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STUDY PROTOCOL

Title: The Effect of Chlorhexidine on the Oral and Lung Microbiota in Chronic Obstructive Pulmonary Disease

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A. Specific Aims

Aim 1. Determine the effect of twice-daily chlorhexidine oral rinse on oral and lung microbiota biomass in subjects with chronic obstructive pulmonary disease (COPD) with chronic bronchitis. Our primary outcome will be to compare the microbiota biomass (number of bacteria as measured by 16S rRNA copy number) of *induced sputum* and the *oral cavity* before and after 8 weeks of twice-daily chlorhexidine oral rinse (n=25) compared to controls (n=25) using qPCR and next-generation sequencing of the bacterial 16S rRNA gene comparing total bacterial biomass.

Our *hypothesis* is that 8 weeks of chlorhexidine oral rinse will decrease microbiota biomass compared to baseline and those on placebo. Furthermore, we hypothesize that chlorhexidine treatment will: i) decrease lung and oral microbiota diversity; ii) alter microbiota taxonomic composition in the lung and oral cavity; iii) decrease systemic inflammation as measured by blood high sensitivity C-reactive protein (hsCRP), fibrinogen and leukocyte count; and iv) demonstrate a trend towards improvement in respiratory health status as measured by the Breathlessness, Cough, and Sputum Scale (BCSS)[1, 2] and St. George's Respiratory Questionnaire (SGRQ).

> **Subaim 1: Determine if chlorhexidine alters the lung and oral rinse microbiota diversity and taxonomic composition.** Our *hypothesis* is that chlorhexidine oral rinse will decrease the diversity (Shannon and inverse Simpson diversity indices) and taxonomic composition of both oral and lung microbiota compared to those on placebo as determined by next-generation sequencing of the bacterial 16S rRNA gene.
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> **Subaim 2: Determine the impact of chlorhexidine on systemic inflammation.** Our *hypothesis* is that the decrease in lung microbiota biomass is associated with a decrease in systemic inflammation as measured by blood hsCRP, fibrinogen, and leukocyte count.
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> **Subaim 3: Determine if respiratory symptoms associate with the lung microbiota biomass.** Our *hypothesis* is that chlorhexidine will demonstrate improved respiratory health status as measured by the BCSS and SGRQ.

B. Background and Significance

COPD and Chronic Obstructive Bronchitis are major health concerns

Chronic obstructive pulmonary disease (COPD) is currently the third leading cause of death in the United States with an estimated prevalence of 23.6 million among all U.S. citizens [3]. **Within our Veteran population the prevalence of COPD is higher than the general population and thus smoking-related diseases are a targeted priority for research within the VA system**. A recent

screening with pulmonary function tests of Veterans in the upper Midwest identified a COPD prevalence of 40%[4]. Within our Veteran population COPD is also associated with a higher rate of all-cause and respiratory-related health care utilization and a high prevalence of co-morbidities[5], thus COPD represents a significant burden to Veterans and to the VA health care system. **Approximately one half of patients with moderate to severe COPD have symptoms of chronic bronchitis, also referred to as chronic obstructive bronchitis (COB)**, defined as chronic cough and phlegm production for $\geq$ 3 months for at least the last two years. When adjustments are made for age, gender, and severity of airflow obstruction, patients with chronic obstructive bronchitis experience more exacerbations, have worse respiratory health status, and have higher mortality than do patients without chronic bronchitis[6, 7], highlighting the need to find therapies that will improve symptoms and other clinical outcomes in this patient subset.

The role of bacteria in COPD

Bacteria are known to be important in COPD with approximately 50% of COPD exacerbations attributed to respiratory pathogens [8, 9]. Patients with COPD are often colonized with bacteria in their lower respiratory tracts even during periods of stable disease. This community of bacteria, or *microbiota*, may be a key factor in the pathogenesis of COPD by causing exacerbations and promoting inflammation. Most studies on the role of bacteria in the pathogenesis of COPD have employed traditional culture-based techniques, which identify only a small fraction (1%) of bacteria present. Current culture-independent methods to describe the microbiota rely on the analysis of the bacterial 16S rRNA gene, which provides a sensitive method of determining which bacterial species are present in complex environmental mixtures [10-14]. Sze and colleagues evaluated the lung tissue microbiomes of eight very severe COPD patients at the time of lung transplantation and noted increased bacterial diversity in the COPD patients compared to controls. **Our group's data show that the microbiome in COPD is more diverse compared to healthy controls and that it is reflective of oral flora,** suggesting that microaspiration and possibly decreased airway clearance plays a role in the lung microbiome[15]. Studies have demonstrated that individuals with COPD are more prone to aspiration due to reduced laryngotracheal mechanosensitivity [16, 17]. **Therefore, we theorize that individuals with chronic obstructive bronchitis have an altered lung microbiota due to increased aspiration of the oral microbiota; this altered lung microbiota contributes to disease manifestations**.

Treatment options for chronic obstructive bronchitis are limited

The most effective medications currently available for COPD are the long-acting inhaled bronchodilators and inhaled corticosteroids. These medications have a modest effect in reducing exacerbations and hospitalizations for COPD, but have not proven to be very effective in improving symptoms or disability and have consistently failed to improve symptoms as measured by health status questionnaires [18, 19]. Approximately one half of patients with moderate to severe COPD have symptoms of COB and these patients have more severe outcomes [6, 7]. Therefore, COB patients are in need of better therapies and also represent a large portion of COPD patients. They also readily produce sputum that can be used for microbiota analyses.

Chlorhexidine

Chlorhexidine is a cationic polybiguanide (bisbiguanide) that is primarily used as a topical antiseptic. The broad-spectrum bactericidal effect of chlorhexidine is primarily a result of the binding of this cationic molecule to negatively charged bacterial walls that results in a bacteriostatic effect in low concentration and disrupts bacterial membranes at higher concentrations. It is active against both gram-postive and –negative organisms and yeast. It is not effective against most viruses. Chlorhexidine is often used as an active agent in oral rinses to reduce dental plaque and oral bacteria and its clinical

efficacy and safety has been documented in a variety of settings such as gingivitis, periodontitis, dental trauma and tooth extraction. Chlorhexidine can be inactivated by anionic surfactants commonly used in toothpastes and mouthwashes. Therefore, chlorhexidine mouth rinses should be used at least 30 minutes after other dental products. Food, drink, smoking and mouth rinses should be avoided for at least one hour after use. This product is safe and only a few side effects have been reported such as rare, mild oral discomfort and a transient decrease in taste[20]. Tooth discoloration[21, 22] has been reported when chlorhexidine treatment was followed by the ingestion of chromogenic beverages, such as tea[23]. Oral bacteria aspiration has been implicated in pulmonary infection as recent studies have demonstrated that oral decontamination with chlorhexidine reduces the incidence of ventilator-associated pneumonia [24].

Efficacy of Oral Decontamination in COPD

The potential benefit of oral decontamination in the treatment of COPD remains unknown. We have compelling evidence that the COPD lung microbiota is more diverse than healthy lungs and is a reflection of the oral microbiota, presumably due to repeated aspiration and decreased clearance. In addition, it is well established that bacteria are a major cause of COPD exacerbations. Therefore, it seems plausible that the oral microbiota is a major contributor in COPD and especially in COPD exacerbations. Chlorhexidine is a broad-spectrum antiseptic that is safe, well-tolerated and shown to decrease respiratory infection associated with oral intubation. We hypothesize that twice daily oral rinses with chlorhexidine will result in a decrease in the lung microbiota biomass. If proven correct, this would be the basis for a large interventional trial to determine if oral decontamination with chlorhexidine will decrease COPD exacerbations.

C. Work Proposed

Overview

The *primary outcome* of this pilot study is to determine if twice-daily chlorhexidine oral rinse decreases the microbiota biomass, as measured by 16S rRNA copy number, in the COPD lung and oral cavity as measured in induced sputum and oral wash. Our *secondary outcome* are to determine if chlorhexidine oral rinse: i) decreases lung and oral microbiota diversity; ii) alters taxonomic composition of the lung and oral cavity microbiota; iii) decreases systemic inflammation as measured by blood hsCRP, fibrinogen and leukocyte count; and iv) changes in respiratory health status as measured by the BCSS and SGRQ. If our hypotheses are supported, this study will form the basis of an interventional trial to determine if altering the oral microbiota with chlorhexidine can prevent COPD exacerbations.

Study Design Overview

This study will determine if the microbiota of the oral cavity and induced sputum is altered by twice-daily chlorhexidine rinse. At *baseline*, the following studies will be done:

1. Spirometry to determine study eligibility and to guide sputum induction protocol.
2. Oral rinse and induced sputum for microbiota analysis. An oral rinse of 15 ml sterile water and induced sputum will be obtained at baseline for microbiota analysis.
3. Inflammatory markers (blood hsCRP, fibrinogen, and leukocyte count).
4. Breathlessness, Cough and Sputum Scale (BCSS). This 3-item self-completed questionnaire focuses on common COPD symptoms.
5. St George's Respiratory Questionnaire (SGRQ). This 50-item self-completed questionnaire quantifies respiratory health status.

After baseline measurements have been obtained, subjects will be randomized to 8 weeks of treatment with either twice daily oral rinse and expectoration of 15 ml of chlorhexidine or twice-daily oral rinse and expectoration of 15 ml of placebo (sterile water with mint flavor and blue dye). At the end of 8 weeks the subjects will have a repeat of the above studies, with the exception of spirometry.

Population and Setting

Participants with a clinical diagnosis of COPD who express willingness to participate in the trial and who fulfill all eligibility criteria will be enrolled in the study. To maximize sputum collection, we will enrich our population with COPD patients with symptoms of chronic bronchitis and a history of a COPD exacerbation in the past year. Patients with these characteristics are prone to having a larger volume of sputum production.

Inclusion and Exclusion Criteria:

- **Inclusion Criteria**
  - Willingness to undergo *sputum induction*
  - Capability to provide written informed consent
  - Age ≥ 40 years and ≤ 85 years
  - FEV_1/FVC ratio (post bronchodilator) ≤70%
  - FEV_1 (post bronchodilator) ≤ 65%
  - Presence or high likelihood of chronic cough and sputum production defined as **one** of the following:
    - Presence of chronic cough and sputum will be defined by responses to the first two questions on the SGRQ. Subjects who respond positively to both question 1 (cough) and question 2 (sputum) on the SGRQ as either “several days per week” or “almost every day” will be eligible.
    - COPD exacerbation within the previous 12 months defined as taking antibiotics and/or prednisone for respiratory symptoms, hospitalization or emergency department visit for respiratory illness.
  - Current or former smoker with lifetime cigarette consumption of $\geq$ 10 pack-years
  - Negative serum pregnancy test at the baseline visit if patient is a pre-menopausal female (menopause defined as absence of a menstrual cycle in the last 12 months)
  - Must be fluent in speaking the English language
  - Have a minimum of four teeth

- **Exclusion Criteria:**
  - Not fully recovered for at least 30 days from a COPD exacerbation.
  - Treated with antibiotics in the last 2 months.
  - The presence of dentures (full plate).
  - Active oral infection being treated by health care professional.
  - Current use of chlorhexidine or over-the-counter mouth washes in the last 2 months.
  - Known allergy or sensitivity to chlorhexidine
  - Unstable cardiac disease
  - Clinical diagnosis of asthma, bronchiectasis, cystic fibrosis, or severe alpha-1 antitrypsin deficiency
  - Active lung cancer or history of lung cancer if it has been less than 2 years since lung resection or other treatment. If history of lung cancer, must have no evidence of recurrence in the 2 years preceding the baseline visit.

- Undergoing active treatment for malignancy except for hormonal therapy (i.e. prostate cancer, breast cancer) or non-metastatic skin cancer and are not symptomatic
- Prisoners or institutionalized patients
- Participation in another study involving an investigational product within 30 days of the baseline visit
- Pregnant or breast-feeding patients.
- Chronically oxygen dependent or room air O_2 saturations < 90%
- Any concomitant condition that might endanger the patient through participation in the study or interfere with study procedures, as assessed by the investigator

Recruitment Strategies

Patients with COPD and chronic cough and sputum production will be drawn from patient populations at the Minneapolis VA HealthCare System. Some study participants will be identified through an Institutional Review Board (IRB)-approved database (3330B). Additional study participants will be identified through the Minneapolis VA Pulmonary Clinic, Pulmonary Function Laboratory or the Minneapolis VA COPD Disease Management Program. Potential participants will receive a letter requesting permission for us to contact them by telephone concerning a study. Upon receipt of the letter, the patient will have the opportunity to opt out by calling the number listed in the letter. If the patient does not opt out after receiving the letter, the study team will contact them to explain the study and determine whether they wish to participate further in the screening process. A copy of this letter is attached to this protocol. In addition, patients' providers will be encouraged to refer patients to the study team for consideration of study participation. Pulmonary and COPD Disease Management providers will be educated about this study during a department meeting. These referred patients will be contacted via phone.

Study Intervention

Study participants will be randomized to receive either 15 ml oral rinse of chlorhexidine twice daily, or matched placebo (placebo consists of sterile water with blue dye and mint flavor). Participants will use the randomly assigned study mouth rinse for 8 weeks. At the initial visit, individuals participating in the study will undergo spirometry, SGRQ and blood sample for hsCRP followed by an oral rinse with sterile saline and sputum induction prior to initiating study drug. After 8 weeks of study drug, subjects will have all samples collected again (spirometry, SGRQ, blood draw, oral rinse, and induced sputum). Briefly, sputum will be induced after pretreatment with albuterol. Subjects will receive nebulized 3% saline (subjects with FEV1 $\geq$ 35%) or normal saline (subjects with FEV < 35%) for 20 minutes and will be encouraged to cough a minimum of every two minutes.

Intervention: The subjects will be instructed to use the study mouth rinse twice daily for 8 weeks. Subjects will be asked to swish 15 ml of study mouth rinse in their mouths for 30 seconds followed by expectoration. The study mouthwash must be used at least 30 minutes after eating and/or tooth brushing. This procedure will be performed in the morning and evening. Subjects will be instructed to not rinse with water or other mouthwashes, brush teeth, or eat immediately after rinsing for 1 hour. The subjects will be instructed not to ingest (swallow) the medication after rinsing. Subjects will be instructed to avoid: i) drinking tea to avoid tooth discoloration; ii) toothpastes containing the antimicrobial agent triclosan (list will be provided) and iii) mouthwashes other than the study drug. The participants will be asked to stop the study medication the night before the last clinic visit and to avoid routine dental clinic visit with cleaning during the study period.

Outcomes

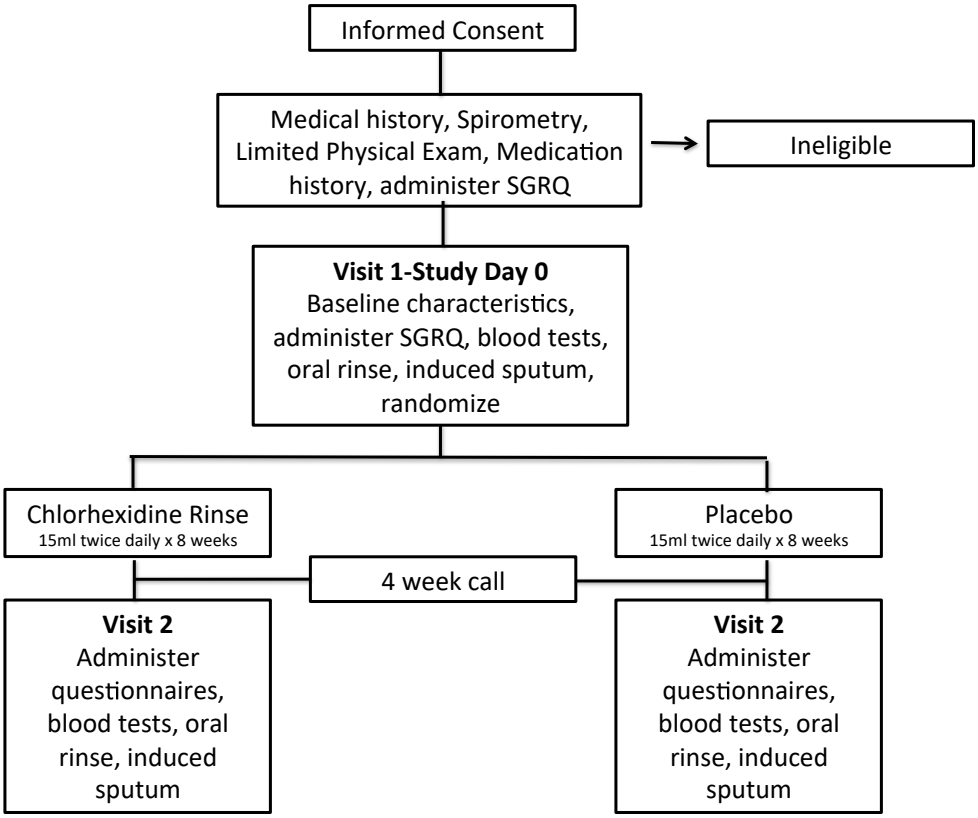
Primary Outcome

Our primary outcome will be to compare the microbiota biomass of induced sputum and the oral cavity before and after 8 weeks of chlorhexidine rinse (n=25) compared to controls (n=25) using next-generation sequencing of the bacterial 16S rRNA gene.

Secondary Outcomes

Secondary outcomes will include: i) sputum and oral microbiota diversity; ii) sputum and oral microbiota taxonomy; iii) inflammatory markers hsCRP, fibrinogen, and leukocyte count; iv) BCSS score; v) SGRQ score; and vi) assessment of adverse effects.

Methods and Data Collection Plan



Medical History

During the baseline visit, after obtaining informed consent, the medical history will be reviewed with the participant to make sure all key components of the past medical history, including dental history, are obtained for eligibility and safety purposes. The participant's electronic medical records will also be reviewed.

Medications/Supplement Use

Participants will be asked to bring all medications (prescription and non-prescription, including inhalers) with them to the study visit. All medications will be recorded and the VA medication dispensing records will also be reviewed electronically. In review of the subject's medications, the study team will be specifically looking for patients on antibiotics that may alter the microbiota. Non-prescription medications will be reviewed to make sure the patient is not taking over-the-counter mouth washes or toothpaste with triclosan that may alter the oral microbiota. The patient's respiratory and non-respiratory medications will not be altered as a part of this study.

Physical Examination

After informed consent and review of both medical and medication history, a member of the study team will perform a focused physical. The physical exam will focus on vital signs including resting oximetry, weight, and the oral and lung examination. At the final study visit the physical examination will be repeated.

St George's Respiratory Questionnaire (SGRQ)

The first two questions of the SGRQ comprise eligibility criteria for the study (see inclusion criteria). If subjects are randomized in the study, they will be asked to complete the entire SGRQ as part of the baseline visit and again at completion of the study intervention. The SGRQ is a self-administered questionnaire that was developed to measure health-related quality of life specifically related to pulmonary disease. Since its development, it has been validated for use in many chronic lung diseases, including COPD[25]. The SGRQ contains 11 questions that ask for a scaled answer (from 1 to 5), 39 questions that ask for true/false answers and one free-text answer. A copy of the SGRQ is attached to this proposal. Subjects can complete the SGRQ in approximately 10-15 minutes. The questions evaluate three areas or domains: respiratory symptoms, activity limitations from breathlessness, and impact of respiratory disease on social and psychological functioning. The SGRQ is scored on a scale of 0 to 100, with a score of 100 reflecting the most severe symptoms, activity limitations and psychosocial impact. The minimum clinically important difference in the SGRQ is widely accepted as being 4 units[26].

Spirometry

Spirometry will be performed prior to randomization to ensure that participants meet eligibility criteria. Spirometry will be performed in compliance with standards jointly published by the American Thoracic Society and the European Respiratory Society[27]. Spirometry will be performed after patient inhales 2 puffs of albuterol (90 mcg/inhalation).

Eligible Subjects:

Laboratory Evaluation

After obtaining informed consent and determining the participant is otherwise eligible for the study, the patient will undergo an oral rinse with sterile water (rinse for 30 seconds and collect expectorant) and sputum induction. The oral rinse and 2 ml of sputum will be used for the microbiota evaluation.

The blood draw will be performed by the VA laboratory or the study personnel. Blood will be sent to the VA laboratory to measure hsCRP, fibrinogen and leukocyte count. These biomarkers have been demonstrated to be predictive of COPD exacerbations[28].

Induced Sputum:

Sputum induction will be performed as outpatients. Subjects will have fasted for at least 2 hours prior to the study. Prior to sputum induction, the subject will rinse, gargle and spit into a sterile cup 15 ml sterile water that will be collected for microbiota studies. Next, all subjects will be pretreated with 360 ug albuterol administered by metered dose inhaler. Prior to initiation of the test peak expiratory flows (PEF) will be obtained and noted. This will constitute the baseline peak flow. To induce sputum the subject will receive nebulized sterile 3% saline (subjects with FEV1 $\geq$ 35%) or normal saline (subjects with FEV1 < 35%) for 20 minutes (150 ml in reservoir). PEF will be monitored every 4 minutes. If the PEF declines to less than 90% of their baseline, monitoring will change to every 2 minutes. If the PEF clines to less than 80% of baseline the nebulization will be discontinued. Subjects will be encouraged to cough throughout the nebulization. In addition, the nebulization treatment will be interrupted every 2 minutes at which time the subject will be encouraged to cough up secretions. Prior to coughing the subject will be asked to spit saliva into a plastic container that will be discarded at the end of the treatment. The sputum will be collected in a separate, sterile container.

Microbiota studies

The sputum sample will be weighed and the oral wash volume will be measured. To identify the microbiota, DNA will be isolated from sputum and oral wash samples. Sputum samples will be treated with dithiothreitol (DTT) prior to isolation. We will use a previously described protocol for DNA isolation that includes bead beating to lyse bacterial cells, followed by precipitation with isopropanol and digestion with RNase. Samples will be subjected to PCR amplification using 16S rRNA gene primers specific to the constant regions flanking the V3 region[15, 29]. Amplicons will be gel purified and sequenced at the University of Minnesota Genomics Center using Illumina MiSeq equipment. To minimize effects of random sequencing errors, we will use RDP Pipeline to eliminate (a) sequences that did not appropriately match the PCR primer and the barcode at the beginning of a read, (b) sequence reads with <50 bases after the proximal PCR primer if they terminated before reaching the distal primer, and (c) sequences that contained more than one undetermined nucleotide (N). Both the proximal and distal primers will be trimmed from high-quality reads before database searches are performed.

Inflammatory Marker
To measure the inflammatory state, hsCRP, fibrinogen, and leukocyte count will be measured in blood.

Sample Banking

The microbiota studies will be performed at the University of MN. All remaining sample will be returned and stored in Dr. Wendt's laboratory at the VAMC. After completion, remaining sample will be stored for a maximum of 10 years for future confirmation studies and studies related to COPD. Samples will be de-identified and will be identified only by a pre-assigned sample ID number. Clinical data will NOT be given to the Univ MN laboratory.

Randomization procedure

Patients will be randomized via permuted block sizes of 2 to active drug or placebo in a ratio of 1:1. Because there is some evidence of an interaction with inhaled corticosteroids and the lung microbiota,

we will stratify randomization according to use of inhaled corticosteroids at baseline. Only the Research Pharmacist will have access to the randomization list. The subjects will be instructed to use the study mouth rinse twice daily for 8 weeks as described above. The subjects will be asked to return all study medications at the last clinic visit. At the last visit the subjects will be asked whether they thought they were taking the active medication or placebo to assess the effectiveness of blinding.

The Breathlessness, Cough and Sputum Scale (BCSS)

The Breathlessness, Cough and Sputum Scale is an easy-to-use instrument for tracking the severity of respiratory symptoms and evaluating the efficacy of treatment in clinical trials in patients with COPD. It is designed as a daily diary in which subjects are instructed to answer three questions on a 0 to 4 scale, targeting breathlessness, cough and sputum. A total daily score is calculated from the question answers. This format allows investigators to assess symptom variability and trajectory over time. This tool has been validated in large clinical trials against the SGRQ[1, 2].

Monthly calls

Participants will be called at 4 weeks by the study coordinator to assess for: i) hospitalizations, ED visits; unplanned clinic visits; ii) new medication use including any systemic antibiotic use; iii) study drug compliance; iv) adverse events.

Effectiveness of blinding

At the last visit the subjects will be asked whether they thought they were taking the active medication or placebo to assess the effectiveness of blinding

Monitoring adverse events

Participants enrolled in this study will be monitored for adverse outcomes including adverse hospitalizations and death. Patients will be questioned during the mid-study telephone call and at the final study visit regarding any adverse drug reactions. In addition, participants will be given contact information for the study team and will be counseled to report any adverse effects. Participants will be questioned during the mid-study telephone call and at the final study visit regarding any hospitalizations. A report of every serious adverse event will be filed with the IRB per institutional policy. Electronic medical records will also be reviewed for 30 days after completing the study to capture any serious adverse outcomes, including hospitalization or death. If necessary, we will contact relatives or household members to obtain additional information.

The risk to the participant is that of the *induced sputum.* Hypertonic saline sputum induction is a routine test performed in many clinical settings and clinical trials. It has been shown to be safe in the setting of COPD. We do not anticipate complications of chlorhexidine treatment since this is an ingredient in several over-the-counter products, including mouth washes, and chlorhexidine mouthwash is commonly prescribed following dental extractions. Listed below are potential complications.

1. Bronchospasm: Bronchospasm is the main reported complication of sputum induction with hypertonic saline and will be treated with albuterol either by metered dose inhaler or nebulized.
2. Intolerance to Chlorhexidine: Oral mucosal irritation and transient decrease in taste have rarely been reported with chlorhexidine. Mild tooth discoloration associated with concurrent tea ingestion has been reported.

A Data Safety and Monitoring Board (DSMB) will not be formed as part of this study since this is a small study with a short follow-up period.

Discontinuation of procedure

The procedure may be terminated at anytime for any of the following reasons:

- If the participant requests to withdraw from the study for any reason.
- If the investigators or the participant's physician believes the patient has experienced a serious adverse event that might be related to the study or that continued participation in the study might jeopardize the participant's medical care.
- The participant becomes so mentally or physically incapacitated that he/she can no longer make an informed judgment about continued participation in the study.

Unmasking of treatment allocation will occur in cases where such information is critical to ongoing care of the participant. At the conclusion of the study, the participants will be informed of treatment allocation.

Data Analysis Plan

At the conclusion of the study, we will censor any subject who reports using systemic antibiotics during the 8-week study period. This will prevent antibiotic use from confounding our microbiota results. As this study is a randomized controlled study, the analysis for the primary outcome will consist of testing for differences in changes in bacterial biomass between the two patient groups using a 2 sample *t*-test with equal variances between groups. While it is widely recognized that the *t*-test is robust with regard to departures from the normality assumption, if the data appear very non-normal (as observed in a *qq*-plot) even after standard transformations are considered (such as the Box-Cox transformation) then Wilcoxon's test will be used. Similarly, if the variance is markedly different between the groups then Welch's 2 sample *t*-test will be used. While randomization should ensure that the groups are balanced on important potential confounders (such as age) all such factors will be examined to check that there are not significant differences between the groups. If there are differences then linear models will be fit to account for these potential confounders (again, standard transformations will be considered to improve model fit and diagnostics will be performed to assess model assumptions).
The analysis for secondary endpoints necessarily takes a different strategy as that investigates the relationship among variables that are not subject to direct randomization. Hence the analysis will consider regression models that investigate how changes in health status (as measured by changes in the SGRQ) depend on microbial diversity and inflammatory markers. Model selection will be guided by scientific plausibility, model diagnostics and measures of model quality (such as AIC).

Sample Size and Power Calculation

We can use the preliminary data on microbial diversity among COPD patients to estimate the power of the study. For concreteness we will assess the power for detecting a difference in the Simpson (1-D) diversity measure (the correlation between this measure and Shannon's measure was 0.96 in our preliminary data). The standard deviation in this measure among moderately affected COPD patients is 0.280 and is 0.298 among the severely affected COPD patients, and as there is no significant difference between the variances (p=0.80 by Bartlett's test) we combine the data from both groups and obtain an estimate of 0.281 for the standard deviation. To obtain an estimate of the magnitude of differences that are reasonable to expect from a drug that decreases the diversity of the lung flora, we fit a linear regression model with diversity as the outcome and age as the predictor variable (the ages range from 55 to 78 among these subjects). Age has a significant positive impact on diversity (p=0.047)

with an estimated slope of 0.017, so that over the course of 20 years the model predicts that there will be a change of 0.34 in diversity. Hence we investigated the power for values of the difference attributable to the effect of chlorhexidine from 0.22 to 0.32 and present the results in Table **1** (these results are from a 2 sample *t*-test with equal variance using the estimated standard deviation from the previous study and 20 subjects per group). These results indicate that the study should have acceptable power if chlorhexidine has an impact on the diversity of the lung microbiota that is comparable to what is observed due to the effect of aging. We will increase the sample size to 25 per group since we anticipate censoring at least 20% of the subjects because of antibiotic use during the study. This population has a demonstrated exacerbation rate of approximately 20% and exacerbations are often treated with antibiotics.

Effect size	0.22	0.24	0.26	0.28	0.30	0.32
Power	0.67	0.75	0.81	0.87	0.91	0.94

Table **1**: The power of the study to detect a difference between the treatment groups as it depends on the magnitude of the difference in diversity between the 2 groups.

Quality Control

Members of the research team will become familiar with the study protocol and be trained in all procedural aspects. Patient recruitment and data acquisition will be performed principally by a trained and experienced study coordinator employed full-time by the Pulmonary Section of the Minneapolis VA Health Care System. Dr. Chris Wendt will supervise all aspect of the study. The study coordinator has expertise in performing spirometry, administering the questionnaires for this study, and in thorough documentation of study procedures.

Source documents will include paper case report forms, printouts from spirometry machines, printouts of laboratory data, original surveys completed by the participants and any outside (non-VA) medical records that are obtained. All data will be entered into a computerized database for statistical analysis, with retention of source documents for verification of all computerized data, should the need arise.

All electronic data will be stripped of personal identifiers and stored in secure network drives that are password-protected. Source documents will be secured in a locked file cabinet within a locked room for security. The only method to link electronic data to individual data will be a key linking participant name to study subject number.

Protection Against Risk

The risk to the participant of this study is small.

Induced Sputum: We will pre-treat with albuterol, either MDI or nebulizer, to prevent bronchospasm. Albuterol is a commercially available, FDA-approved drug. The common side effects associated with albuterol are mild, transient tachycardia, tremulousness, and nervousness. In addition, we will monitor peak flows and terminate the procedure for a significant drop. All bronchospasm will be treated with additional doses of albuterol.

Spirometry. The risks of spirometry are minimal. Some participants may experience temporary shortness of breath or lightheadedness as a result of deep and rapid breathing that is required to perform spirometry. These risks are small and spirometry is routinely performed in this patient population. Administration of albuterol prior to testing is also a standard procedure and is known to be

very safe. Albuterol is a commercially available, FDA-approved drug. The common side effects associated with albuterol are mild, transient tachycardia, tremulousness, and nervousness.
Laboratory evaluation. Some patients will experience mild to moderate pain at the needle insertion site. Other minimal risks include: risk of bleeding/hematoma at needle puncture site, risk of infection by going through the skin, risk of lightheadedness or fainting. The participants of this study will have their blood drawn by a study physician with experience performing blood draws or by a trained phlebotomist. The study physician will use the proper protective equipment including gloves, lab coat and eye protection. Blood will be collected in a standard fashion to minimize the above risks.

SGRQ and BCSS. Participants may feel uncomfortable answering questions about their respiratory symptoms or the impact of their lung disease on their lives. In such cases, participants have the right to refuse to answer questions contained in these questionnaires.

Medication assignment. Chlorhexidine has a good safety profile as evidenced in the background section of this proposal. The Chlorhexidine product being used in this study is 0.12% solution concentration. Patients will be asked to perform an oral rinse for 30 seconds twice daily. They will be instructed to avoid tooth brushing or eating for 30 minutes prior and one hour after the rinse. Participants will be educated about potential adverse effects as part of the informed consent process. Participants will be screened for adverse effects at the 4 week telephone call and at the last clinic visit. Participants can elect to stop the study medication and withdraw from the study at any point for any reason.

Protection of female participants. Pregnant and breast-feeding women will be excluded. There is insufficient data to determine if chlorhexidine is safe for a fetus or nursing child.

Risk to patient confidentiality. Confidential information will be collected as part of this study. Safeguards will be taken to protect this information. Source documents will be kept in a locked cabinet in a locked room. All electronic data will be stripped of personal identifiers. The database will be password-protected and stored in a secured network drive behind the VA firewall. Only the study team will have access to the source documents and the electronic data.

Adequacy of Protection against risks:

- **Recruitment and Informed consent:** Study participants will be asked to sign an informed consent approved by the Institutional Review Board of the VAMC. This includes a clear description of the study and its objectives, the number of patient contacts and procedures, the information to be collected, the tests (induced sputum) to be performed, possible benefits and risks to the patient, and permission to use the patient's health plan medical record data. The subjects will be informed that remaining sample would be used for future studies.
- **Protections against Risk:** We will take special precautions to protect the security of the research data. These include authorization to send and receive data, verification of identity, and encryption of all data. We will follow institutional guidelines on data access and security. Rigorous policies will be followed for maintaining secure backups for all research data and complete documentation of data files, both locally and centrally. All data processing procedures will be carefully documented and reviewed periodically.
  All patients identified for inclusion in the research project will be assigned a unique study identification number. Prior to any data transfer, all identifiers (e.g., names, medical record numbers, health plan enrollee numbers, etc.) will be removed. We will also utilize password protection programs for all computerized records. In no instances will identifying information, either for patients or health plans, be publicly disclosed.

All study staff will be trained and reminded to appreciate the confidential nature of research data. Each investigator and staff member involved in the proposed study will sign a confidentiality agreement annually and be required to have participated in required IRB training.

Risk to patient confidentiality. Confidential information will be collected as part of this study. Safeguards will be taken to protect this information. Source documents will be kept in a locked cabinet in a locked room. All electronic data will be stripped of personal identifiers. The database will be password-protected and stored in a secured network drive behind the VA firewall. Only the study team will have access to the source documents and the electronic data.

Potential Benefits of the Research to the Subject and Others

A possible future benefit is that the research may improve health care for themselves and other patients with similar diseases.

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