Sputum Induction	A,C	X	X		X		X	
Oral Wash	A,C	X	X		X		X	
Complete Blood Count with Platelet Count and Differential Blood Sample	A,C	X			X			
Serum IgE Blood Sample	A,C	X						
RAST Blood Analysis	A,C	X						
Research Blood Samples	A,C	X			X			
Adverse Events Review	A,C		X	X	X	X	X	X
Compliance/Adherence to Diary and Study Drugs	A	X	X	X	X	X	X	X

1. Dispensation of albuterol to asthmatic subjects (if required at Visit 2 and Visit 3)
2. Dispensation of ICS/LABA to asthmatic subjects
3. Dispensation of azithyromycin/placebo to asthmatic subjects
4. Diary is completed twice daily by asthmatic subjects at home. Compliance will be reviewed at every visit (clinic or telephone).
5. Peak flow meter readings are done twice daily. Compliance will be reviewed at every visit (clinic or telephone).
6. Reversibility only conducted on those subjects with an FEV1% < 60% predicted.
7. Only required for females of child-bearing potential
8. Methacholine challenge only conducted on subjects whose FEV1% > 60% predicted.

Inclusion criteria (All participants):

1. Age 18 - 55 years
2. Able to provide informed consent
3. QTc < 440 ms on ECG
4. Smoking history < 10 pack-years

For subjects with poorly controlled asthma

1. Methacholine PC20 ≤ 16 mg/ml
2. Albuterol response ≥ 12% on FEV1 after 4 puffs of albuterol
3. Ability to use combination ICS + LABA
4. Meet definition for Th2-low asthma: peripheral blood eosinophil count < 300 and exhaled nitric oxide level < 30 ppb.

Exclusion criteria:

1. History of allergy or intolerance to any medications used in this study
2. Medication exclusions:
    a. Current use of medications that prolong QTc interval
    b. Current use of omalizumab or other ant-IgE therapies
    c. Current use of anti IL 5 therapies
    d. Current use of anticoagulants
    e. Prednsione or other oral steroids within past 3 months
3. Pregnancy or lactation
4. Other respiratory or inflammatory disorders (e.g., sarcoidosis, emphysema)
5. Pre-existing liver disease (AST or ALT >10% above the upper limit of normal)
6. Smoking within the last 6 months
7. Exacerbation of asthma in past 3 months
8. Affected by a hearing disorder
9. Clinically significant medical condition (e.g., heart failure, seizure disorder) which may increase risk as determined by study investigator
10. Corrected QT interval > 450 msec. Patients with known cardiac history or prolonged QT interval on a screening EKG are excluded given the small but real potential for macrolide-related side effects (49-54).

Recruitment

Subjects will be recruited from the Pulmonary and General Medicine clinics at the University of Chicago and at Northwestern University. We will also use advertisements including online, flyer and posters.

Duration of protocol

Participation may be up to 14 weeks.

Location where research is to be conducted

For subjects recruited at the University of Chicago, research is conducted in the Clinical Research Center (CRC) on W5 of the Gilman-Smith building. Some methods (e.g., methacholine) may be done in the Pulmonary Function Lab on the 5th floor of the DCAM building. For subjects recruited at Northwestern University, research is conducted at the Clinical Research Unit on the 11th floor of the Galter Pavilion. Samples and data collected at Northwestern will be sent to the University of Chicago.

Explanation of Tests and Procedures

1. Physical examination: Includes vital signs and examination of the cardiorespiratory system.
2. Asthma control test (ACT). This is a series of six questions (appended) that allow a patient to provide a numeric rating to key asthma symptoms. This is a research- and clinically-validated tool (61,62) and is a standard in the asthma research community.
3. Questionnaires. Subjects will be asked to complete six questionnaires. These questionnaires address subjects' home exposures, work exposures, family history, etc. These questionnaires should be completed by subjects at the visit 1.
4. Spirometry. The patient will be instructed at the time their visit is scheduled to hold beta agonist therapy and avoid caffeinated beverages for 12 hours prior to their study visit. If the subject's baseline FEV1 is below 60% of predicted value, the subject will receive 4 puffs of albuterol delivered by a metered dose inhaler. Spirometry will be repeated after 15 minutes in order to assess reversibility of obstruction.
5. Exhaled nitric oxide measurement. Subjects inhale deeply and then exhale slowly through the mouth into a tube. The exhaled gas is sampled and eNO measured.
6. Methacholine aerosol challenge test. Subjects whose baseline FEV1>60% will inhale increasing concentrations of methacholine starting with saline diluent followed by: 0.025 mg/ml, 0.1 mg/ml, 0.25 mg/ml, 1.0 mg/ml, 2.5 mg/ml, 10 mg/ml and 25 mg/ml. Spirometry will be performed after each dose until a ≥ 20% fall in the forced expiratory volume in one second is achieved or the highest dose of methacholine is administered. No further doses of methacholine will be administered once this threshold has been reached. The patient will then receive two puffs of albuterol via a metered dose inhaler and spirometry reassessed to ensure that the FEV1 has returned to within 10% of baseline. Participants will not be allowed to leave the center unless their FEV1 is within 10% of that visit's baseline.
7. Venipuncture to obtain 85ml of blood for safety and research samples.
   a. RAST: The test is used to check for allergic sensitivity to specific substances. In the test, the sample of blood is mixed with substances known to trigger allergies. The test measures the level of allergy antibodies (specific IgE antibodies) in the blood which are present if there is an allergic reaction.
8. Electrocardiogram (ECG). This is done to obtain a corrected QT interval (QTc). If the QTc is longer than 450 ms, patients will not be eligible for study given the very small but real risk of azithromycin-related cardiac events (54,55).

9. Sputum induction. The subject will be asked to breathe in a salty mist for up to 12 minutes. Every two minutes the subject will be asked to cough deeply and strongly in order to bring up mucus. Subjects may clear their saliva at any time during the procedure. If the subject produces an adequate specimen in the first four minutes the procedure may be terminated early. Subjects are advised that their active participation is important and that they should provide us with a vigorous, deep cough to produce sputum. Sputum samples will be sent to either University of Chicago or Northwestern for cell counts.
10. Oral wash. Subjects will be asked to rinse saline around in their mouth and spit it into a cup.
11. Electronic Diary. Asthmatic subjects will be asked to complete twice daily diaries in RED-Cap, a HIPAA-compliant, research oriented database (56). Subjects will receive a hyperlink via email at 7AM and 7PM. The hyperlink directs the subject to the electronic diary contained within REDCap. The diary is designed to require less than 2 minutes of the subjects' time and addresses the following:

    a. Symptoms from the night before and from the day of

    b. Physical limitations from the night before and from the day of

    c. Confirmation of study product dosing

    d. Peak flow value

12. Peak Flow Meter Readings. Asthmatic subjects will be asked to exhale forcefully into a peak flow meter once in the morning and once in the evening every day. The subject will record the value from the device into the electronic diary.

Asthma medications during the study (Asthma Subjects Only)

Before we begin, we will go over current asthma medications. During this study, we will be providing two asthma medications in a single inhaler, which is guideline-based treatment for the disease severity of the participants in this study. This may be a change from the asthma medicines the subject takes. We will take care that the subjects tolerate the change-over to the new medications. After their participation in this study is finished, we will ask them to see their primary care doctor who, with the subject, will decide which asthma medicines they should be taking at that point.

Starting on the day of visit 1 and continuing through the end of the study, subjects with asthma will receive a standard dosing of two asthma medications in a single inhaler as noted above.

Controller therapy. This will be done using a standard combined single inhaler that contains both an inhaled corticosteroid (fluticasone, 250 mcg) and a long-acting beta-adrenergic agonist (salmeterol, 50 mcg). This is provided in the Advair disk inhaler device and is a device that is familiar to most asthmatic subjects. Subjects use this medication twice daily, generally at about 8 am and 8 pm. The Advair disk has a counter which demonstrates the number of inhalations used by the patient; this number will be recorded by research staff at each in-person visit.

Rescue therapy. This will be done using a standard preparation of albuterol, 90 mcg per inhalation, per the device ProAir or equivalent metered dose inhaler. This device is familiar to most asthmatic subjects. Subjects use this medication when required for relief of acute asthma symptoms. Subjects will keep a diary card of the use of albuterol and provide this card to research staff at each visit.

Initial, thorough training in the use of each device is done during visit #1 by research staff using the Teach To Goal method implemented by the Asthma and COPD research team at the University of Chicago (63-65). Adherence to and proper technique in the use of each medication will be assessed at each visit, and will be recorded on forms by research staff. Subjects have a 24/7 access to one of the study physicians or other member of the research team for any problems associated with the use of these medications.

Subjects are instructed not to use other asthma medications during the study without informing research staff. The research lead physicians (Dr. White, Dr. Smith) or other member of the research team will contact primary care providers when necessary to ensure appropriate asthma care throughout the study. Subjects return to their prescribed asthma medication use at the end of the trial.

Study Drug

Azithromycin. We have received an exemption from the U.S. Food and Drug Administration (FDA) for the use of azithromycin in this protocol. The dose, 250 mg daily for 8 weeks, is a dose and regimen used in other lung diseases such as bronchiectasis, COPD, and cystic fibrosis (33-40).

Participants with asthma who complete the four week run-in phase (visit 1 to visit 2) will enroll at visit 2 into the study drug phase. They will receive the study drug, azithromycin (250 mg once a day for 8 weeks) or blinded placebo through the end of visit 4. They will be provided with a study drug diary. When they return at Visit 4 the study staff will collect the study drug bottle and count drug remaining. At the time of the telephone review (visit 5) subjects with asthma will be asked about any side-effects from azithromycin/placebo use.

Subjects are cautioned about potential side-effects of the drug and reminded about how to contact a study physician or other member of the research team.

Payment

Subjects will receive $100 at the end of each visit for a total of $400. This will come in a form of a check which will be mailed to their home.

Risks

Azithromycin

The use of azithromycin is contraindicated in patients with known hypersensitivity to any macrolide or ketolide, or in patients with a history of cholestatic jaundice or hepatic dysfunction associated with prior use of azithromycin.

Common:

-Mild Diarrhea
-Nausea
-Abdominal pain
-Vomiting
-Mild rash
-Nervous feeling

-Sleep problems

Very Uncommon:

-Diarrhea that is watery or bloody
-Headache with chest pain and severe dizziness
-Ototoxicity (hearing loss)
-Fainting
-Fast or pounding heartbeats
-Nausea
-Upper stomach pain
-Itching
-Loss of appetite
-Dark urine
-Clay-colored stool
-Jaundice (yellowing of the skin or eyes); or
-Severe skin reaction -- fever, sore throat, swelling in face or tongue, burning in eyes, skin pain, followed by a red or purple skin rash that spreads (especially in the face or upper body) and causes blistering and peeling

Rare:

Azithromycin is an antibiotic. Like with any other antibiotic, bacteria in the body may develop resistance to the medicine over time. Because of this the body may not fight off these bacteria as well as it should fight them off if these bacteria were to cause an infection. While the practical risk of this is very low, given what we know about azithromycin and resistant bacteria, if the subject were to develop an infection during or immediately after this study such as a bronchitis (infection of the breathing tubes), ear infection, throat infection, or sinus infection, the doctors should treat them with an antibiotic other than azithromycin.

There are known drug interactions with nelfinavir and with warfarin. Previous asthma-related studies using azithromycin have demonstrated few severe adverse events (20,41,44,57,58).

Spirometry:

The spirometry test may cause some study subjects to feel short of breath, lightheaded or dizzy. Treatment will be available if this occurs.

Methacholine challenge testing:

This involves the intentional narrowing of the airways by breathing the drug methacholine. This is likely to occur in subjects with asthma, but much less so in non-asthma controls. As a result of airway narrowing, participants may experience chest tightness, shortness of breath and/or coughing. It is possible that excessive airway narrowing may occur. However, this is unlikely because methacholine is delivered in incremental steps starting from very low doses. Spirometry is conducted between each dose-step and no more methacholine is delivered if the FEV1 is reduced by more than 20% from the post-saline baseline. In addition, bronchodilator medications that reverse airway narrowing will be immediately available. Participants will not be allowed to leave the center unless their FEV1 is within 10% of that visit's baseline.

Exhaled nitric oxide testing:

There is no significant risk to performing this test.

Sputum induction:

Breathing in a salty mist may cause an unpleasant salty taste after the procedure. Coughing hard to produce sputum can cause a sore throat. Other risks that rarely occur include: shortness of breath, wheeze, chest tightness, lightheadedness, nausea and headache. Extremely rarely, a subject may have a significant episode of shortness of breath and wheezing. These symptoms are usually prevented by the albuterol nebulizer treatment given as part of the spirometry shortly before sputum induction.

Blood Draw:

Includes momentary discomfort, bruising, bleeding, inflammation, or (rarely) infection at the site of needle insertion. On rare occasion fainting can occur as well. Both discomfort and bruising should disappear in a few days. A qualified member of the study team will draw the subject's blood to avoid or reduce these risks.

***Electrocardiogram (ECG)*:**

Includes momentary discomfort from application and removal of the pads used for testing.

Oral wash:

There are no risks with this test.

Azithromycin and Pregnancy / breastfeeding:

There are no adequate and well-controlled studies in pregnant women. It is considered FDA category B. Azithromycin'suse in pregnant or breast-feeding women is not recommended unless clearly needed.

Asthma exacerbation: This is covered in substantial detail in the DSMP, provided in the Appendix.

Inhaled corticosteroid therapy (fluticasone): Common: headache, back pain, sore throat sneezing, cough and nausea.

Long-acting beta agonist therapy (salmeterol): Common: headache, dizziness, lightheadness insomnia, nausea, dry mouth and throat irritation.

Short-acting beta agonist therapy (albuterol): Common: nervousness, shaking of a part of the body, headache, irritation of the throat, cough, and muscle, bone or back pain.

Pregnancy

Women of childbearing potential will undergo a pregnancy test on the day of study entry and at each visit. They must have a negative urine pregnancy test to be eligible for study entry. If the

test is positive, the study physician will discuss the matter with the participant. If a participant becomes pregnant before completing the study, they will be asked to notify one of the study doctors immediately. The investigator shall counsel the subject and discuss the risks of continuing with the pregnancy and the possible effects on the fetus. Monitoring of the pregnant subject shall continue until the conclusion of the pregnancy. Pregnancies will be reported to the data and safety monitoring board (DSMB) within one day of the site's awareness. Should the pregnancy result in a congenital abnormality or birth defect, a SAE will be submitted to the IRB, DSMB and NHLBI using the SAE reporting procedures described below.

Confidentiality

Each subject will be assigned a sequential identification number. This number will be linked to the subjects name and data.

We maintain strict confidentiality of all data to minimize the risk of inappropriate access or dissemination of such information. All protected health information including genotype data is saved on a password-protected computer or CRI server in a locked office with limited access. Only staff listed on this protocol will have access to the subjects name and data.

Data sent outside the University of Chicago or Northwestern University, will be sent using an identification number and not the participant's name.

Outcome Measures

Primary:

- ✓ ACT score change from 0 (baseline) to 8 weeks

Secondary:

- ✓ Change in FEV1 from 0 (baseline) to 8 weeks
- ✓ Sputum eosinophils change from 0 (baseline) to 8 weeks
- ✓ Sputum neutrophils change from 0 (baseline) to 8 weeks
- ✓ COMPEX score of asthma severity (from daily diary)
- ✓ Microbiome Shannon alpha-diversity score change from 0 (baseline) to 8 weeks
- ✓ Microbiome beta-diversity score change from 0 (baseline) to 8 weeks
- ✓ Microbiome change in relative proportion of top 10 genera from 0 (baseline) to 8 weeks

Data management

For demographic and clinical data we use a HIPAA compliant, web-based REDCap data management system residing on a password-protected network. REDCap is a web based, metadata-driven software application developed at Vanderbilt University (Harris et al, 2009) that is maintained by the Center for Research Informatics (CRI) at the University of Chicago. Northwestern University uses the same REDCap system. It allows for range and consistency checks during initial data entry to detect errors, and has built-in features for exporting data into Excel, SAS, STATA, or R files. Files exported from REDCap will be linked to the microbiome data using a unique patient ID number. Double-entry systems will be used to minimize coding error: our experience with our U19 AADCRC and with AsthmaNet has found that such double-entry reduces significantly the error-rate in getting information into accessible web forms, and retention

of the paper CRFs ensures proper audit procedures. For biospecimen tracking, we use a web based application with a MYSQL database and a robust security model.

Data and Safety Monitoring Board (DSMB)

The Board will consist of three members at the University of Chicago and at Northwestern University who are not part of this study. Members are Dr. Robert Guzy, Dr. Manu Jain (Chair), and Dr. Borko Javonavich (biostatistician). The DSMB will meet every 6 months to review all safety data and make recommendations to the NHLBI regarding continuation of the trial. A copy of the Data Safety and Monitoring Plan (DSMP) is located in Appendix 1.

Statistical analysis

Effect of treatment on clinical outcomes. Descriptive statistics will be generated to summarize the demographic and clinical characteristics of the asthma patients at baseline stratified by treatment arm. To compare changes in clinical parameters of interest, we will perform two-sample t-tests comparing the mean within-subject changes from baseline to 8 weeks. The primary and secondary outcomes measures are listed above.

For the COMPEX scores, we will determine the time to deterioration as defined in Fuhlbrigge et al (2017). This criteria defines a "diary event" as reaching a predefined change from baseline (threshold) for at least 2 consecutive days, or deterioration in all six variables comprising the COMPEX score over at least a 5-day period plus at least one variable reaching a threshold criterion for at least 2 consecutive days. A "CompEx event" is then defined as the first occurrence of either a diary event or a severe asthma exacerbation (deterioration of asthma leading to oral corticosteroid use, emergency room admission, or hospital admission). Patients who do not experience a CompEx event will be censored as of the last negative assessment. The time from baseline to a CompEx event in days will be compared in the two treatment arms using a logrank test.

Data analysis will be performed using R and STATA software. Linear mixed effects models will be fit for continuous longitudinal outcomes and ordinal, logit, and Poisson mixed effects models will be employed for ordinal, binary, and count responses, respectively (59).

Initial microbiome analysis. Microbiome analysis is done in collaboration with Jack Gilbert, PhD, who is Director of The Microbiome Center at the University of Chicago, which houses an advanced sample processing and sequencing core. Samples are partitioned into two aliquots: one for PCR amplification and analysis of the 16S rRNA V4-V5 hypervariable region using amplicon sequencing. We will independently do qPCR of total 16S rRNA of each sputum, oral and nasal sample to determine total load. We will use the Illumina MiSeq platform and barcoded primers to sequence PCR-libraries, and always include non-template and blank controls. The remarkable simplicity of airway microbiota allows a high level of multiplexing (>300 samples per MiSeq flow cell) and ensures 99.99% sequencing coverage after 10,000 reads.

We will limit sources of and account for potential contamination in our data pipeline, such as in reagents and kits (60,61) and in processing MiSeq-generated sequences (62,63). We will handle contamination issues by: 1) eliminating OTUs that are substantially increased when total 16S is low, 2) randomize samples to different extraction kits and re-run with a different lot-numbered kit to confirm findings, 3) do phenol-chloroform extractions instead of relying exclusively on kits, 4) maintain consistency in kits and lots for each analysis, and 5) cycle bar codes between runs.

We will use FASTX-Toolkit for quality trimming of Illumina files. The QIIME pipeline is used for primer trimming, chimera checking and sequence alignment, taxonomic classification and calculation of α and β diversity indices, and generation of rarefaction curves. We construct an abundance matrix of reference OTUs and phylogenetic trees derived from the GreenGenes reference set (64). We will employ a suite of multivariate statistical tools to compare amplicon data between and within sample types by canonical correlation analysis, regression profiling, and visualization (e.g. non-metric multi-dimensional scaling [NMDS], Principal Coordinates of Analysis, and Principal Components Analysis). We will also look at the species diversity of each sample and observe if there is any variation between the cohorts. In addition, we will use nonparametric statistics (ANOSIM, ADONIS, ANOVAR, PERMNOVA, etc.) to determine the statistical significance of participant factors. Significance of differences in relative percentage and diversity indices between groups will be calculated using Student's t-test. The Pearson correlation coefficient will be used to assess correlations among continuous variables.

Microbial bioinformatics. We will employ a suite of multivariate statistical techniques, network modeling and machine learning approaches to characterize the composition and structure of the microbial community between and within the normal subjects and asthma experimental arms, and determine microbial biomarkers that correlate with ACT score, albuterol use, changes in FEV1, and markers of airway inflammation. All amplicon sequencing data will be quality filtered and de-multiplexed and then subjected to de novo operational taxonomic unit (OTU) picking, and sub-OTU characterization using DeBlur (https://github.com/biocore/deblur.) Microbial diversity will be summarized using Chao1 estimator and Shannon index, and association with clinical and immune variables will be tested by regression analysis and generalized linear modeling. UniFrac distances (between-sample beta diversity) will be used to visualize and test the overall microbiota structure difference between study arms using multivariate methods such as principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA). DESeq2, which allows correction for multiple testing, will be used to perform differential abundance analysis to identify clinically relevant taxa (65). Based on these results, logit models will be generated using dichotomized clinical and immune variables as outcomes, and the microbiome data as independent variables. Variable selection methods will be integrated to avoid over-fitting. Since we expect that the number of genera will be approximately equal to 300, a dimension reduction will be performed so that efficient statistical inference can be conducted, using either the LASSO (66) or thresholding (67) methods within the context of high-dimensional inference for generalized linear mixed effects models. Classification performance will be evaluated using ROC curve and the 0.632+ bootstrap method (68). The machine learning approach, Random Forest, will also be applied to determine whether the microbiome is predictive of clinical outcomes (69). We will also examine whether there are natural grouping patterns in the microbial community data and, if so, how these grouping patterns relate to asthma phenotypes. To do this, we will compute Bray-Curtis dissimilarity measures between subjects and perform hierarchical cluster analysis and nonmetric multidimensional scaling to visualize if the groupings determined by the hierarchical clustering algorithm relate to asthma phenotypes. We will also fit multivariate analysis of variance (70) and ordination models for the relative abundance of the microbiome response against the treatment and time effects using the R package vegan (71).

We will analyze changes from the pre- to post-azithromycin treatment, and between azithromycin and placebo subjects, in 1) abundance of key microbial genera (e.g., Pseudomonas, on which our first power calculations are generated, as well as Streptococcus and Hemophilus), 2) change in alpha-diversity, richness and evenness indices, 3) principal coordinate analysis (PCoA) and non-metric multi-dimensional scaling (NMDS) of beta-diversity indices, and 4) com-

parisons of key changes to clinical parameters of most compelling interest: ACT score, methacholine responsiveness, FEV1, markers of airway inflammation such as BAL eosinophils and neutrophils, BAL concentrations (measured by multiplex assay) of Th2 related cytokines such as IL-5 and IL-13, IL-17 related cytokines such as IL-17a, and TGF-β1. The measurement of Th17-related cytokines is particularly important as we anticipate that 70% of our subjects will have a blood eosinophil count < 300 per μl or BAL eosinophil proportion < 3%, and azithromycin may be more effective in patients with eosinophil-low asthma (20).

Poisson modeling of relative abundance. To test for differences in relative abundance of key microbial genera between the placebo and azithromycin groups at each time point and over time, we will fit a Poisson regression model with treatment and time as fixed effects with offset (for the total abundance) and with subjects as random effects for the microbial genera of interest (72). Since missing data may be present in the data set, we will create a multiple imputation simulation function in R for generalized linear mixed effects models to improve efficiency of inference and to assess sensitivity of the model over multiple imputations using the R package mice. We will use the specific Poisson regression model to impute the missing values to obtain further efficiency in the data analysis. Based on the Poisson regression model fit, we will make inference for alpha, beta, and gamma diversity measures. These measures will be calculated using either Shannon entropy or the inverse Simpson index (in this context, the habitats of the microbiome genera are the samples collected on the treatment groups over time). Power and sample size calculations for the U01 RCT will be performed based on the fitted Poisson and multivariate regression models. If a test statistic is not significant in the pilot study, we will use mathematical statistical properties of the test statistics (such as the likelihood-ratio, the Wald test, or the score test statistics) under the alternative hypothesis which will allow us to generate power as functions of the sample size. We will create an R package in which we implement all statistical methods in the data analysis work-flow and create R markdown files with fully reproducible data analyses and description of statistical methods. In this way, the data analysis can be easily replicated. If new statistical methods are developed as a result of this complex multifacted data analysis, we will write a methodological paper for submission to a statistical journal.

Power calculations. Power calculations are difficult given the paucity of data about changes in the airway microbiome. This is explicitly an exploratory protocol to ascertain whether, in the short term, treatment of azithromycin in a small cohort of asthmatics changes the lower airway microbiome. We can generate potential power calculations based on the difference in (for example) relative abundance of pathogens in lower airway samples. For this protocol, a key goal is to obtain a good estimate of just how much the microbiome changes in response to azithromycin: if substantial, this trial will aid greatly our ability to make power calculations for larger trials. If changes are small, we will have a clear answer at a modest cost. For example, based on our preliminary published data (25), *Pseudomonas* abundance among asthma patients using ICS was 10.4 ± 1.8%. Assuming 20 evaluable patients on azithromycin in a before and after analysis, we will have 80% power (two-sided $\alpha=0.05$) to detect a change in abundance of $\Delta=0.9$ to $\Delta=1.2$ if the within-patient correlation between pre- and post-treatment abundance is $\rho=0.7$ or $\rho=0.5$. The half-width of the 95% confidence interval for the change in abundance will be 0.7 to 0.84 if $\rho=0.7$ or $\rho=0.5$.

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