Title: Vitamin D and Prebiotics for Intestinal Health in Cystic Fibrosis

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IRB Research Protocol

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Short title: Vitamin D and Prebiotics in Cystic Fibrosis

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2. Abstract

Patients with CF have dysfunctional intestinal microbiota. We hypothesize that administration of high-dose vitamin D and a potent prebiotic will be effective and simple strategies to reduce CF-related intestinal dysbiosis and improve critical intestinal functions. We further hypothesize that the combination of vitamin D + inulin will be additive or synergistic in this regard. We therefore propose a pilot and feasibility, 2 x 2 factorial design, placebo-controlled, randomized controlled trial to test the feasibility of our approach in a larger study. We will test our hypothesis with the following aims: 1. Determine the impact of vitamin D and the prebiotic inulin on gut microbiota richness and diversity in stable outpatient adults with CF. We will assess the change in major gut microbiome characteristics from baseline and targeted microbiome-dependent metabolites (secondary endpoints) in 40 randomized CF adults block-randomized to either: 1) high-dose vitamin D3 (50,000 IU weekly plus prebiotic placebo; 2) prebiotic (inulin) plus placebo vitamin D3); 3) combined vitamin D3 and inulin; and 4) double vitamin D and prebiotic placebo. Aim 2. Our primary endpoint will be changes in gut and sputum microbiome.

3. Introduction and Background:

Cystic fibrosis (CF) is the most common life-shortening genetic condition among Caucasians in the United States. Individuals with CF have an altered gastrointestinal (GI) microbiota, which may be a result of chronic systemic inflammation and infection, frequent use of antibiotics, and/or medically prescribed and habitual high-fat/high-calorie diets[1]. Limited studies to date document that the GI microbiota of CF individuals is disordered—resulting in a

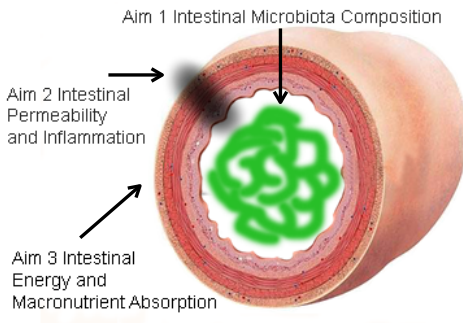
less diverse, pro-inflammatory GI microbiota profile (dysbiosis) compared to a healthy, non-inflammatory GI microbiota composition in individuals without CF. Further, the severity of the CFTR mutation impacts the degree of the GI dysbiosis, which begins early in childhood and persists into adulthood[2]. In addition, GI dysbiosis has been associated with significant CF morbidity, including impaired growth in children[3], nutrient malabsorption[4], GI tract inflammation[4], decreased pulmonary function[5], and increased need for IV antibiotics[5]. Thus, efforts to correct or improve GI microbiota dysbiosis may result in improved CF clinical outcomes.

Studies designed to correct GI dysbiosis with probiotics to reduce pulmonary exacerbation and GI tract inflammation have been inconclusive to date, in part due to limited sample sizes and study quality issues[6]. Prebiotics, which are non-digestible fermentable oligosaccharides, such as fructo-oligosaccharides (e.g. inulin) and resistant starches (e.g. pectin), occur naturally in various plants and are routinely used as dietary supplements and effectively modulate and diversify the GI microbiota in humans[7]. Also, relevant to this proposal, vitamin D deficiency has been associated with GI dysbiosis in rodent models of CF and in clinical studies with individuals with CF[8]. We recently conducted a pilot, double-blind, randomized controlled trial (RCT) examining the impact of high-dose vitamin D_3 supplementation on GI microbiota in adults with CF and concomitant vitamin D deficiency.

Vitamin D_3 supplementation decreased indexes of GI dysbiosis, particularly in subjects with prevalent vitamin D deficiency. Subjects receiving vitamin D_3 (vs. placebo) demonstrated decreased abundance of potentially pathogenic bacteria while less pathogenic bacteria were enriched[9]. In a separate study using high-resolution plasma metabolomics, high-dose vitamin D in adults with CF during an acute pulmonary exacerbation altered metabolic pathways potentially related to gut microbial metabolism[10]. Favorable changes to the GI microbiota have also been observed with vitamin D supplementation in adults without CF[11]. *Therefore, we hypothesize that administration of high-dose vitamin D and a commonly used prebiotic (inulin) will be effective strategies to reduce GI dysbiosis and to improve critical intestinal functions in CF with the additive or synergistic effects of the combination of vitamin D + inulin.*

To address our hypotheses, we propose a pilot and feasibility, placebo-controlled, 2 x 2 factorial, double blind, randomized controlled trial (RCT) of vitamin D ± prebiotic in adults with CF and insufficient vitamin D status.

<u>Aim 1.</u> Determine the impact of high-dose oral vitamin D and the prebiotic inulin on gut microbiota richness and diversity, and associated metabolomic changes, in stable outpatient adults with CF. In a 12-week RCT, we will assess the feasibility of a 2x2 factorial design RCT (primary-endpoint) to change major gut microbiome characteristics from baseline (Shannon diversity index) and targeted microbiome-dependent metabolites in 40 CF adults block-randomized to either: 1) high-dose vitamin D_3 50,000 IU weekly (plus prebiotic placebo); 2) inulin (12 g/day) prebiotic (plus vitamin D placebo); 3) combined vitamin D_3 and inulin; and 4) vitamin D placebo and prebiotic placebo. At baseline and



12 weeks, we will determine changes in GI microbiota composition, diversity, and richness using 16S rRNA gene sequencing and microbiome-dependent metabolites pathways in stool and plasma using high-resolution metabolomics analysis.

3.1 Scholarly rationale and prior literature

Intestinal Microbiota in CF

Patients with CF have aberrant intestinal microbiota, as evidenced by decreased bacterial diversity and overabundance of pathogenic bacteria starting in infancy[12],[13]. Many factors in CF may lead to aberrant intestinal microbiota including frequent use of antibiotics, small bowel bacterial overgrowth, disturbed bowel motility, alterations in mucin clearance, pancreatic insufficiency, and intestinal inflammation[14,15,16]. It is also possible that the habitual high-fat CF diet further promotes dysbiosis[17]. In a study of adolescents with CF, the severity of the CFTR mutation (presence of F508del) was associated with more abundant pathogenic bacteria such as *E. coli* and *E. biforme,* less abundant beneficial bacteria, and less microbial diversity[18], suggesting an inherent dysbiosis caused by the CFTR defect. Rodent models of CF have mirrored the GI dysbiosis found in patients with CF in that rodents with mutations in the CFTR have less bacterial diversity and richness compared to wild type (WT) animals[19,20]. *It is, therefore, clear that GI dysbiosis exists in patients with CF; however, the clinical significance of this state is unknown.*

Antibiotics alter intestinal microbiota

Patients with CF often receive combination antibiotic therapy to ensure coverage against the highly pathogenic bacteria *Pseudomonas aeruginosa[21]*. Broad spectrum antibiotic therapy alters the intestinal microbiota by disrupting local host-microbe communities, leading to decreased bacterial richness and diversity[22]. In a study of adults with CF during a period of stability, the number of IV antibiotic courses was inversely related to the intestinal microbiota diversity[23]. A longitudinal study of a single patient with CF found that courses of antibiotic therapy decreased intestinal diversity and richness[24]. *Therefore, wide spectrum antibiotic therapy remains a major factor in the intestinal dysbiosis found in patients with CF.*

Probiotics in cystic fibrosis

Early, generally small, studies suggested that probiotics may improve GI health and nutrition in patients with CF. A 6-month RCT of a *Lactobacillus reuteri*-based probiotic given to adults with CF found increased GI diversity and decreased abundance of the potentially pathogenic Proteobacteria phylum compared to placebo[25]. Bruzzese et al found *Lactobacillus GG* given to children with CF experienced reduced abundance of potentially pathogenic bacteria and improved intestinal inflammation as assessed by fecal calprotectin[26]. Jafari et al also found that probiotics given to children improved quality of life and rate of pulmonary exacerbations compared to placebo[27]. In contrast, a large study of *Lactobacillus GG* in children up to age 16 found no impact of probiotics on pulmonary exacerbations or other clinical outcomes such as BMI and FEV1[28]. There are currently too few well designed RCTs investigating the role of probiotics in patients with CF to recommend this therapy for all patients with CF[29,30]. Furthermore, probiotics have been reported to cause bacteremia in immunocompromised patients

which is of concern in patients with CF[31,32]. *Thus, the efficacy and safety of probiotics to improve GI dysbiosis and subsequent clinical CF outcomes is not well-established.*

Prebiotics, gut microbiota and risk respiratory infections

Prebiotics are non-digestible dietary fibers that include inulin-type fructans and oligosaccharides that promote the formation of beneficial bacteria in the gut[33]. Prebiotics do not contain bacteria and thus *are not* associated with bacteremia. A number of RCTs have confirmed that prebiotics can change gut microbiota in adults and children without CF[34,35,36]. Additionally, some RCTs have demonstrated that changes in gut microbiota were associated with improved lung health. Ranucci et al reported in infants without CF enrolled in a 24 month RCT that an oligosaccharide-enriched formula protected against respiratory infections and increased the abundance of Bifidobacteria, a genera associated with protection against respiratory infections[37]. Another RCT examining the prebiotics galacto-oligosaccharides and polydextrose found in children without CF found that the prebiotic formula was associated with fewer episodes of respiratory infections compared to children ingesting a standard formula[38]. *Although prebiotics have demonstrated efficacy in promoting beneficial gut microbiota and improving respiratory infections in children without CF, no studies have been published using prebiotics in children and adults with CF. Prebiotics potentially have a better safety profile than probiotics since they are not associated with risk of bacteremia.*

Preliminary Results: Vitamin D status and intestinal microbiota in CF

Others have shown that vitamin D deficient mice or vitamin D receptor KO mice, have increased intestinal dysbiosis and GI inflammation[39]. We explored the role for vitamin D in altering the intestinal microbiota in a pilot randomized placebo-controlled trial of adults with CF. We evaluated 41 adults with CF who were clinically stable and were not on recent IV antibiotics. Prior to any intervention, we found that vitamin D insufficient subjects displayed a higher abundance of potentially more pathogenic bacteria in the stool from the class of Gammaproteobacteria (class representing gram- bacteria such as Pseudomonas) (**Figure 2**).

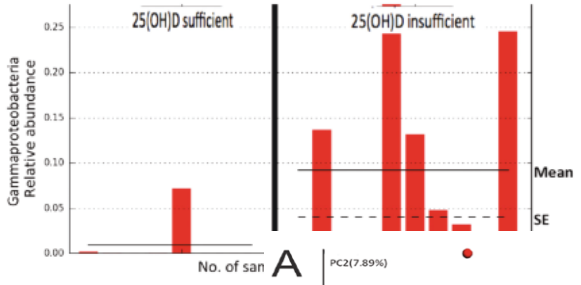


Figure 2 Relative abundance of Gammaproteobacteria in the stool of adults with CF based on vitamin D status: vitamin D (25(OH)D) insufficient (<30 ng/mL) or vitamin D sufficient (≥30 ng/mL). Subjects with vitamin D insufficiency had more abundance of this class of potentially pathogenic bacteria compared to those with vitamin D sufficiency.

We then randomized 23 subjects were vitamin D insufficient to vitamin D_3 50,000 IU weekly for 12 wks or placebo. We found that treatment with vitamin D_3 demonstrated significant changes in the clustering of microbial populations compared to placebo (**Figure 3**). Subjects who received vitamin D had significant

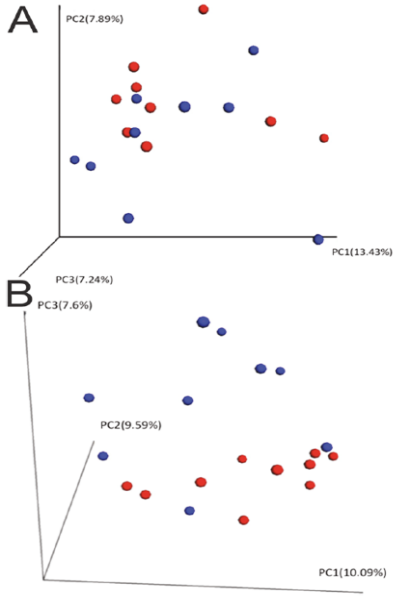
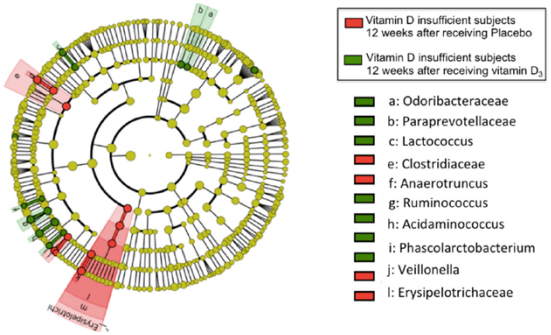


Figure 3 Principal coordinate (PC) analysis based on unweighted UniFrac distance matrices generated with stool 16S rRNA gene sequencing. Adults with CF and vitamin D insufficiency (25(OH)D < 30 ng/mL) were randomized to vitamin D (blue) or placebo (red). Stool was collected at baseline (panel A) and 12 weeks of intervention (panel B). Principal coordinate analysis demonstrates differential clustering of bacterial communities before and after treatment.

increases in the taxa of *Lactococcus* (generally accepted as beneficial bacteria) and significant decreases in *Veillonella* and *Erysipelotrichaceae* (potentially more pathogenic bacteria) (**Figure 4**)

In a separate pilot study of CF patients hospitalized for acute pulmonary exacerbation, high-resolution plasma metabolomics indicated that butanoate and tryptophan metabolism, among several other pathways, were altered by high-dose vitamin D compared to placebo[40]. Butanoate (butyrate) is a major metabolite arising from the bacterial fermentation of dietary fiber, and tryptophan metabolism is heavily influenced by the gut microbiota[41,42]. *Together, these data provide strong preliminary data for a potential effect of vitamin D treatment on gut microbiota, as well as for the utility of high-resolution metabolomics to assess metabolic responses to an intervention.*

Figure 4 Linear discriminant analysis Effect Size (LefSe) in adults with CF and vitamin D deficiency randomized to vitamin D (green) or placebo (red). The LefSe analysis demonstrates genera and species substantially and differentially abundant in the stool samples from subjects randomized with vitamin D compared to those randomized to placebo.



4.1) Specific Aims and Objectives

We hypothesize that administration of high-dose vitamin D and a commonly used prebiotic (inulin) will be effective strategies to reduce GI dysbiosis and to improve critical intestinal functions in CF with the additive or synergistic effects of the combination of vitamin D + inulin.

To address our hypotheses, we propose a pilot and feasibility, placebo-controlled, 2 x 2 factorial, double blind, randomized controlled trial (RCT) of vitamin D ± prebiotic in adults with CF and insufficient vitamin D status.

4.2) Specific aims:

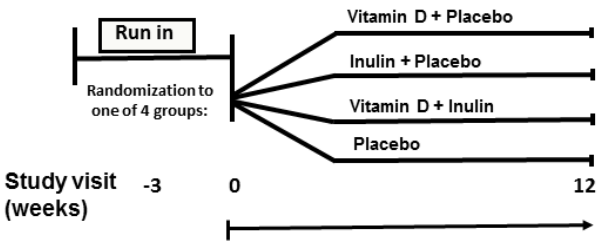
Aim 1. Determine the impact of high-dose oral vitamin D and the prebiotic inulin on gut microbiota richness and diversity, and associated metabolomic changes, in stable outpatient adults with CF. In a 12-week RCT, we will assess the feasibility of a 2x2 factorial design RCT (primary-endpoint) to change major gut microbiome characteristics from baseline (Shannon diversity index) and targeted microbiome-dependent metabolites in 40 CF adults block-randomized to either: 1) high-dose vitamin D3 50,000 IU weekly (plus prebiotic placebo); 2) inulin (12 g/day) prebiotic (plus vitamin D placebo); 3) combined vitamin D3 and inulin; and 4) vitamin D placebo and prebiotic placebo.

At baseline and 12 weeks, we will determine changes in GI microbiota composition, diversity, and richness using 16S rRNA gene sequencing and microbiome-dependent metabolites pathways in stool and plasma using high-resolution metabolomics analysis.

Primary outcome measure: To compare phylotype richness of the respiratory and gastrointestinal tract microbiota before and after supplementation with high dose vitamin D ± inulin. These will be measured using ecological diversity measures including the Shannon index and the Species Richness index.

5. Study design and methods

5. 1 Overview of study design (Figure)



Study synopsis: **60 adults will sign consent for screening with 40 randomized** with CF and vitamin D intake less than 2000 IU daily will be randomized in a 12-week RCT to: **1**) high-dose vitamin D_3 (50,000 IU weekly plus 12 g corn-derived maltodextrin/day as the prebiotic placebo); **2**) Chicory-derived inulin (12 g/day) prebiotic plus placebo vitamin D_3); **3**) combined vitamin D_3 and inulin; and **4**) placebo vitamin D_3 and placebo prebiotic. Stool and serum will be collected at baseline and at 12 weeks in the Clinical Research Center (CRC) of the NIH-funded Georgia Clinical and Translational Science Alliance (Georgia CTSA). We will assess the feasibility of this approach and collect preliminary data on changes in gut microbiota diversity (Shannon Index as primary), composition, and richness from baseline over time using 16S rRNA gene sequencing to inform future studies. Additional measures will include high-resolution stool and plasma metabolomics.

a. Enrollment:

1. **Patient Selection:** We will screen for study patients from adults with CF seen for routine care at the Emory CF Center. *Inclusion Criteria:* 1) male and female patients (age > 18 years) with confirmed CF by genetic mutation and/or sweat chloride testing, 2) ~~stable pulmonary function (no greater than 5% difference in FEV1% predicted from previous measurement within the past year)~~, 3) not currently on oral or systemic antibiotics for pulmonary exacerbation 4) use of CFTR modulator therapy is allowed *Exclusion Criteria:* 1) active GI disease, abdominal pain and/or diarrhea, 2) chronic kidney disease worse than stage 3 (eGFR < ml/min per 1.73 m^2), 3) any vitamin D supplement use >2,000 IU or vitamin D analogue, (**patients who are taking more than 2,000 IU of vitamin D must agree to stop the vitamin D for 6 weeks and take less than 2,000 IU of vitamin D during the study**) 4) use of immunosuppressants or history of organ transplantation, or 5) current use of probiotics or prebiotics.

2. **Subject identification and initial screening: We propose to consent 60 subjects at least to randomize 40 subjects in the following arms.** We propose to study **40 subjects (10 in each arm)** in this 2x2 factorial design study (statistical considerations below).

Patients with CF who are scheduled for routine outpatient appointments will be pre-screened for eligibility by reviewing the electronic medical record.

3. **Informed consent and subject enrollment:** Informed consent will be obtained from the subject or the legally authorized representative. Amid the coronavirus crisis, informed consent will be obtained via secure videochat via Emory Zoom. The patient's identity will be confirmed with two forms of identification with full name and date of birth. The research coordinator or Drs. Alvarez, Ziegler, or Tangpricha will obtain informed consent from the eligible subjects. The informed consent will be signed securely via DocuSign and returned via encrypted email. If the subject can not return via DocuSign, the subject will take a photo of the signed informed consent and return via encrypted email and mail in the wet signature. Once the DocuSign form is received or the photo/wet signature is received, the subject will be mailed the kits for stool and sputum collection as well as the vitamin D/prebiotic/inulin/placebo.

4. **Baseline studies**: Stool samples will be collected using a stool kit provided to the subject. Subjects will be provided in person or via return mail with a stool kit and a prepaid envelope to have the sample mailed directly to the study lab within the next 48 hours. These will be stored at -80 degree C. Subjects will provide a sputum sample in person or via return mail. Sputum samples will be collected via a sputum kit on day of visit and stored at -80 degree C. Relevant medical information will be extracted from the medical record and from questions to the participant.

5. **Intervention:**

Vitamin D dose: We have conducted a number of studies demonstrating very effective use of vitamin D_3 at a dose of 50,000 IU weekly in the adult CF population. Our study in 2009 demonstrated that vitamin D_3 was superior to vitamin D_2 or UV lamp therapy in CF[43]. Subjects receiving weekly doses of oral vitamin D_3 50,000 IU for 12 weeks raised mean serum 25(OH)D concentrations from 21 ± 10 to 47 ± 20 ng/mL ($p<0.001$) with 100% of subjects achieving a serum 25(OH)D greater than 30 ng/mL. We conducted a more recent study examining our dosing regimen of vitamin D3 50,000 IU vs placebo weekly for 12 weeks on gut microbiota in patients with CF and found that serum 25(OH)D increased significantly in the vitamin D_3 group from a mean of 24.99 ± 5.05 to 45.29 ± 21.01 ($p<0.05$) compared to no change in placebo. Therefore, for this study we will use our established vitamin D dosing regimen of vitamin D_3 50,000 IU once weekly for 12 weeks. As in past studies, we will obtain the vitamin D_3 and matching placebo capsules from Bio-Tech Pharmacal, Inc.

Inulin: Inulin is non-digestible fiber found in many food products. Inulin is alsoavailable as an over the counter supplement. Inulin will be provided from the brand name "Now Foods". This is a commercially available inulin supplement that is available in powder form. We have been in contact with the investigational pharmacy who will make sachets of 12 grams of Jetsu inulin for the active inulin group and 12 grams of

maltodextrin which is a food additive and commonly used as a control to inulin. Subjects will be asked to dissolve a sachet daily into a beverage of their choice (coffee, tea, water) and to ingest this daily.

All study drugs will be dispensed by the Investigational Drug Pharmacy.

6. **Follow-up studies:** Patients will be followed throughout the duration of the study for 3 months. Subjects will provide their stool and sputum sample either in person or return by mail. For those subjects seen in person, participants will be seen for study follow-up visits at 3 months (+/- 4 weeks) as scheduled for their usual medical care (routine out-patient follow-up visits). Interim phone calls will be made to participants on a bi-weekly basis after week 1 to remind about taking pills. This will be done by the co-investigator. At the end of 3 months, we will obtain repeat stool and sputum samples from the entire cohort.

Procedures to be performed:

a. A history and physical exam will be performed as routine standard of care for patients presenting to the Cystic Fibrosis Center. Relevant medical information will be extracted from the medical record and from questions to the participant

b. Stool collection: Participants will be asked to provide a stool sample in a collection container for analysis of stool microbiota. This will be done upon enrollment (baseline) and at 3 month follow-up.

c. Sputum collection**:** Participants will be asked to expectorate sputum into a collection container for analysis of airway microbiota. This will be done upon enrollment (baseline) and at 3 month follow-up.

d. Dietary history will be taken at baseline and at the end of the study to determine if there are any changes in dietary pattern pre and post intervention.

7. **Risk/benefits:**

Potential risk: The risk of participating in this study is minimal. There is the question of withholding treatment in subjects who are vitamin D insufficient and randomized to receive placebo however, there is currently no evidence that short duration (3 month) period of not receiving supplemental vitamin D affects morbidity and mortality in CF. Moreover, at the end of the 3 months, patients in both groups will be treated with 1000 IU daily of vitamin D to ensure that the group that didn't get vitamin D will be treated. Inulin is not systematically absorbed so there should be no systemic side effects. However, inulin can cause

gastrointestinal side effects such as bloating, diarrhea and excess gas. We will monitor for this potential side effect during our biweekly calls for adherence to the study drug.

Additional risks will be associated with confidentiality issues surrounding administration of the patient questionnaire and collection/recording of data, but steps will be taken to minimize these risks as discussed below.

Potential benefits: There may be no specific benefit to patients participating in the study. If our hypotheses are confirmed, and vitamin D plus/minus inulin supplementation improves the beneficial microbiota in CF. Future studies will investigate the cause of dysbiosis and other potential clinical outcomes. Therefore, the anticipated benefits of the proposed research study outweigh the potential risks to participating subjects.

8. Data collection

The following chart demonstrates when and from where data items will be collected. All of the sputum, and stool samples that are collected at enrollment and at the 3 month follow-up visit will be stored for future analysis of microbiota. Samples will be stored for long term storage for future studies only if subjects provide written informed consent with a second informed consent to grant long term storage of samples.

Data Items	**Source and timeline of collection**
Demographic Information	Self-report; Electronic Medical Record
Medical history (e.g., CF genotype , Dietary supplement and medication intake and antibiotic treatment and pulmonary exacerbations in the past 3 months)	Completed at enrollment. Self-report; Electronic Medical Record
Sputum microbiota analysis	Sputum Sample collected at baseline and month 3 during standard follow-up visit to the CF center for routine care.
Stool microbiota analysis	Stool Sample collected at baseline and month 3 during standard follow-up visit to the CF center for routine care.
Spirometry (including FEV1 and FVC) – as standard of care by inpatient and outpatient hospital team.	Obtained from electronic medical Record as pulmonary function tests are measured at baseline and 3 months as part of routine care.

8.1 Specimens to be collected: Specimens collected at baseline and follow-up visits will include stool and sputum.

8.2 Randomization: Subjects will be randomized to two interventions (vitamin D3 vs placebo) and (prebiotic vs placebo). Treatment assignments will be performed by blinded allocation according a schedule maintained by biostatisticians of the Georgia CTSA. The subjects' physicians will be informed of their participation in the trial, and the electronic medical record at Emory will be flagged indicating that the patient is participating in this trial to prevent the use of vitamin D or pre/probiotics. Our primary analysis will be intention-to-treat. If any subjects receive therapies that impact the microbiome such as antibiotics or immunosuppressant drugs, they will be analyzed in the groups to which they were randomized. In a secondary analysis we will make note of the subjects who these therapies and determine if there were any between group differences in the initiation of these therapies. The Investigational Drug Pharmacy will provide the randomization scheme.

8.3 Archived specimens: Study subjects may sign an additional consent form for long term storage of samples. In addition to immune function, vitamin D has been linked to several non-skeletal disease states and conditions in the general population that may be applicable to CF. One example is diabetes, a common co-morbidity in patients with CF. It is, therefore, possible that remaining specimens will be banked for future study related to vitamin D in CF but beyond the specific scope of the current study. Any stored samples will be de-identified with a specific code whose identity can only be accessed by authorized study personnel appointed by the PI. Specimens will be stored in a -80° freezer at the Vitamin D and Bone Research Laboratory at the Emory University School of Medicine Division of Endocrinology and Metabolism.

9. Participant selection

9.1 Requested sample size and expected refusal or withdrawal rate: We propose to recruit 60 subjects to sign consent in order to have 40 randomized at the study site. This is a pilot study which will provide critically needed data to adequately power a larger study. The CF Foundation funded this as a pilot and feasibility study.

9.2 Inclusion/exclusion criteria:

We will screen for study patients from adults with CF seen for routine care at the Emory CF Center.

Inclusion Criteria: 1) male and female patients (age > 18 years) with confirmed CF by genetic mutation and/or sweat chloride testing, 2) stable pulmonary function (no greater than 5% difference in FEV1% predicted from previous measurement within the past year), 3) not currently on oral or systemic antibiotics for pulmonary exacerbation, and 4) use of CFTR modulator therapy is allowed

Exclusion Criteria: 1) active GI disease, abdominal pain and/or diarrhea, 2) chronic kidney disease worse than stage 3 (eGFR < ml/min per 1.73 m^2), 3) any vitamin D supplement use >2,000 IU or vitamin D analogue, 4) use of immunosuppressants or history of organ transplantation, or 5) current use of probiotics or prebiotics.

9.3 Subject recruitment plan: Recruitment will be performed through referral from the outpatient CF Clinic.

9.4 Screening for eligibility: All patients with CF will be screened for eligibility at presentation to the CF clinic for routine visit by reviewing the subject's vitamin D intake. Screening will be completed daily by the research coordinator. Patients who have met the above inclusion criteria and have met none of the exclusion criteria on our initial assessment will be offered the opportunity to participate in our study. The study personnel will explain the study protocol in detail in the consent form as well as verbally. Any additional information regarding questions or concerns from potential or confirmed study participants will be provided by the study coordinator.

9.5 **Withdrawal from study:** Participants may withdraw from the study at any time. Automatic withdrawal occurs when a participant moves away from the center or refuses providing further blood, stool or sputum sample.

10. Statistical analysis

Statistical Considerations for Pilot Study:
We received this funding from the CF Foundation to test the feasibility of this 2x2 study design.

We will evaluate the feasibility of recruitment, retention, assessment and implementation of the proposed interventions. We wish to identify any potential flaws in the study design and potential challenges in the subsequent large-scale study. Our measures of feasibility will focus on metrics of recruitment, retention, acceptability & completion as adopted from the NIH/NCCIH . Our target metric of success is compared to our previous single intervention study with vitamin D (Table).

Feasibility Question	Feasibility Metric	Prior Rate	Target Metric of Success
Recruitment	Number of subjects screened a month	16.2/month	>15/month
Recruitment	Number of Enrolled/Screen Fails	1:4.05	1:4 Enrolled/Screened
Retention	Number of dropouts and reasons for dropout	13% (3 out of 23 enrolled)	<15%
Adherence	Number of subjects who adhere to assignment	Not recorded	>80%
Acceptability	Subject ratings for acceptability during and after participation	Not recorded	>3 on 5 point Likert scale
Completion	Number of subjects who complete all study procedures	73%	>70%
Completion	Duration from initiation to completion of pilot study	52 subjects in 15 months	40 randomized subjects in 15 months

The pilot sample size of 40 randomized patients is based on the pragmatics of recruitment, patient flow, and budgetary constraints. We have preliminary data on vitamin D; however, we do not have data on prebiotics which necessitates this pilot study to adequately power a larger study.

For the vitamin D arm, our preliminary data suggest a standard deviation of 1.2 of the diversity index. In our proposed pilot study consisting of 20 subjects in each vitamin D arm, we have 29% power to detect a between-group difference of 0.5 and 56% power to detect a difference of 0.75 in the diversity index using a significance level of 0.05. For the prebiotic arm, we do not have preliminary data. However, if vitamin D and pre-biotic have a synergistic effect, there may be greater power to detect a between-group difference of 0.5 (or 0.75).

The CF Foundation acknowledges that we are underpowered as a pilot study. However, our goal is not to evaluate efficacy of the proposed interventions but to examine the feasibility of conducting this pilot in a 2x2 factorial study of a larger scale. Moreover, the pilot data from this study will give us a first impression of the variability and effect sizes of the Shannon diversity index in the patient population of interest in each of the 4 treatment arms and thus assist in the sample size and power calculation of the subsequent larger study. In addition, these pilot data will inform power calculations for our secondary outcomes. Finally, the data that we collect from this pilot will help identify modifications needed in the planning and design of the subsequent study. We will also determine between-group differences, but given that low power is characteristic of pilot studies, statistical significance is not the focus of the analysis. We will also summarize patient characteristics at baseline and during follow-up using mean ± SD (or median and interquartile range, as appropriate) for continuous variables and frequency (percentage) for categorical variables. Statistics on the diversity index will also be reported at baseline and at 12 wks for each of the 4 treatment combinations. A qualitative review will also be provided, and this will include, among others, an evaluation of the representativeness of the subjects, a discussion on how to refine the data collection tools, how to track patients better, and how to improve implementation of the proposed interventions.

11. Adverse event reporting:

All adverse events will be documented and reviewed by the PI. These will be enumerated and reported according to IRB policies. Any serious adverse events will be reported within 24 hours to the IRB. They will be reviewed by all investigators and a disposition determined before proceeding with further enrollment.

12. Data and safety monitoring plan (DSMP)

The procedures associated with this research trial are associated with minimal risk. Therefore, no formal data safety monitoring board will be appointed. Our data safety and monitoring plan will be as follows: The physical risks associated with this study are associated with venipuncture, any adverse events associated with venipuncture will be monitored by the site study coordinator and reported to the study PI, Dr. Tangpricha. Adverse events will then be reported to the Emory IRB within the timeframe stipulated by IRB regulations.

Oversight of the progress and safety of the trial will be provided by the PI. Adverse events are not anticipated, but any occurring will be documented and reported according to Emory IRB policies and procedures. Cumulative adverse events and study progress summary will be communicated to the IRB at the time of continuing review.

Anticipated Adverse Events:

Untreated vitamin D deficiency: Subjects enrolled in the placebo arm of this study may have 25(OH)D less than 30 ng/mL which is considered vitamin D insufficient by the present CFF guidelines. Vitamin D deficiency does not generally result in clinical symptoms. The study will be completed over the course of 3 months and thus long term consequences of low vitamin D would be unlikely. There is currently no evidence that short duration (3 month) period of not receiving supplemental vitamin D affects morbidity and mortality in CF. Moreover, at the end of the 3 months, both groups will be treated with 1000 IU Vitamin D daily to ensure that the group that did not get vitamin D will be treated.

Vitamin D Toxicity/Hypercalcemia: One of the potential risks of taking high amounts of vitamin D is vitamin D toxicity. This would manifest as hypercalcemia. The risk of this will be minimal since the dose of vitamin D that has been proposed in the intervention arm is 50,000 IU once a week for 3 months which has been recommended by the CF foundation as standard of care for treatment of vitamin D deficiency.

Gastrointestinal Discomfort from Inulin: This is a dietary fiber and there could be some side effects as a result of the inulin administration including diarrhea, bloating, excess gas. We will record these reports as part of the study.

Patient Monitoring: will be performed by the P.I. and Co-Investigators

Plans for protecting subject confidentiality:

Recruitment and Informed Consent: Potential subjects will be approached during routine clinic visit in person or via telemedicine at the Emory cystic fibrosis center after discussion and approval of the subject's care team. Participation in our study is voluntary and their decision does not affect their healthcare in their CF center. The site-PI or designee will discuss the study with each potential subject and ask questions to determine whether the subject (and legal guardian) comprehends the risks and benefits of the research. If the subject (and legal guardian) decides to participate, they will provide written informed consent.

Confidentiality: All paper records and case report forms will be kept in locked file cabinets prior to data entry into the electronic database. All electronic data will be coded, de-identified and stored on password protected computers and will be accessible only to the investigative team. All data maintained in the computerized database will be accessible only with a login and protected password by members of the investigative team. After the study is completed, all data will be kept according to NIH and FDA regulations in a locked file.

13. If applicable: pharmaceutical, biologic, and device information

We will obtain the vitamin D and matching placebo capsules from BioTech Pharmacal Inc and inulin from Now Foods. At the initiation and at the end of 3 months, we will test the capsule for vitamin D content by an independent laboratory to ensure that the dosage is correct and remains stable throughout the duration of the study.

Our clinical trial using vitamin D and inulin is exempt from IND regulations [21 CFR 312.2 (b)]. These is based on the fact that we are using standard supplementation protocols that have been recommended by the CF foundation for treatment of vitamin D Deficiency. There are no guidelines for inulin dosing. Moreover, our trial is not intended to support a new indication for use, will not support a change in advertising, does not change the route of administration, will be conducted in compliance with an IRB and informed consent and will not be represented as safe or effective for the purposes under investigation in this proposal.

11. References and appendices

APPENDIX 1: REFERENCES

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