PROTOCOL

Cathelicidin and Vitamin D: Impact on Populations At-Risk and with COPD

ClinicalTrials.Gov Registration NCT02464059

UNC PROTOCOL

Version 1.2

Updated September 6 2017

SECTION 1: Vitamin D Study Overview

1.1: Introduction and Background: Understanding mechanisms leading to decrements in lung function, the physiologic hallmark of obstructive lung diseases including chronic obstructive pulmonary disease (COPD), are necessary to inform interventions to improve lung health. The antimicrobial peptide cathelicidin, and its primary regulator vitamin D, has been implicated in the development and progression of chronic lung disease. Cathelicidin has bactericidal and inflammatory activities in the lung and is regulated by vitamin D levels.

1.2: Purpose of the Study: The purpose of the study is to determine the effect of vitamin D replacement on blood and lung cathelicidin levels in populations at-risk or with established COPD. The antimicrobial peptide cathelicidin, and its primary regulator vitamin D, has been implicated in the development and progression of chronic lung disease. We hypothesize that oral vitamin D supplementation will raise cathelicidin levels in the pulmonary compartment, thereby potentially restoring relative lung cathelicidin deficiency. To test this hypothesis, we will recruit from two sites: 1) Johns Hopkins (via the Subpopulations and Intermediate Outcome Measures in COPD Study (SPIROMICS) study of individuals with established COPD (NA_00035701: PI Hansel) and 2) UNC COPD Clinic for individuals at-risk for COPD (smokers with preserved lung function). This MOP reflects procedures for the UNC site.

At each site, we will measure blood and lung lavage cathelicidin levels in 20 vitamin D insufficient individuals (10 participants with serum 25-OH Vitamin D<10 ng/ml and 10 participants with serum 25-OH vitamin D between 10-20) before and after eight weeks of oral vitamin D supplementation to determine the effect of vitamin D supplementation on cathelicidin levels. Completion of this project directly or indirectly works towards ultimately understanding if a readily measurable blood marker, cathelicidin, can inform who may benefit from vitamin D supplementation to prevent chronic lung disease. The results from this application would lead to a therapeutic intervention study that will provide further opportunities to improve our understanding of the relationship between vitamin D, cathelicidin and lung function.

1.3 Objectives:

To evaluate the effect of oral vitamin D supplementation on plasma and lung cathelicidin measures in individuals with vitamin D insufficiency. Our primary analysis will include comparison between pre- and post-supplementation cathelicidin levels using paired t-tests for repeated measures

The study procedures outlined in this proposal specifically do not include any assessment of outcomes related to disease treatment, mitigation, cure, or prevention. We do not assess change in lung function over time, symptom scores, exacerbation risk, sputum production, pneumonia outcomes or any other assessment related to treatment/mitigation of COPD. The only outcome collected if BAL and blood samples to measure cathelicidin pre- and post-vitamin D supplementation (along with standard safety outcomes that are not related to any disease process). The lack of any assessment of outcomes related to diagnosis, cure, mitigation, treatment or prevention of disease demonstrates that such outcomes are not the scope of the proposed study.

1.4 Overall Project Design:

The design of the study to complete this aim will be in the format of a baseline and repeat bronchoscopy after oral supplementation with 50,000 IU vitamin D3 weekly for eight weeks in individuals with reduced vitamin D levels. The specific study data collection is outlined in the table below.

- Participants will undergo a screening assessment (visit S0) to determine the presence of vitamin D insufficiency (serum 25OH-VitD <20 ng/ml) as well as normal renal function and calcium levels. Pre- and post-bronchodilator spirometry will be performed to document normal lung function.
- If eligible based on S0 data, participants will undergo baseline bronchoscopy with bronchoalveolar lavage (BAL) for collection of lung cathelicidin levels (visit B1). Blood for cathelicidin, vitamin D binding protein, vitamin D and PBMC will be collected at this visit.
- They will then return to the Meadowmont research clinic weekly for eight weeks of directly observed vitamin D supplementation as well as assessment of adverse events (visits V0-V7).
    - After the initial four weeks of treatment (V4), all patients will be evaluated and have blood work obtained for calcium level to monitor for hypercalcemia.
    - Patients will be screened at each weekly visit for symptoms of hypercalcemia.
- After 8 weeks of vitamin D treatment, serum 25OH-VitD levels will be measured (visit V8) to ensure increased level prior to undergoing bronchoscopy. Individuals not increasing vitamin D> 20 after 8 weeks will not be eligible for B2 bronchoscopy and will be deemed to have completed study. Blood for BMP/ calcium level will also be collected.
- A second BAL collection will occur after the eight week of vitamin D treatment to measure cathelicidin in the plasma and the BAL (visit B2). Blood for cathelicidin, vitamin D binding protein, vitamin D and PBMC will also be collected at this visit.
- A 24 hour post-bronchoscopy follow-up phone call will occur after B1 and B2
- All procedures will be research related. A visit window of ±1 day is acceptable for all study visits except B1 and B2, where a 28 day window is permissible given potential delay in laboratory results and bronchoscopy scheduling.

Table. Study Data Collection Schedule

Visit	S0	B1	V0	V1	V2	V3	V4	V5	V6	V7	V8	B2
Time (weeks)	-2	-1	0	1	2	3	4	5	6	7	8	9
Window (+/- days)	0	28	1	1	1	1	1	1	1	1	1	28
Visit Duration (hrs)	2	5	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	5
Informed Consent	•											
Spirometry	•											
Vitamin D	•						•				•	
Vit D binding prot/cathelicidin	•										•	

PBMC		•										•
BMP/ calcium	•						•				•	
BAL		•										•
Drug administration			•	•	•	•	•	•	•	•		
Adverse events & interval history				•	•	•	•	•	•	•	•	
Key:Time= nominal visit time; S=screening visit, Bn= Bronchoscopy visit n, Vn=Study visit												

SECTION 2: RECRUITMENT

2.1 Definition of Recruitment & Consent: Recruitment is establishing eligibility of the potential candidate and informing the candidate of their eligibility and signing of the informed consent form by the potential candidate.

2.2 Sources of Recruitment: Potential participants will be identified from three sources: 1) patients referred by providers in the pulmonary subspecialty clinic, 2) patients referred by providers in the general medicine outpatient clinic, 3) self-referral from recruitment flyers.

2.3 Process of Recruitment: Study team members will approach providers during clinic sessions for identification of potential participants. If the clinician is agreeable and obtains the approval for the patient, the recruitment team will approach the patient. For recruitment via face-to-face encounter, these conversations will occur in a private office with a trained study team member. Study information will be reviewed with the patient and a copy of the IRB approved consent form will be provided to them to review at their leisure (taking it home, if desired). After having the chance to consider the trial, a time/date will be scheduled for the consent process and screening visit. For participants self-referring from recruitment flyers, the study team will make contact via phone call using a telephone screening script.

2.4 Study Inclusion and Exclusion Criteria:

i. Inclusion
    i. Post-BD FEV1/FVC>=0.70
    ii. Post-BD FEV1> 70% predicted
    iii. Age 18-80
    iv. Current smoker (current history of smoking at least 5 cigarettes daily)
    v. Serum calcium<10.5mg/dl
    vi. 25-OH VitD<20 ng/ml at screening
        1. Note that target recruitment is at least 10 participants with 25-OH VitD <10 ng/ml
    vii. CrCl ≥60 mL/min as estimated by the Cockcroft-Gault equation
    viii. Women of reproductive potential with negative serum or urine pregnancy test and be willing to refrain from participating in a conception process and subject/partner must use at least 2 reliable forms of contraceptives for the duration of the study

ii. Exclusion

i. Current use of vitamin D supplements
ii. Oxygen use >2L/min at rest
iii. Known allergy/sensitivity or any hypersensitivity to components of study drugs or their formulations
iv. Pregnancy or currently breast-feeding
v. History of nephrolithiasis (kidney stones)
vi. Self-reported HIV positive serostatus
vii. Any condition that, in the opinion of the site investigator, would compromise the subject's ability to participate in the study.

SECTION 3: DETAILED STUDY VISIT PROCEDURES

3.1 Chart/Phone Pre-Screening Process

i. The **telephone screening script** will be used to determine potential eligibility for all participants contacted by phone.
ii. If participant is eligible and interested, they will be scheduled for an in-person visit for informed consent review/signing as well as screening laboratory data. If screening is occurring in person, informed consent and blood work can be completed same day provided the participant has had adequate time to review the consent form.

3.2 Screening Visit (S0)

i. Review/sign consent form
ii. Assignment of study ID
    a. Using "VitDXXX" where

       XXX= sequential numbers from 501-999

iii. Completion of **demographics form**
iv. Urine pregnancy test (for women of childbearing age)
v. Performance of pre- and post-bronchodilator spirometry (4 puffs albuterol)
vi. Completion of **screening form** to determine eligibility
vii. If eligibility confirmed, obtain blood testing for screening visit (see **laboratory processing section**)
    a. One-3.5 ml SST to UNC McLendon Lab for eligibility screening
        i. Serum 25OH-Vitamin D
        ii. Serum Basic metabolic panel
    b. One 8.5 ml SST for serum storage
        i. Serum for 25OH-Vitamin D and vitamin D binding protein
    c. Two 4 ml EDTA Lavender for plasma storage
        i. Plasma for cathelicidin
viii. Confirm contact number to reach participant after 25OH-VitD and BMP resulted.
ix. Payment to participant
x. After labs resulted, PI to review vitamin D/BMP results and determine eligibility
xi. MD to contact person to arrange bronchoscopy

3.3 Bronchoscopy (B1)

i. Before scheduling the first of the two bronchoscopy procedures, it should be established that
        i. Patient has signed IRB-approved consent for the research protocol
        ii. Has met the inclusion criteria for the study.
        iii. The benefits and risks of the procedure have been explained to the participant and patient has given consent for the procedure.
ii. The target windows for scheduling the bronchoscopy is 28 days after S0 visit for the first procedure and 1 week after V8 for the second procedure.

iii. Day of bronchoscopy, the following bloods will be collected (processed at Alexis lab):
    a. Two 10 ml Green Top Lithium tubes for PBMC storage

See **"BRONCHOSCOPY PROCEDURES"** for bronchoscopy procedure details

3.4 Initial medication administration visit (V0)

i. Participant will present for first drug administration. At the time of visit, a bottle of 25-OH vitD will be removed for secured storage and label completed with:
    a. Participants study number (VitDXXX)
    b. Initials of the participant
    c. Date of V0 visit
ii. Participant will be given one pill of Vitamin D 50,000 IU and asked to take pill with water at that time
iii. Complete **Vitamin D Subject Specific Accountability Record**
iv. Provide participant with **Vitamin D informational handout**
v. Schedule V1 in 7 days
vi. Payment to participant:

3.5 Follow-up visits

i. Visits V1-V3
    a. **Adverse events form** is completed
    b. Participant will be given one pill of Vitamin D 50,000 IU and asked to take pill with water at that time
    c. Complete **vitamin D administration log**
    d. Schedule next visit in 7 days
    e. Payment to participant:

ii. Visit V4
    a. **Adverse events form** is completed
    b. Participant will be given one pill of Vitamin D 50,000 IU and asked to take pill with water at that time
    c. Complete **Vitamin D Subject Specific Accountability Record**
    d. Schedule next visit in 7 days
    e. Obtain blood (see **laboratory processing section**)
        i. One-3.5 ml SST to UNC McLendon Lab for eligibility screening
            1. Serum 25OH-Vitamin D
            2. Serum Basic metabolic panel
    f. Confirm contact number to reach participant after 25OH-VitD and BMP resulted.
    g. Payment to participant:
    h. Notify study MD when lab results completed

iii. Visit V5-V7
   a. **Adverse events form** is completed
   b. Participant will be given one pill of Vitamin D 50,000 IU and asked to take pill with water at that time
   c. Complete **Vitamin D Subject Specific Accountability Record**
   d. Schedule next visit in 7 days
   e. Payment to participant:

iv. Visit V8
   a. **Adverse events form** is completed
   b. Schedule next visit in 7 days
   c. Obtain blood (see **laboratory processing section**)
      i. One-3.5 ml SST to UNC McLendon Lab for eligibility screening
         1. Serum 25OH-Vitamin D
         2. Serum Basic metabolic panel
      ii. One 8.5 ml SST for serum storage
         1. Serum for 25 OH-Vitamin D and vitamin D binding protein
      iii. Two 4 ml EDTA Lavender for plasma storage
         1. Plasma for cathelicidin
   d. Confirm contact number to reach participant after 25OH-VitD and BMP resulted.
   e. Payment to participant:
   f. Notify study MD when lab results completed
      a. If vitamin D not >20 ng/ml, participant not eligible for bronchoscopy and will have completed study
      b. If vitamin D>=20 ng/ml, study staff contact person to arrange bronchoscopy

3.6 Bronchoscopy (B2)

i. The target windows for scheduling the bronchoscopy is 1 week after V8 for the second procedure.
ii. On day of bronchoscopy, the following blood will be obtained (processed at Alexis lab):
   a. Two 10 ml Green Top Lithium tubes for PBMC storage

See **"BRONCHOSCOPY PROCEDURES"** for procedure details

SECTION 4: REPORTING EVENTS

The proposed intervention study involves treatment of participants with vitamin D with the goal of determining if this intervention is efficacious in altering lung cathelicidin levels, and is therefore classified as a clinical trial. Because this is a clinical trial, the PI will designate an independent monitor to perform independent review of ongoing study progress and safety. Adverse events (AE) will be reported both to our IRB and the Independent Monitor through annual progress reports or more frequently as described below. AE reports and annual summaries will not include subject- or group-identifiable material. Each report will only include the identification code.

The Independent Monitor for this study is Dr. Sonali Bose at Johns Hopkins (sbose7@jhmi.edu)

For this application, an adverse event is defined as any untoward medical occurrence in a subject during participation in the clinical study or with use of the intervention (vitamin D) being studied. An adverse finding can include a sign, symptom, abnormal assessment (laboratory test value, vital signs, etc.), or any combination of these. A serious adverse event (SAE) is any adverse event that results in one or more of the following outcomes:

- Death
- A life-threatening event
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability/incapacity
- A congenital anomaly or birth defect
- An important medical event based upon appropriate medical judgment

AEs will be labeled according to severity, which is based on their impact on the patient. An AE will be termed "mild" if it does not have a major impact on the patient, "moderate" if it causes the patient some minor inconvenience, and "severe" if it causes a substantial disruption to the patient's well-being. AEs will be categorized according to the likelihood that they are related to the study intervention. Specifically, they will be labeled definitely unrelated, definitely related, probably related, or possibly related to the study intervention.

The PI will review AE rates at least monthly.

When an AE occurs, the AE will be reported to the Institutional Review Board at UNC, to the funding agency and to the Independent Monitor. Our procedure is for the study coordinator to notify the Principal Investigators and for the program staff to make necessary arrangements for the participant to see a medical provider. Most assessments will occur in research space adjacent to the Meadowmont clinic, and referrals would be made to the clinic acute provider. The Emergency Room is the first choice for emergent care, and we would arrange for transport or accompany the participant as needed. SAEs that are unanticipated, serious, and possibly related to the study intervention will be reported to the Independent Monitor and IRB in accordance with requirements.

Unexpected fatal or life-threatening AEs related to the intervention will be reported to the IRB and Independent Monitor within 3 days. Other serious and unexpected AEs related to the

intervention will be reported to the IRB and Independent Monitor within 14 days. Anticipated or unrelated SAEs will be handled in a less urgent manner but will be reported to the Independent Monitor and IRB. In the annual AE summary, the Independent Monitor Report will be required to state that they have reviewed all AE reports.

Study progress and safety will be reviewed monthly by the PI. Progress reports, including patient recruitment, retention/attrition, and AEs, will be provided to the Independent Monitor annually. An Annual Report will be compiled and will include a list and summary of AEs. In addition, the Annual Report will address (1) whether AE rates are consistent with pre-study assumptions; (2) reason for dropouts from the study; (3) whether all participants met entry criteria; (4) whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims of the study; and (5) conditions whereby the study might be terminated prematurely. The Annual Report will be sent to the Independent and included in the Progress Report to the NIH. The IRB and other applicable recipients will review progress of this study on an annual basis.

During the funding of this study, any action by the IRB, the Independent Monitor, or one of the study investigators that results in a temporary or permanent suspension of the study will be reported to the NHLBI Program Official within 1 business day of notification.

SECTION 5: STUDY VISIT CHECKLISTS

The following pages include a separate page for each study visit with a checklist of items that must be completed at each visit. These pages should be printed and used as a step-by-step guide of each visit.

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

S0- Screening Visit Checklist

☐ Review and sign consent form

☐ Assign study ID

☐ Complete demographics form

☐ Urine pregnancy test (for women of childbearing age)

☐ Pre- and post-bronchodilator spirometry

☐ Complete screening form

☐ If eligibility confirmed, obtain blood testing

- ☐ One-3.5 ml SST to UNC McLendon Lab for eligibility screening (BMP, 25-OH vitamin D)
- ☐ One 8.5 ml SST for serum storage (25-OH vitamin D, vitamin D binding protein)
- ☐ Two 4 ml EDTA lavender tube for plasma storage (cathelicidin)

☐ Confirm contact phone number

☐ Provide participant with "Bronchoscopy Handout"

☐ Payment to participant:

☐ Notify MD of blood results for review and scheduling of bronchoscopy

Review total recruitment:

Eligibility: 25OH VitD <20ng/ml (with targeted recruitment of at least 10 participants with level <10 ng/ml)

Bronchoscopy scheduling: Target 1 week ± 28 days

☐ Once bronchoscopy is scheduled, V0 should be scheduled within 1 week ±1 day of bronchoscopy)

☐ Notify pharmacy of medication pickup

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

B1- Bronchoscopy #1 Visit Checklist

☐ Review consent form for completion

☐ Obtain blood testing

- ☐ Two 10 ml Green Top Lithium tubes for PBMC storage

☐ Review labs to confirm vitamin D <20 ng/ml

☐ Escort patient to bronchoscopy

☐ Payment to participant:

☐ MD to complete bronchoscopy form

☐ Staff to complete bronchoscopy sample processing form

☐ Confirm V0 is scheduled (within 1 week ±1 day of bronchoscopy)

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V0- Initial medication administration checklist

☐ Go to pharmacy

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Provide participant informational flyer regarding vitamin D side effects

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V1 in 7±1 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V1- Follow-up visit checklist

☐ Go to pharmacy

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V2 in 7±1 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___	**Participant Initials**: ___ ___ ___	Date: _____

V2- Follow-up visit checklist

☐ Go to pharmacy

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V3 in 7±1 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___	**Participant Initials**: ___ ___ ___	Date: _____

V3- Follow-up visit checklist

☐ Go to pharmacy

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V4 in 7±1 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V4- Follow-up visit with lab draw checklist

☐ Go to pharmacy

☐ Complete adverse events form

☐ Obtain blood testing

☐ One-3.5 ml SST to UNC McLendon Lab for eligibility screening

(BMP, 25-OH vitamin D)

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V5 in 7±1 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___	**Participant Initials**: ___ ___ ___	Date: _____

V5- Follow-up visit checklist

☐ Go to pharmacy

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V6 in 7±1 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V6- Follow-up visit checklist

☐ Go to pharmacy

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V7 in 7±1 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V7- Follow-up visit checklist

☐ Go to pharmacy

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V8 in 7±1 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V8- Follow-up visit with lab draw checklist

☐ Complete adverse events form

☐ Obtain blood testing

- ☐ One-3.5 ml SST to UNC McLendon Lab for eligibility screening

  (BMP, 25-OH vitamin D)
- ☐ One 8.5 ml SST for serum storage (25-OH vitamin D, vitamin D binding protein)
- ☐ Two 4 ml EDTA lavender tube for plasma storage (cathelicidin)

☐ Provide participant with "Bronchoscopy Handout"

☐ Confirm contact phone number

☐ Review labs to determine vitamin D level

If 25-OH-VitD≥20 mg/ml: Notify MD to schedule bronchoscopy

If 25-OH-VitD<20 mg/ml: Participant not eligible for bronchoscopy; study is completed

Bronchoscopy scheduling: Target 1 week ± 5 days

☐ Payment to participant:

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

B2- Bronchoscopy #2 Visit Checklist

☐ Review consent form for completion

☐ Obtain blood testing

- ☐ Two 10 ml Green Top Lithium tubes for PBMC storage

☐ Review labs to confirm vitamin D ≥20 ng/ml

☐ Escort patient to bronchoscopy

☐ Payment to participant:

☐ MD to complete bronchoscopy form

☐ Staff to complete bronchoscopy sample processing form

SECTION 7: LABORATORY PROCEDURES

1. Plasma processing for cathelicidin

1. Collect two 4 ml EDTA lavender top for plasma, invert 6-12 times after collection
2. Place sample on ice after collection
3. Centrifuge lavender top at 4°C at 1500rpm for 15 minutes.
4. Remove vacutainer tube from the centrifuge, carefully, protecting the layer and not mixing.
5. Remove stopper from the vacutainer tube.
6. Remove plasma and transfer to fresh polypropylene tube. Be careful to not disturb white cells in the buffy coat.
7. Recentrifuge the transferred plasma in order to avoid every contamination with white blood cells: 1500xg at 4°C for 15 minutes
8. Aliquot into 250 microliter aliquots in polypropylene tubes labeled "plasma" (see table below) with study ID
    a. Individual labels should also include study ID (VitDXXX) and date
9. Storage store at -80°C in polypropylene tubes.

2. Serum processing for 25-OH vitamin D and vitamin D binding protein

1. Collect one 8.5 ml SST for serum.
2. Allow samples to clot for 30 minutes at room temperature
3. Centrifuge for 15 minutes at 1000 x g.
4. Remove serum and aliquot into 250 microliter aliquots in polypropylene tubes labeled "serum" (see table below) with study ID
    a. Individual labels should also include study ID (VitDXXX) and date
5. Store at -80°C in polypropylene tubes

3. PBMC Processing for storage

PBMC Processing

1. Obtain the two 10 ml green-top tubes of heparinized whole blood. Samples should be processed within two hours, and stored at room temperature prior to processing.

2. Pour each tube into 2 empty 50mL conicals such that each conical contains approximately 10mL of whole blood.

3. Place the emptied green top tubes back on test tube rack. **DO NOT discard vacutainer tubes yet**

4. Wash each vacutainer tube with about 3mL of Dulbecco's Phosphate Buffered Saline (DPBS) consecutively to ensure the collection of any residual cells along the tube walls. Dispense the DPBS containing the residual cells into one of the 50mL conicals.

5. Repeat step 4 for all tubes.

    a. If the volume of each conical is less than 20mL, then proceed to step 9.

    b. If the volume of each conical is 20mL or more, then proceed to step 6.

6. Dilute the blood to 50mL with DPBS and mix 2 times with a pipette.

7. Take conicals that have been pre-filled with 18mL of DPBS and place them behind the sample conicals.

8. From the 50mL sample volume, separate out approximately 18mL into pre-filled conicals.

9. Bring the volume of each conical to approximately 35mL of whole blood and DPBS.

10. Underlay Ficoll-Hypaque (F/H) by performing the following method:

    a. Insert a 10 ml pipette into a F/H bottle and draw up F/H to orange line of pipette.

    b. Release the pipette from pipette gun to allow F/H to dispense slowly into each conical.

    c. It is essential that the F/H be dispensed slowly so that there is no mixing of the whole blood with the F/H layer.

11. Make sure the centrifuge break is <u>OFF</u> and centrifuge all tubes for 20 minutes at 2400 RPM at 22^{O}C.

12. Harvest the interface which contains the mononuclear cells, with a 10 ml serological pipette.

13. Pool the harvested cells into a clean, sterile 50 ml conical. Cells may be dispensed in two clean 50mL conical if blood volume was received from more than 6 green top tubes. Bring volume of each conical to 50 ml with DPBS.

14. Centrifuge 10 minutes at 500 G (1500 RPM)

15. Aspirate the DPBS with a 2 ml serological pipette into waste system. Leave approximately 120µl of supernatant and vortex pellet gently.
16. If there are 2 conicals of harvested cells, combine into **one** conical.
17. Follow protocol for counting cells.
18. For the final wash, bring volume up to 35mL with sterile DPBS and centrifuge 5 minutes at 1500 RPM.

PBMC Freezing

1. After the final wash, aspirate and discard the supernatant from the PBMC pellet.
2. Re-suspend in Recovery Cell Culture media (freezing media) according to the PBMC count (please refer to the Processing Table):
    a. Freeze in 10 million cells per mL of Recovery Cell Culture media using the appropriate freezing racks
    b. Aliquot 1mL of 10 million cells plus freezing media into the appropriately labeled cryovial
    c. Place vials immediately into Mr. Frosty Cell Freezing Cans and into -80ºC overnight
    d. On the following day, transfer PBMCs from cans in -80 ºC to the -140 ºC freezer

3. Bronchoalveolar lavage processing

A. BAL Supernatant

1. Spin the conical(s) filled with a volume of up to 80mL of BAL supernatant and cells at 1600 rpm for 6 minutes (4 ºC)
2. After centrifugation, remove and save all the supernatant into the following (labeled "Sup"):
    a. Aliquot 1mL into 6 vials. Store at -80 ºC
    b. Aliquot 10mL into 5-8 conicals depending upon remaining volume.Store at -80 ºC
    c. Individual labels should include "Sup", study ID (VitDXXX) and date
3. Re-suspend BAL Cells in 4mL HBSS media.
4. Add 35mL HBSS media to the BAL Cell conical, spin at 1500rpm for 5 minutes (22 ºC)
5. After centrifugation, aspirate and discard the HBSS supernatant from the BAL Cells.

6. Re-suspend the cells in Recovery Cell Culture freezing media:
    a. Freeze in 10 million cells per mL of freezing media using freezing racks.
    b. Aliquot 1mL (10 million cells and freezing media) into appropriately labeled cryovial
        i. Individual labels should include "cells", study ID (VitDXXX) and date
    c. Place vials immediately into Mr. Frosty Cell Freezing Cans and into -80^{o}C for overnight incubation.
    d. On the following day, transfer cells from cans in -80 ^{o}C to the -140 ^{o}C freezer

Processing Table

Biospecimen (Parent tube)	**Volume**	**Aliquot sample type**	**Label Type**	**Aliquot #**	**Aliquot conc. & Volume**	**Freezing Temp. (Celsius)**
BAL conical	80 mL	Supernatant (1mL aliquots)	Sup	6	1.0mL	minus 80 º
		Supernatant (10mL aliquots)	Sup	Depends on Supernatant volume	10.0mL	minus 80 º
		Bal Cells	cells	Depends on Bal Cell count	$10x10^{6}$ Bal Cells/ 1.0mL	minus 80º overnight to minus 140º
Green top tube	20 ml	PBMC	PBMC	Depends on PBMC count	$10x10^{6}$ PBMC/ 1.0mL	minus 80º overnight to minus 140º
Lavender top EDTA tube for plasma	10 ml	Plasma (250 microliter aliquots)	Plasma	As many as possible	250 microlit	minus 80 º
SST top for serum	8 ml	Serum (250 microliter aliquots)	Serum	As many as possible	250 microlit	minus 80 º

SECTION 8: BRONCHOSCOPY PROCEDURES

i. **Patient preparation**

a. The participant should be interviewed prior to the procedure to ensure that there are no contraindications to performing the bronchoscopy such as
   i. recent acute or serious illness
   ii. anticoagulation or bleeding disorder
   iii. fever
   iv. oxygen desaturation on room air
   v. allergy to local anesthetics or sedation
   vi. food intake within 6 hours.

b. Patients should be instructed to take their usual morning oral medications with a small sip of water.

c. Patients should also have a companion or reliable escorted means of transportation home after the procedure so that they can safely return home after the procedure.

d. The patient should be informed that the procedure is being performed for research purposes and is not for clinical care.

e. The details of the procedure should be explained to the participant and consent obtained in accordance with local hospital or clinic practices and procedures.

f. Risks related to the procedure:
   i. allergic or adverse reaction to sedation or local anesthesia
   ii. nose-bleed
   iii. sore-throat
   iv. coughing
   v. chest soreness from coughing
   vi. fever
   vii. infection

g. Participant will be reimbursed in the form of a check, given to participant immediately prior to procedure

ii. **Conduct of the procedure**

a. **Sedation**
   i. Before sedation, a “time-out” should be performed to verify that the patient is correctly identified and is undergoing a bronchoscopy.
   ii. The bronchoscopy insertion orifice should be ascertained (right or left nostril or mouth) and local anesthetic applied in accordance with local practice.
   iii. The patient is moderately sedated during monitoring of vital signs and oxygenation according to local procedures.
   iv. Typically a benzodiazepine (e.g midazolam) and a narcotic (e.g. fentanyl) are used.

1. Upper limits of sedation
    a. Fentanyl 400 mcg IV
    b. Versed 6 mg IV

b. **Method**
    i. The bronchoscope is inserted and the upper airway is anesthetized with 1-2 mL aliquots of lidocaine 1% or 2% solution.
    ii. The lower airways are anesthetized with aliquots of lidocaine to diminish or prevent coughing.
    iii. The amount of lidocaine instilled should be monitored. Typically *no more than 500 mg* should be administered in a single procedure.
    iv. In order to minimize the use of lidocaine, the inspection of the left lung airways should be brief or limited.

c. **Collection of specimens**
    i. The bronchoscope is advanced into the right mainstem bronchus and the BAL is collected by instillation of 50 mL x 2 in the right middle lobe (either segments). If necessary to obtain at least 50 cc BAL return, additional 50 cc aliquots can be instilled in alternative segments of the RML or anterior RUL, to a maximum of 300 cc BAL instillation.
    ii. Return of BAL fluid can sometimes be facilitated by ensuring that the return suction is not too high (< 100 cm H2O), by turning the patient with the right lung superior, or by asking the patient to take a deep breath and cough.

d. **Completion of procedure**
    i. The BAL form is completed which records the results of the BAL.
    ii. The research coordinator should be notified that the procedure is completed.
    iii. If the patient has a post-bronchoscopy fever, the use of anti-pyretics are advised.

e. **Post bronchoscopy care**
    i. The patient should be monitored in supervised environment until they have recovered from sedation, and vital signs and oxygenation are stable.
    ii. The patient should be instructed not to take any food or liquid by mouth for at least two hours after the procedure or until the gag-reflex has returned.
    iii. The patient should be notified to contact the clinic or study physician if any symptoms of concern are noted after the procedure.

i. **Sample Handling**

a. BAL samples should be transported within 30 minutes at room temperature. If transport time will be 30-60 min, samples should be placed on ice. Transport time should not exceed 60 minutes to maximize cell viability.

PROTOCOL

Cathelicidin and Vitamin D: Impact on Populations At-Risk and with COPD

ClinicalTrials.Gov Registration NCT02464059

SPIROMICS/BAYVIEW PROTOCOL

Version 1.7

Updated April 20 2016

SECTION 1: Vitamin D Study Overview

1.1: Introduction and Background: Understanding mechanisms leading to decrements in lung function, the physiologic hallmark of obstructive lung diseases including chronic obstructive pulmonary disease (COPD), are necessary to inform interventions to improve lung health. The antimicrobial peptide cathelicidin, and its primary regulator vitamin D, has been implicated in the development and progression of chronic lung disease. Cathelicidin has bactericidal and inflammatory activities in the lung and is regulated by vitamin D levels.

1.2: Purpose of the Study: The purpose of the study is to determine the effect of vitamin D replacement on blood and lung cathelicidin levels in populations at-risk or with established COPD. The antimicrobial peptide cathelicidin, and its primary regulator vitamin D, has been implicated in the development and progression of chronic lung disease. We hypothesize that oral vitamin D supplementation will raise cathelicidin levels in the pulmonary compartment, thereby potentially restoring relative lung cathelicidin deficiency. To test this hypothesis, we will recruit from two ongoing cohort studies at Johns Hopkins, the SHIELD study of African-American individuals at high risk for development of obstructive lung diseases (OLDs) (NA_00019960: PI Kirk), and the Subpopulations and Intermediate Outcome Measures in COPD Study (SPIROMICS) study of individuals with established COPD (NA_00035701: PI Hansel). We will also recruit participants from prior COPD studies NA_00009880, NA_00035381, NA_00085617, NA_00068035, and IRB00047215. This MOP reflects procedures for the SPIROMICS site.

We will measure blood and lung lavage cathelicidin levels in 20 vitamin D insufficient individuals (10 participants with serum 25-OH Vitamin D<10 ng/ml and 10 participants with serum 25-OH vitamin D between 10-20) before and after eight weeks of oral vitamin D supplementation to determine the effect of vitamin D supplementation on cathelicidin levels Completion of this project directly or indirectly works towards ultimately understanding if a readily measurable blood marker, cathelicidin, can inform who may benefit from vitamin D supplementation to prevent chronic lung disease. The results from this application would lead to a therapeutic intervention study that will provide further opportunities to improve our understanding of the relationship between vitamin D, cathelicidin and lung function.

1.3 Objectives:

To evaluate the effect of oral vitamin D supplementation on plasma and lung cathelicidin measures in individuals with vitamin D insufficiency. Our primary analysis will include comparison between pre- and post-supplementation cathelicidin levels using paired t-tests for repeated measures

1.4 Overall Project Design:

The design of the study to complete this aim will be in the format of a baseline and repeat bronchoscopy after oral supplementation with 50,000 IU vitamin D3 weekly for eight weeks in individuals with reduced vitamin D levels. The specific study data collection is outlined in the table below.

- Participants will undergo a screening assessment (visit S0) to determine the presence of vitamin D insufficiency (serum 25OH-VitD <20 ng/ml) as well as normal renal function and calcium levels. Pre- and post-bronchodilator spirometry will be performed.
- If eligible based on S0 data, participants will undergo baseline bronchoscopy with bronchoalveolar lavage (BAL) for collection of lung cathelicidin levels (visit B1). Blood for cathelicidin, vitamin D binding protein, vitamin D and PBMC will be collected at this visit.
- They will then return to the SPIROMICS research clinic weekly for eight weeks of directly observed vitamin D supplementation as well as assessment of adverse events (visits V0-V7).
    - After the initial four weeks of treatment (V4), all patients will be evaluated and have blood work obtained for calcium level to monitor for hypercalcemia.
    - Patients will be screened at each weekly visit for symptoms of hypercalcemia.
- After 8 weeks of vitamin D treatment, serum 25OH-VitD levels will be measured (visit V8) to ensure increased level prior to undergoing bronchoscopy. Individuals not increasing vitamin D> 20 after 8 weeks will not be eligible for B2 bronchoscopy and will be deemed to have completed study. Blood for BMP/ calcium level will also be collected.
- A second BAL collection will occur after the eight week of vitamin D treatment to measure cathelicidin in the plasma and the BAL (visit B2). Blood for cathelicidin, vitamin D binding protein, vitamin D and PBMC will also be collected at this visit.
- All procedures will be research related. A visit window of ±1 day is acceptable for all study visits except B1 and B2, where a 28 day window is permissible given potential delay in laboratory results and bronchoscopy scheduling.

Table. Study Data Collection Schedule

Visit	S0	B1	V0	V1	V2	V3	V4	V5	V6	V7	V8	B2
Time (weeks)	-2	-1	0	1	2	3	4	5	6	7	8	9
Window (+/- days)	0	28	1	1	1	1	1	1	1	1	1	28
Visit Duration (hrs)	2	5	1	0.5	0.5	0.5	1	0.5	0.5	0.5	1	5
Informed Consent	•											
Spirometry	•											
Vitamin D	•						•				•	
Vit D binding prot/cathelicidin/ PBMC		•										•
BMP/ calcium	•						•				•	
BAL		•										•
Drug administration			•	•	•	•	•	•	•	•		
Adverse events & interval history				•	•	•	•	•	•	•	•	

Key:Time= nominal visit time; S=screening visit, Bn= Bronchoscopy visit n, Vn=Study visit

SECTION 2: RECRUITMENT

2.1 Definition of Recruitment & Consent: Recruitment is establishing eligibility of the potential candidate and informing the candidate of their eligibility and signing of the informed consent form by the potential candidate.

2.2 Sources of Recruitment: Participants of the SPIROMICS study (Protocol NA_00035701- PI Hansel) additional approved studies including prior COPD studies NA_00009880, NA_00035381, NA_00085617, NA_00068035, and IRB00047215. Total goal N=20. Recruitment happens at the Bayview Lung Health Study (SPIROMICS)

2.3 Process of Recruitment: Study team members from this protocol will receive a list of research participants from the above listed protocols who have previously indicated willingness to be contacted for future studies. This study team will contact research participants. These contacts will be made via phone call or in face-to-face encounters during scheduled study visits for the ongoing approved protocols. For recruitment via face-to-face encounter, these conversations will occur in a private office with a trained study team member.

2.4 Study Inclusion and Exclusion Criteria:

i. Inclusion
    i. Post-BD FEV1/FVC<0.70
    ii. Post-BD FEV1> 50% predicted
    iii. Age 18-80
    iv. Current or former smokers
    v. Serum calcium<10.5mg/dl
    vi. 25-OH VitD<20 ng/ml at screening
        1. Note that target recruitment is at least 10 participants with 25-OH VitD <10 ng/ml
    vii. CrCl ≥60 mL/min as estimated by the Cockcroft-Gault equation
    viii. Women of reproductive potential with negative serum or urine pregnancy test and be willing to refrain from participating in a conception process and subject/partner must use at least 2 reliable forms of contraceptives for the duration of the study

ii. Exclusion
    i. Current use of vitamin D supplements
    ii. Oxygen use >2L/min at rest
    iii. Known allergy/sensitivity or any hypersensitivity to components of study drugs or their formulations
    iv. Pregnancy or currently breast-feeding
    v. History of nephrolithiasis (kidney stones)
    vi. Self-reported HIV positive serostatus
    vii. Any condition that, in the opinion of the site investigator, would compromise the subject's ability to participate in the study.

SECTION 3: DETAILED STUDY VISIT PROCEDURES

3.1 Chart/Phone Pre-Screening Process

i. Potential individuals will be identified through screening database for following eligibility
    a. Post-BD FEV1/FVC<0.70
    b. Post-BD FEV1> 50% predicted
    c. Age 18-80
    d. Current or former smokers
ii. Individuals will be contacted via phone or in person during in-person visit.
iii. The **telephone screening script** will be used to determine further eligibility for all participants.
iv. If participant is eligible and interested, they will be scheduled for an in-person visit for informed consent review/signing as well as screening laboratory data. If screening is occurring in person, informed consent and blood work can be completed same day.

3.2 Screening Visit (S0)

i. Review/sign consent form
ii. Assignment of study ID
    a. Using "VitDXXX" where

       XXX= sequential numbers from 001-999

iii. Completion of **demographics form**
iv. Urine pregnancy test (for women of childbearing age)
v. Performance of pre- and post-bronchodilator spirometry (4 puffs albuterol)
vi. Completion of **screening form** to determine eligibility
vii. If eligibility confirmed, obtain blood testing for screening visit (see **laboratory processing section**)
    a. Two 3.5 ml SST to JHH for eligibility screening
        i. Serum 25OH-Vitamin D
        ii. Serum Basic metabolic panel
viii. Confirm contact number to reach participant after 25OH-VitD and BMP resulted.
ix. Payment to participant
x. After labs resulted, PI to review vitamin D/BMP results and determine eligibility
xi. MD to contact person to arrange bronchoscopy

3.3 Bronchoscopy (B1)

i. Before scheduling the first of the two bronchoscopy procedures, it should be established that
        i. Patient has signed IRB-approved consent for the research protocol
        ii. Has met the inclusion criteria for the study.
        iii. The benefits and risks of the procedure have been explained to the participant and patient has given consent for the procedure.

ii. The target windows for scheduling the bronchoscopy is 1 week after S0 visit for the first procedure and 1 week after V8 for the second procedure.
iii. Day of bronchoscopy, the following bloods will be collected:
    a. Two 3.5 ml SST for serum storage
        i. Serum for 25OH-Vitamin D and vitamin D binding protein
    b. Two 4 ml EDTA Lavender for plasma storage
        i. Plasma for cathelicidin
    c. Two 10 ml Green Top Lithium tubes for PBMC storage
        i. PBMC to be processed at Mark Liu's lab

See **"BRONCHOSCOPY PROCEDURES"** for bronchoscopy procedure details

3.4 Initial medication administration visit (V0)

i. Participant will present for first drug administration. At the time of visit, a bottle of 25-OH vitD will be removed for secured storage and label completed with:
    a. Participants study number (VitDXXX)
    b. Initials of the participant
    c. Date of V0 visit
ii. Participant will be given one pill of Vitamin D 50,000 IU and asked to take pill with water at that time
iii. Complete **Vitamin D Subject Specific Accountability Record**
iv. Provide participant with **Vitamin D informational handout**
v. Schedule V1 in 7 days
vi. Payment to participant:

3.5 Follow-up visits

i. Visits V1-V3
    a. **Adverse events form** is completed
    b. Participant will be given one pill of Vitamin D 50,000 IU and asked to take pill with water at that time
    c. Complete **vitamin D administration log**
    d. Schedule next visit in 7 days
    e. Payment to participant

ii. Visit V4
    a. **Adverse events form** is completed
    b. Participant will be given one pill of Vitamin D 50,000 IU and asked to take pill with water at that time
    c. Complete **Vitamin D Subject Specific Accountability Record**
    d. Schedule next visit in 7 days
    e. Obtain blood (see **laboratory processing section**)
        i. Two 3.5 ml SST to JHH for safety screening
            1. Serum 25OH-Vitamin D

2. Serum Basic metabolic panel

f. Confirm contact number to reach participant after 25OH-VitD and BMP resulted.
g. Payment to participant
h. Notify study MD when lab results completed

iii. Visit V5-V7

a. **Adverse events form** is completed
b. Participant will be given one pill of Vitamin D 50,000 IU and asked to take pill with water at that time
c. Complete **Vitamin D Subject Specific Accountability Record**
d. Schedule next visit in 7 days
e. Payment to participant:

iv. Visit V8

a. **Adverse events form** is completed
b. Schedule next visit in 7 days
c. Obtain blood (see **laboratory processing section**)
    i. Two 3.5 ml SST to JHH for safety screening
        1. Serum 25OH-Vitamin D
        2. Serum Basic metabolic panel
d. Confirm contact number to reach participant after 25OH-VitD and BMP resulted.
e. Payment to participant
f. Notify study MD when lab results completed
    a. If vitamin D not >20 ng/ml, participant not eligible for bronchoscopy and will have completed study
    b. If vitamin D>=20 ng/ml, study staff contact person to arrange bronchoscopy

3.6 Bronchoscopy (B2)

i. The target windows for scheduling the bronchoscopy is 1 week after V8 for the second procedure.
ii. On day of bronchoscopy, the following blood will be obtained:
    a. Two 3.5 ml SST for serum storage
        i. Serum for 25OH-Vitamin D and vitamin D binding protein
    b. Two 4 ml EDTA Lavender for plasma storage
        i. Plasma for cathelicidin
    c. Two 10 ml Green Top Lithium tubes for PBMC storage
        i. PBMC to be processed at Mark Liu's lab

See **"BRONCHOSCOPY PROCEDURES"** for procedure details

SECTION 4: REPORTING EVENTS

The proposed intervention study involves treatment of participants with vitamin D with the goal of determining if this intervention is efficacious in altering lung cathelicidin levels, and is therefore classified as a clinical trial. Because this is a clinical trial, the PI will designate an independent monitor to perform independent review of ongoing study progress and safety. Adverse events (AE) will be reported both to our IRB and the Independent Monitor through annual progress reports or more frequently as described below. AE reports and annual summaries will not include subject- or group-identifiable material. Each report will only include the identification code.

The Independent Monitor for this study is Dr. Sonali Bose at Johns Hopkins (sbose7@jhmi.edu)

For this application, an adverse event is defined as any untoward medical occurrence in a subject during participation in the clinical study or with use of the intervention (vitamin D) being studied. An adverse finding can include a sign, symptom, abnormal assessment (laboratory test value, vital signs, etc.), or any combination of these. A serious adverse event (SAE) is any adverse event that results in one or more of the following outcomes:

- Death
- A life-threatening event
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability/incapacity
- A congenital anomaly or birth defect
- An important medical event based upon appropriate medical judgment

AEs will be labeled according to severity, which is based on their impact on the patient. An AE will be termed "mild" if it does not have a major impact on the patient, "moderate" if it causes the patient some minor inconvenience, and "severe" if it causes a substantial disruption to the patient's well-being. AEs will be categorized according to the likelihood that they are related to the study intervention. Specifically, they will be labeled definitely unrelated, definitely related, probably related, or possibly related to the study intervention.

The PI and Independent monitor will review AE rates at least monthly.

When an AE occurs, the AE will be reported to the Institutional Review Board at Johns Hopkins Hospital and Bloomberg School of Hygiene, to the funding agency and to the Independent Monitor. Our procedure is for the study coordinator to notify the Principal Investigators and for the program staff to make necessary arrangements for the participant to see a medical provider. Most assessments will occur in research space adjacent to the Hopkins clinics, and referrals would be made to the clinic acute provider. The Johns Hopkins Emergency Room is the first choice for emergent care, and we would arrange for transport or accompany the participant as needed. SAEs that are unanticipated, serious, and possibly related to the study intervention will be reported to the Independent Monitor and IRB in accordance with requirements.

Unexpected fatal or life-threatening AEs related to the intervention will be reported to the IRB and Independent Monitor within 3 days. Other serious and unexpected AEs related to the

intervention will be reported to the IRB and Independent Monitor within 14 days. Anticipated or unrelated SAEs will be handled in a less urgent manner but will be reported to the Independent Monitor and IRB. In the annual AE summary, the Independent Monitor Report will be required to state that they have reviewed all AE reports.

Study progress and safety will be reviewed monthly by the PI. Progress reports, including patient recruitment, retention/attrition, and AEs, will be provided to the Independent Monitor following each of the quarterly reviews. An Annual Report will be compiled and will include a list and summary of AEs. In addition, the Annual Report will address (1) whether AE rates are consistent with pre-study assumptions; (2) reason for dropouts from the study; (3) whether all participants met entry criteria; (4) whether continuation of the study is justified on the basis that additional data are needed to accomplish the stated aims of the study; and (5) conditions whereby the study might be terminated prematurely. The Annual Report will be sent to the Independent Monitor and will be forwarded to the IRB and included in the Progress Report to the NIH. The IRB and other applicable recipients will review progress of this study on an annual basis.

During the funding of this study, any action by the IRB, the Independent Monitor, or one of the study investigators that results in a temporary or permanent suspension of the study will be reported to the NHLBI Program Official within 1 business day of notification.

SECTION 5: STUDY VISIT CHECKLISTS

The following pages include a separate page for each study visit with a checklist of items that must be completed at each visit. These pages should be printed and used as a step-by-step guide of each visit.

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

S0- Screening Visit Checklist

☐ Review and sign consent form

☐ Assign study ID

☐ Complete demographics form

☐ Urine pregnancy test (for women of childbearing age)

☐ Pre- and post-bronchodilator spirometry

☐ Complete screening form

☐ If eligibility confirmed, obtain blood testing

- ☐ Two 3.5 ml SST to JHH (BMP, 25-OH vitamin D)

☐ Confirm contact phone number

☐ Provide participant with "Bronchoscopy Handout"

☐ Payment to participant

☐ Notify MD of blood results for review and scheduling of bronchoscopy

Review total recruitment:

Eligibility: 25OH VitD <20ng/ml (with targeted recruitment of at least 10 participants with level <10 ng/ml)

If 10 participants have been recruited with level ≥ 10 and <20, then a participant with level ≥10 and <20 is no longer eligible. At that point, only individuals with level <10 are eligible.

Bronchoscopy scheduling: Target 1 week ± 5 days

☐ Once bronchoscopy is scheduled, V0 should be scheduled within 1 week ±1 day of

bronchoscopy)

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

B1- Bronchoscopy #1 Visit Checklist

☐ Review consent form for completion

☐ Obtain blood testing

- ☐ Two 3.5 ml SST for serum storage (25-OH vitamin D, vitamin D binding protein)
- ☐ Two 4 ml EDTA lavender tube for plasma storage (cathelicidin)
- ☐ Two 10 ml Green Top Lithium tubes for PBMC storage (Mark Liu's lab)

☐ Review labs to confirm vitamin D <20 ng/ml

☐ Escort patient to bronchoscopy

☐ Payment to participant

☐ MD to complete bronchoscopy form

☐ Staff to complete bronchoscopy sample processing form

☐ Confirm V0 is scheduled (within 1 week ±1 day of bronchoscopy)

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V0- Initial medication administration checklist

☐ Label new bottle of vitamin D with participant study ID, initials, date

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Provide participant informational flyer regarding vitamin D side effects

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V1 in 7±1 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V1- Follow-up visit checklist

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V2 in 7±1 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V2- Follow-up visit checklist

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V3 in 7±1 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___	**Participant Initials**: ___ ___ ___	Date: _____

V3- Follow-up visit checklist

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V4 in 7±1 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___	**Participant Initials**: ___ ___ ___	Date: _____

V4- Follow-up visit with lab draw checklist

☐ Complete adverse events form

☐ Obtain blood testing

- ☐ Two 3.5 ml SST to JHH (BMP, 25-OH vitamin D)

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V5 in 7±1 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V5- Follow-up visit checklist

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V6 in 7±1 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V6- Follow-up visit checklist

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V7 in 7±1 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V7- Follow-up visit checklist

☐ Complete adverse events form

☐ Provide participant one pill of 50,000 IU vitamin D to take with water at visit

☐ Complete Vitamin D Subject Specific Accountability Record

☐ Schedule V8 in 7±1 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___ **Participant Initials**: ___ ___ ___ Date: _____

V8- Follow-up visit with lab draw checklist

☐ Complete adverse events form

☐ Obtain blood testing

- ☐ Two 3.5 ml SST to JHH (BMP, 25-OH vitamin D)

☐ Provide participant with "Bronchoscopy Handout"

☐ Confirm contact phone number

☐ Review labs to determine vitamin D level

If 25-OH-VitD≥20 mg/ml: Notify MD to schedule bronchoscopy

If 25-OH-VitD<20 mg/ml: Participant not eligible for bronchoscopy; study is completed

Bronchoscopy scheduling: Target 1 week ± 5 days

☐ Payment to participant

Assigned ID: VitD ___ ___ ___	**Participant Initials**: ___ ___ ___	Date: _____

B2- Bronchoscopy #2 Visit Checklist

☐ Review consent form for completion

☐ Obtain blood testing

- ☐ Two 3.5 ml SST for serum storage (25-OH vitamin D, vitamin D binding protein)
- ☐ Two 4 ml EDTA lavender tube for plasma storage (cathelicidin)
- ☐ Two 10 ml Green Top Lithium tubes for PBMC storage (Mark Liu's lab)

☐ Review labs to confirm vitamin D ≥20 ng/ml

☐ Escort patient to bronchoscopy

☐ Payment to participant

☐ MD to complete bronchoscopy form

☐ Staff to complete bronchoscopy sample processing form

SECTION 7: LABORATORY PROCEDURES

1. Plasma processing for cathelicidin

1. Collect two 4 ml EDTA lavender top for plasma, invert 6-12 times after collection
2. Place sample on ice after collection
3. Centrifuge lavender top at 4°C at 1500rpm for 15 minutes.
4. Remove vacutainer tube from the centrifuge, carefully, protecting the layer and not mixing.
5. Remove stopper from the vacutainer tube.
6. Remove plasma and transfer to fresh polypropylene tube. Be careful to not disturb white cells in the buffy coat.
7. Recentrifuge the transferred plasma in order to avoid every contamination with white blood cells: 1500xg at 4°C for 15 minutes
8. Aliquot into 250 microliter aliquots in polypropylene tubes labeled "plasma" (see table below) with study ID
    a. Individual labels should also include study ID (VitDXXX) and date
9. Storage store at -80°C in polypropylene tubes.

2. Serum processing for 25-OH vitamin D and vitamin D binding protein

1. Collect two 3.5 ml SST for serum.
2. Allow samples to clot for 30 minutes at room temperature
3. Centrifuge for 15 minutes at 1000 x g.
4. Remove serum and aliquot into 250 microliter aliquots in polypropylene tubes labeled "serum" (see table below) with study ID
    a. Individual labels should also include study ID (VitDXXX) and date
5. Store at -80°C in polypropylene tubes

3. PBMC Processing for storage (Hui Qing in Mark Liu's lab)

PBMC Processing

1. Obtain the two 10 ml green-top tubes of heparinized whole blood. Samples should be processed within two hours, and stored at room temperature prior to processing.

2. Pour each tube into 2 empty 50mL conicals such that each conical contains approximately 10mL of whole blood.

3. Place the emptied green top tubes back on test tube rack. **DO NOT discard vacutainer tubes yet**

4. Wash each vacutainer tube with about 3mL of Dulbecco's Phosphate Buffered Saline (DPBS) consecutively to ensure the collection of any residual cells along the tube walls. Dispense the DPBS containing the residual cells into one of the 50mL conicals.

5. Repeat step 4 for all tubes.

    a. If the volume of each conical is less than 20mL, then proceed to step 9.

    b. If the volume of each conical is 20mL or more, then proceed to step 6.

6. Dilute the blood to 50mL with DPBS and mix 2 times with a pipette.

7. Take conicals that have been pre-filled with 18mL of DPBS and place them behind the sample conicals.

8. From the 50mL sample volume, separate out approximately 18mL into pre-filled conicals.

9. Bring the volume of each conical to approximately 35mL of whole blood and DPBS.

10. Underlay Ficoll-Hypaque (F/H) by performing the following method:

    a. Insert a 10 ml pipette into a F/H bottle and draw up F/H to orange line of pipette.

    b. Release the pipette from pipette gun to allow F/H to dispense slowly into each conical.

    c. It is essential that the F/H be dispensed slowly so that there is no mixing of the whole blood with the F/H layer.

11. Make sure the centrifuge break is <u>OFF</u> and centrifuge all tubes for 20 minutes at 2400 RPM at 22^{O}C.

12. Harvest the interface which contains the mononuclear cells, with a 10 ml serological pipette.

13. Pool the harvested cells into a clean, sterile 50 ml conical. Cells may be dispensed in two clean 50mL conical if blood volume was received from more than 6 green top tubes. Bring volume of each conical to 50 ml with DPBS.

14. Centrifuge 10 minutes at 500 G (1500 RPM)

15. Aspirate the DPBS with a 2 ml serological pipette into waste system. Leave approximately 120µl of supernatant and vortex pellet gently.

16. If there are 2 conicals of harvested cells, combine into **one** conical.

17. Follow protocol for counting cells.

18. For the final wash, bring volume up to 35mL with sterile DPBS and centrifuge 5 minutes at 1500 RPM.

PBMC Freezing

1. After the final wash, aspirate and discard the supernatant from the PBMC pellet.
2. Re-suspend in Recovery Cell Culture media (freezing media) according to the PBMC count (please refer to the Processing Table):
    a. Freeze in 10 million cells per mL of Recovery Cell Culture media using the appropriate freezing racks
    b. Aliquot 1mL of 10 million cells plus freezing media into the appropriately labeled cryovial
    c. Place vials immediately into Mr. Frosty Cell Freezing Cans and into -80ºC overnight
    d. On the following day, transfer PBMCs from cans in -80 ºC to the -140 ºC freezer (SHIELDLUNG BAL PBMC)

3. Bronchoalveolar lavage processing (Hui Qing in Mark Liu's lab)

A. BAL Supernatant

1. Spin the conical(s) filled with a volume of up to 80mL of BAL supernatant and cells at 1600 rpm for 6 minutes (4 ºC)
2. After centrifugation, remove and save all the supernatant into the following (labeled "Sup"):
    a. Aliquot 1mL into 6 vials. Store at -80 ºC
    b. Aliquot 10mL into 5-8 conicals depending upon remaining volume.Store at -80 ºC
    c. Individual labels should include "Sup", study ID (VitDXXX) and date
3. Re-suspend BAL Cells in 4mL HBSS media.

4. Add 35mL HBSS media to the BAL Cell conical, spin at 1500rpm for 5 minutes (22 ^{o}C)
5. After centrifugation, aspirate and discard the HBSS supernatant from the BAL Cells.
6. Re-suspend the cells in Recovery Cell Culture freezing media:
    a. Freeze in 10 million cells per mL of freezing media using freezing racks.
    b. Aliquot 1mL (10 million cells and freezing media) into appropriately labeled cryovial
        i. Individual labels should include "cells", study ID (VitDXXX) and date
    c. Place vials immediately into Mr. Frosty Cell Freezing Cans and into -80^{o}C for overnight incubation.
    d. On the following day, transfer cells from cans in -80 ^{o}C to the -140 ^{o}C freezer

Processing Table

Biospecimen (Parent tube)	**Volume**	**Aliquot sample type**	**Label Type**	**Aliquot #**	**Aliquot conc. & Volume**	**Freezing Temp. (Celsius)**
BAL conical	80mL	Supernatant (1mL aliquots)	Sup	6	1.0mL	minus 80 ^{o}
		Supernatant (10mL aliquots)	Sup	Depends on Supernatant volume	10.0mL	minus 80 ^{o}
		Bal Cells	cells	Depends on Bal Cell count	$10x10^{6}$ Bal Cells/ 1.0mL	minus 80^{o} overnight to minus 140^{o}
Green top tube	20 ml	PBMC	PBMC	Depends on PBMC count	$10x10^{6}$ PBMC/ 1.0mL	minus 80^{o} overnight to minus 140^{o}
Lavender top EDTA tube for plasma	10 ml	Plasma (250 microliter aliquots)	Plasma	As many as possible	250 microlit	minus 80 ^{o}
SST top for serum	8 ml	Serum (250 microliter aliquots)	Serum	As many as possible	250 microlit	minus 80 ^{o}

SECTION 8: BRONCHOSCOPY PROCEDURES

i. **Patient preparation**

a. The participant should be interviewed prior to the procedure to ensure that there are no contraindications to performing the bronchoscopy such as
   i. recent acute or serious illness
   ii. anticoagulation or bleeding disorder
   iii. fever
   iv. oxygen desaturation on room air
   v. allergy to local anesthetics or sedation
   vi. food intake within 6 hours.

b. Patients should be instructed to take their usual morning oral medications with a small sip of water.

c. Patients should also have a companion or reliable escorted means of transportation home after the procedure so that they can safely return home after the procedure.

d. The patient should be informed that the procedure is being performed for research purposes and is not for clinical care.

e. The details of the procedure should be explained to the participant and consent obtained in accordance with local hospital or clinic practices and procedures.

f. Risks related to the procedure:
   i. allergic or adverse reaction to sedation or local anesthesia
   ii. nose-bleed
   iii. sore-throat
   iv. coughing
   v. chest soreness from coughing
   vi. fever
   vii. infection

g. Participant will be reimbursed in the form of a check, given to participant immediately prior to procedure

ii. **Conduct of the procedure**

a. **Sedation**
   i. Before sedation, a "time-out" should be performed to verify that the patient is correctly identified and is undergoing a bronchoscopy.
   ii. The bronchoscopy insertion orifice should be ascertained (right or left nostril or mouth) and local anesthetic applied in accordance with local practice.
   iii. The patient is moderately sedated during monitoring of vital signs and oxygenation according to local procedures.

iv. Typically a benzodiazepine (e.g midazolam) and a narcotic (e.g. fentanyl) are used.
    1. Upper limits of sedation
        a. Fentanyl 400 mcg IV
        b. Versed 6 mg IV

b. **Method**
    i. The bronchoscope is inserted and the upper airway is anesthetized with 1-2 mL aliquots of lidocaine 1% or 2% solution.
    ii. The lower airways are anesthetized with aliquots of lidocaine to diminish or prevent coughing.
    iii. The amount of lidocaine instilled should be monitored. Typically *no more than 500 mg* should be administered in a single procedure.
    iv. In order to minimize the use of lidocaine, the inspection of the left lung airways should be brief or limited.

c. **Collection of specimens**
    i. The bronchoscope is advanced into the right mainstem bronchus and the BAL is collected by instillation of 50 mL x 2 in the right middle lobe (either segments). If necessary to obtain at least 50 cc BAL return, additional 50 cc aliquots can be instilled in alternative segments of the RML or anterior RUL, to a maximum of 300 cc BAL instillation.
    ii. Return of BAL fluid can sometimes be facilitated by ensuring that the return suction is not too high (< 100 cm H2O), by turning the patient with the right lung superior, or by asking the patient to take a deep breath and cough.

d. **Completion of procedure**
    i. The BAL form is completed which records the results of the BAL.
    ii. The research coordinator should be notified that the procedure is completed.
    iii. If the patient has a post-bronchoscopy fever, the use of anti-pyretics are advised.

e. **Post bronchoscopy care**
    i. The patient should be monitored in supervised environment until they have recovered from sedation, and vital signs and oxygenation are stable.
    ii. The patient should be instructed not to take any food or liquid by mouth for at least two hours after the procedure or until the gag-reflex has returned.
    iii. The patient should be notified to contact the clinic or study physician if any symptoms of concern are noted after the procedure.

i. **Sample Handling**

a. BAL samples should be transported within 30 minutes at room temperature. If transport time will be 30-60 min, samples should be placed on ice. Transport time should not exceed 60 minutes to maximize cell viability.