Bolus Weekly Vitamin D_3 Supplementation Impacts Gut and Airway Microbiota in Adults With Cystic Fibrosis: A Double-Blind, Randomized, Placebo-Controlled Clinical Trial

Mansi Kanhere,[1] Jiabei He,[2] Benoit Chassaing,[3,4] Thomas R. Ziegler,[5,6] Jessica A. Alvarez,[5] Elizabeth A. Ivie,[5] Li Hao,[5] John Hanfelt,[2] Andrew T. Gewirtz,[3] and Vin Tangpricha[5,6]

[1]Division of Endocrinology, Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30322; [2]Rollins School of Public Health, Emory University, Atlanta, Georgia 30322; [3]Center for Inflammation, Immunity & Infection, Institute for Biomedical Sciences, Georgia State University, Atlanta, Georgia 30303; [4]Neuroscience Institute, Georgia State University, Atlanta, Georgia, 30303; [5]Division of Endocrinology, Metabolism, and Lipids, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia 30322; and [6]Atlanta VA Medical Center, Decatur, Georgia 30300

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

Context: Disruption of gut microbiota may exacerbate severity of cystic fibrosis (CF). Vitamin D deficiency is a common comorbidity in patients with CF that may influence composition of the gut microbiota.

Objectives: Compare microbiota of vitamin D-sufficient and -insufficient CF patients and assess impact of a weekly high-dose vitamin D_3 bolus regimen on gut and airway microbiome in adults with CF and vitamin D insufficiency (25-hydroxyvitamin D < 30 ng/mL).

Design: Forty-one subjects with CF were classified into two groups: vitamin D insufficient (n = 23) and vitamin D sufficient (n = 18). Subjects with vitamin D insufficiency were randomized to receive 50,000 IU of oral vitamin D_3 or placebo weekly for 12 weeks. Sputum and stool samples were obtained pre- and postintervention and 16S ribosomal RNA genes sequenced using Illumina MiSeq technology.

Results: Gut microbiota differed significantly based on vitamin D status with *Gammaproteobacteria*, which contain numerous, potentially pathogenic species enriched in the vitamin D-insufficient group. Principal coordinates analysis showed differential gut microbiota composition within the vitamin D-insufficient patients following 12 weeks treatment with placebo or vitamin D_3 (permutation multivariate analysis of variance = 0.024), with *Lactococcus* significantly enriched in subjects treated with vitamin D_3, whereas *Veillonella* and *Erysipelotrichaceae* were significantly enriched in patients treated with placebo.

Conclusion: This exploratory study suggests that vitamin D insufficiency is associated with alterations in microbiota composition that may promote inflammation and that supplementation with vitamin D has the potential to impact microbiota composition. Additional studies to determine the impact of vitamin D on microbiota benefit clinical outcomes in CF are warranted. ***(J Clin Endocrinol Metab* 103: 564–574, 2018)**

Cystic fibrosis (CF) is a chronic disease, principally characterized by abnormal mucosa in the digestive and respiratory systems as a result of a mutation in the CF transmembrane conductance regulator (CFTR) gene. The CFTR gene encodes an ion channel that regulates chloride transport on the epithelial surface of the airway,

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA
Copyright © 2018 Endocrine Society
Received 6 September 2017. Accepted 13 November 2017.
First Published Online 16 November 2017

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CONSORT, Consolidated Standards of Reporting Trials; $FEV_{1\%}$, forced expiratory volume; LDA, linear discriminate analysis; LEfSE, linear discriminate analysis effect; PCR, polymerase chain reaction; permanova, permutation multivariate analysis of variance; QIIME, Quantitative Insights Into Microbial Ecology; rRNA, ribosomal RNA; SD, standard deviation.

pancreas, and gastrointestinal tract; hence, CFTR mutations result in impaired clearance of secretions in the lungs, decreased bicarbonate secretion from the pancreas, and malabsorption in the gastrointestinal tract (1). As a result, patients with CF develop recurrent sinus and pulmonary infections, CF-related diabetes as a result of chronic scarring and destruction of the pancreas, fat malabsorption, and eventually, respiratory decline and failure (2, 3). There is evolving evidence that the chronic proinflammatory intestinal environment in individuals with CF leads to and/or is a result of intestinal dysbiosis (disruption of the microbiota) with a substantial decrease in microbial diversity compared with healthy controls (4–6). Likewise, studies show that the airway microbiota is also affected in CF, with alterations beginning in childhood (7), showing a decrease in microbial diversity associated with advancing age and declining lung function (8, 9). In addition, there appear to be changes in specific bacterial groups that herald the onset of pulmonary exacerbations and initial *Pseudomonas* species colonization (10). Therefore, intestinal dysbiosis may have important implications for clinical symptoms and management in CF. Burke *et al.* (6) found a correlation among percent-predicted forced expiratory volume in 1 second ($FEV_{1\%}$) and reduced gut microbial diversity, reduced α diversity (a measure of species richness and even-ness and a marker of a healthy gut microbiota), and lower relative abundance of beneficial bacteria, such as *Roseburia*, in those with most severe lung disease.

Patients with CF commonly develop vitamin D insufficiency for myriad reasons, including fat malabsorption, decreased vitamin D intake, and decreased sunlight exposure (11). An important extraskeletal function of vitamin D is its role in immune regulation, such as upregulation of antimicrobial peptide expression (cathelicidins and B-defensins) and inhibition of effector T cell Th1 and Th17 function (12–14). Recent studies in murine models identified vitamin D metabolism as an important factor influencing the gut microbiota, through the vitamin D receptor, which is abundantly expressed in the ileum (15, 16). Vitamin D receptor is proposed to maintain the integrity of the intestinal mucosal barrier through the inhibition of inflammation-induced epithelial cell apoptosis and enhancement of intercellular junctions (17, 18). Recent studies have demonstrated that vitamin D supplementation may be important to maintain normal gut microbial homeostasis in healthy subjects with a reduction in typical opportunistic pathogens and an increase in species richness (a parameter associated with a healthy gut microbiota) (19, 20).

Although there is robust evidence to support the hypothesis that microbial dysbiosis is predominant in individuals with CF, clinical significances of such dysbiosis are not well understood. Moreover, there have been no studies, to date, to evaluate if vitamin D insufficiency predisposes CF patients to a greater dysbiosis. The aim of the current study was to evaluate the role of vitamin D insufficiency on the airway and gut microbiota in patients with CF and explore the association with pulmonary outcomes. Furthermore, with the use of a randomized placebo-controlled trial, we sought to explore our hypothesis that supplementation with high-dose vitamin D might restore a more healthful microbiota and thus, improve clinical outcomes in patients with CF and vitamin D insufficiency.

Materials and Methods

The study was approved by the Emory University Institutional Review Board. Patient enrollment began in November 2015; follow-up for the last patient was completed in February 2017. The study was registered at www.clinicaltrials.gov (NCT02589444). Potential study subjects were identified among those presenting for routine visits at the CF Clinic at the Emory University Cystic Fibrosis Center, and interested subjects were recruited after written, informed consent. Subject enrollment and allocation are outlined in the Consolidated Standards of Reporting Trials (CONSORT) diagram (Fig. 1).

Trial design

This was a double-blind, randomized, placebo-controlled, interventional pilot study in 41 adults with CF. Patients with CF who were ≥18 years of age without contraindication to oral high-dose vitamin D met inclusion criteria. Exclusion criteria included the following: 1) use of immunosuppressants, 2) pregnancy or plans to become pregnant in the next 3 months, 3) disorders associated with hypercalcemia, 4) current hypercalcemia (albumin-corrected serum calcium >10.8 mg/dL, or ionized calcium >5.2 mg/dL), 5) history of nephrolithiasis with active symptoms within the past 2 years, 6) chronic kidney disease worse than stage III (<60 ml/min), 7) current substantial hepatic dysfunction total bilirubin >2.5 mg/dL, with direct bilirubin >1.0 mg/dL, 8) history of HIV/AIDS, 10) history of illicit drug abuse. In addition, we amended our study with three additional criteria: 11) systemic antibiotic use in the last 4 weeks, 12) use of probiotics, and 13) inflammatory bowel disease, 4 months after the start of the study and after 12 subjects were randomized, as we considered that these factors may also influence our study endpoints. Of the 12 subjects who were randomized, only 4 would have been excluded.

Intervention

Subjects who were screened and enrolled were classified into two groups: those with serum 25-hydroxyvitamin D [25(OH)D] level < 30 ng/mL (vitamin D insufficient) and those with serum 25(OH)D levels ≥ 30 ng/mL (vitamin D sufficient), based on levels obtained at baseline and per the CF Foundation Vitamin D Guidelines (21). Baseline assessment included demographic data, weight, height, CF mutation, history of current medications, history of pulmonary exacerbations (defined as new or worsening respiratory signs or symptoms reported by the subject or picked up by the clinician, requiring start of oral or

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

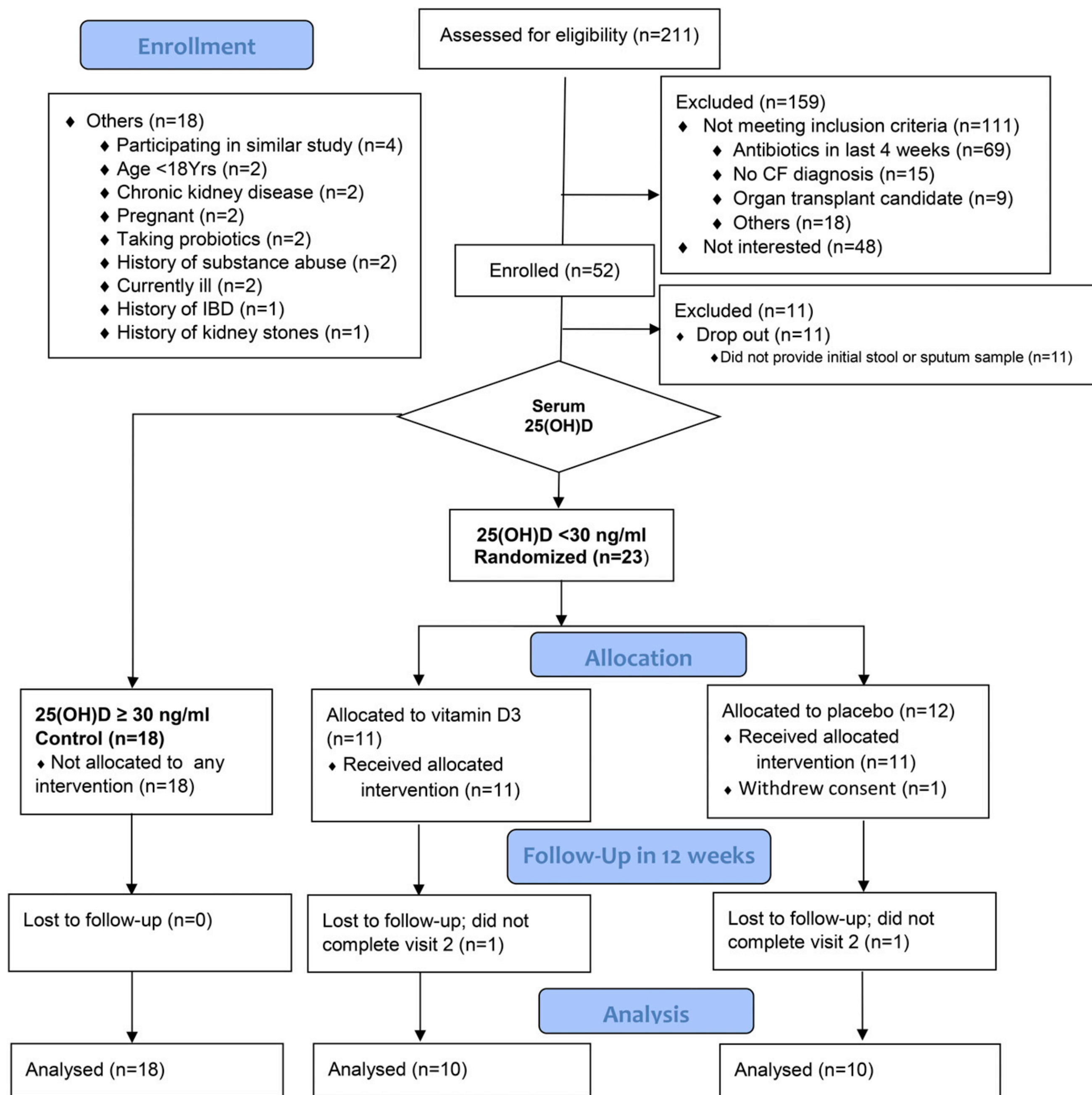

Figure 1. CONSORT diagram showing recruitment and allocation of subjects. IBD, inflammatory bowel disease.

intravenous antibiotics), hospitalizations in the past 3 months, and spirometry, including FEV_1% and forced vital capacity for measurement of lung function, performed as routine standard of care for CF outpatients.

Subjects who were vitamin D sufficient (n = 18 subjects) were followed longitudinally for 12 weeks and did not receive any further intervention. Subjects who were vitamin D insufficient (n = 23 subjects) were randomly assigned to either receive placebo or 50,000 IU of oral vitamin D_3, once weekly for 12 weeks, in random blocks of two or four by the Investigational Drug Pharmacy. The study drug and placebo were dispensed by the Investigational Drug Pharmacy and mailed to the subjects' residence. The study team, clinician, and subjects were blinded to the treatment assignment until trial completion. Randomized subjects were asked to limit any additional supplemental vitamin D to <3000 IU/day and could continue their usual vitamin D supplementation at the time of screening and randomization. Participants were seen for study follow-up visits at 12 weeks in the clinic or at the Clinic Research Center at Emory University Hospital of the Atlanta Clinical and Translational Science Institute (±4 weeks). Three subjects had scheduled follow-up visits sooner than 12 weeks. We asked those subjects to take an additional vitamin D or placebo pill per week to ensure that all 12 study pills were completed before the follow-up visit. Follow-up data collection included history of medications and antibiotic use, in addition to any pulmonary exacerbations and hospitalizations in the interim.

Weekly phone calls were made to the randomized participants after week 1 as a reminder to take weekly study pills. Symptoms of vitamin D toxicity (excessive thirst, frequent

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

urination, constipation, and confusion) were assessed by patient questionnaire serially and at the final study visit.

Sample collection and storage

Collection of stool and sputum samples for characterization of gut and airway microbiota was done at baseline and at the follow-up visit at 12 weeks (±4 weeks) for the entire cohort. Participants were asked to provide noninduced expectorated sputum into a collection container on the day of the study visit, and it was immediately stored at −80°C for future airway microbiota analysis. For collection of stool samples, subjects were provided with an OMNIgene-GUT stool kit (DNA Genotek, Ottawa, ON, Canada) and a prepaid envelope to have the sample mailed directly to the study laboratory within the next 48 hours. Each sample was homogenized, and three, 200 mg aliquots were placed in sterile Eppendorf tubes and stored at −80°C until future microbiota analysis. OMNIgene-GUT has been shown to stabilize DNA for microbiome profiling and minimize bias in microbial composition compared with methods requiring immediate refrigeration at −20°C (22).

Analytical methods

Serum 25(OH)D was measured using a chemiluminescent-based, automated method (IDS-iSYS; Immunodiagnostic Systems, Scottsdale, AZ). To ensure accuracy of the serum 25(OH)D measurements, our laboratory participates in the Vitamin D External Quality Assessment Scheme (site 606) and the National Institute of Standards and Technology/National Institutes of Health Vitamin D Metabolites Quality Assurance Program.

16S Ribosomal RNA gene sequencing and processing

OMNIgene-GUT-preserved samples were prepared for 16S ribosomal RNA (rRNA) gene amplification and sequencing using MiSeq technology (Illumina, San Diego, CA), following the protocol of the Earth Microbiome Project (www.earthmicrobiome.org/emp-standard-protocols), with its modifications to the PowerSoil DNA Isolation Kit (Qiagen, Germantown, MD) procedure for extracting DNA, as previously done (23, 24). DNA was extracted from frozen feces using a PowerSoil-htp Kit (Qiagen) with mechanical disruption (bead beating). The V4 region of the 16S rRNA genes was amplified with polymerase chain reaction (PCR) from each sample using the composite forward primer and the reverse primers 515FB-806RB (23, 25). PCR reactions consisted of 5PRIME HotMasterMix (Quantabio, Beverly, MA), 0.2 μM of each primer, and 10 to 100 ng template, and reaction conditions were 3 minutes at 95°C, followed by 30 cycles of 45 seconds at 95°C, 60 seconds at 50°C, and 90 seconds at 72°C on a thermocycler (Bio-Rad Laboratories, Hercules, CA). Two independent PCRs were performed for each sample and then combined and purified with Agencourt AMPure magnetic purification beads, and products were visualized by gel electrophoresis. Products were then quantified (fluorescence spectrophotometer; BioTek, Winooski, VT), and a master DNA pool was generated from the purified products in equimolar ratios. The pooled products were quantified and then sequenced using an Illumina MiSeq sequencer (paired-end reads, 2 × 250 bp) at Cornell University (Ithaca, NY). Sequences were demultiplexed and quality filtered using the Quantitative Insights Into Microbial Ecology (QIIME; version 1.8.0) software package (26). Forward and reverse Illumina reads were joined using the fastq-join method. We used the QIIME default parameters for quality filtering, as described in detail by Caporaso *et al.* (26) [reads truncated at the first low-quality base and excluded if 1) there were more than three consecutive low-quality base calls, 2) <75% of read length was consecutive high-quality base calls, 3) at least one uncalled base was present, 4) >1.5 errors were present in the bar code, 5) any Phred qualities were below 20, or 6) the length was <75 bases]. Sequences were clustered using the UCLUST algorithm (27) (*i.e.*, a 97% threshold of pairwise identity) and assigned to operational taxonomic units using the Greengenes reference database 13_8. (28). New clusters were created with sequences that did not match any reference sequences. A single representative sequence for each operational taxonomic unit was aligned, and a phylogenetic tree was built using FastTree (29). The phylogenetic tree described previously was used to assess beta and alpha diversity. Unweighted UniFrac distances between samples were computed, as done previously, to measure beta diversity (30, 31). Principal coordinates analysis plots were used to assess and visualize beta diversity further. Linear discriminate analysis (LDA) effect (LEfSE) was used to investigate bacterial members that drive differences between groups by comparing the abundance of specific taxa between each experimental group (32). Groups were compared for distinct clustering using the permutation multivariate analysis of variance (permanova) method through QIIME. Unprocessed sequencing data are deposited in the European Nucleotide Archive under Accession Number PRJEB23120.

Outcome measures

The primary outcome was the change in phylotype richness of the airway and gut microbiota before and after supplementation with high-dose vitamin D_3. Secondary endpoints included serum 25(OH)D levels and lung function, as measured by FEV_1% in the 3 months preceding treatment and after completion of vitamin D_3 supplementation or placebo.

Statistical analysis

Patients were analyzed according to the study group to which they were assigned. Continuous variables were presented as the means with the standard deviation (SD). Normality assumptions were checked by Shapiro-Wilk tests. Differences among the control, placebo, and vitamin D groups, respectively, for continuous variables were assessed with one-way analysis of variance with Tukey methods or Kruskal-Wallis, Student's t, or Mann-Whitney U tests, as appropriate. Categorical variables were presented as the percentage of frequency and differences among groups were assessed using the Fisher exact test. An unadjusted general linear regression model and a linear model adjusted by baseline serum 25(OH)D levels were used to evaluate the association between vitamin D treatment and the absolute change in serum 25(OH)D concentrations.

Results

Subject protocol and recruitment

Out of the 211 subjects screened, a total of 52 subjects were eligible and provided consent for participation in the study (Fig. 1). Of the 52 subjects who provided consent, 11 subjects were excluded, as a result of lack of collection

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

of a baseline stool sample. Of the total of 41 eligible subjects, 23 were classified as vitamin D insufficient, and 18 subjects were classified as vitamin D sufficient. The 23 vitamin D-insufficient subjects were randomized to receive vitamin D_3 or placebo. One patient withdrew from the study in the placebo group, and one patient was lost to follow-up in each of the placebo and vitamin D_3 groups, respectively. All other randomized patients received the study medication, and 38 patients were included in the final analysis (Fig. 1; CONSORT diagram).

Baseline characteristics and vitamin D status

Baseline clinical and demographic characteristics were comparable across all three groups (Table 1). The majority of vitamin D-insufficient patients were homozygous for the ΔF508Del allele, and all had pancreatic insufficiency. By study design, the vitamin D-sufficient group had higher mean serum 25(OH)D concentration compared with the vitamin D-insufficient group. There was no difference in baseline mean serum 25(OH)D levels in the vitamin D-insufficient groups randomized to vitamin D or placebo (Table 1). Vitamin D-insufficient subjects who were randomized to vitamin D had a higher absolute change of 25(OH)D concentrations compared with the subjects randomized to placebo [$P = 0.02$ (unadjusted)], and this remained significant ($P = 0.03$) in a multivariate linear regression mode adjusted for baseline 25(OH)D level (Fig. 2).

As expected, the mean daily oral habitual vitamin D intake in the vitamin-insufficient group was lower [1435 IU/d ± 1300 compared with 3500 ± 200 IU/d in the vitamin D-sufficient group ($P = 0.0002$)]. All but two subjects in the vitamin D-insufficient group, who were randomized to placebo, maintained serum 25(OH)D concentrations <30 ng/mL at the end of 12 weeks of intervention.

Lung function

Before randomization, the vitamin D-insufficient subjects receiving placebo or vitamin D_3 had similar FEV_1% values (Supplemental Table 1). There was no statistically significant change in the pulmonary outcomes (as

Table 1. Baseline Characteristics of Study Participants

Variable Name	Vitamin D Sufficient Control (n = 18)	Vitamin D Insufficient Randomized to Placebo (n = 10)	Vitamin D Insufficient Randomized to Vitamin D3 (n = 10)	P Value[a]	P Value[b]
Age, years, means ± SD	43 ± 17	32 ± 11	34 ± 10	0.18[c]	0.35[d]
Race, n (%)					
White	17 (94%)	7 (70%)	10 (100%)	0.16[e]	0.21[e]
Hispanic	0 (0%)	1 (10%)	0 (0%)		
Black	1 (6%)	1 (10%)	0 (0%)		
Others	0 (0%)	1 (10%)	0 (0%)		
Sex, n (%)					
Male	9 (50%)	8 (80%)	6 (60%)	0.31[e]	0.58[e]
BMI, means ± SD; n,%	23 ± 3	22 ± 3	23 ± 6	0.99[f]	0.99[d]
Meet CF nutrition target (BMI ≥ 22 in female; ≥ 23 in male)	10 (56%)	5 (50%)	4 (40.00%)		
CFRD status, n (%)					
CFRD	2 (11%)	3 (30%)	1 (10%)	0.51[g]	0.58[e]
Pancreatic insufficiency, n (%)					
Yes	18 (100%)	10 (100%)	10 (100%)	NA	NA
CF mutation					
ΔF508 homo	5 (28%)	7 (70%)	4 (40%)	0.01[g]	0.006[e]
ΔF508 hetero	8 (44%)	2 (20%)	1 (10%)		
Others	1 (56%)	1 (10%)	5 (50%)		
Unknown	4 (22%)	0 (0%)	0 (0%)		
Serum 25(OH)D level, ng/ml, means ± SD	37 ± 6	22 ± 6	25 ± 5	<0.001[f]	0.22[d]
Vitamin D supplementation, IU/day, means ± SD	3519 ± 2418	1770 ± 1643	1100 ± 849	0.001[c]	0.56[d]

Abbreviation: CFRD, CF-related diabetes.

[a] P value comparing all three groups.

[b] P value comparing the 25(OH)D-insufficient group randomized to placebo with weekly high-dose vitamin D_3.

[c] Kruskal-Wallis test.

[d] Mann-Whitney U test.

[e] Fisher exact test.

[f] One-way analysis of variance Tukey method.

[g] Freeman-Halton test.

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

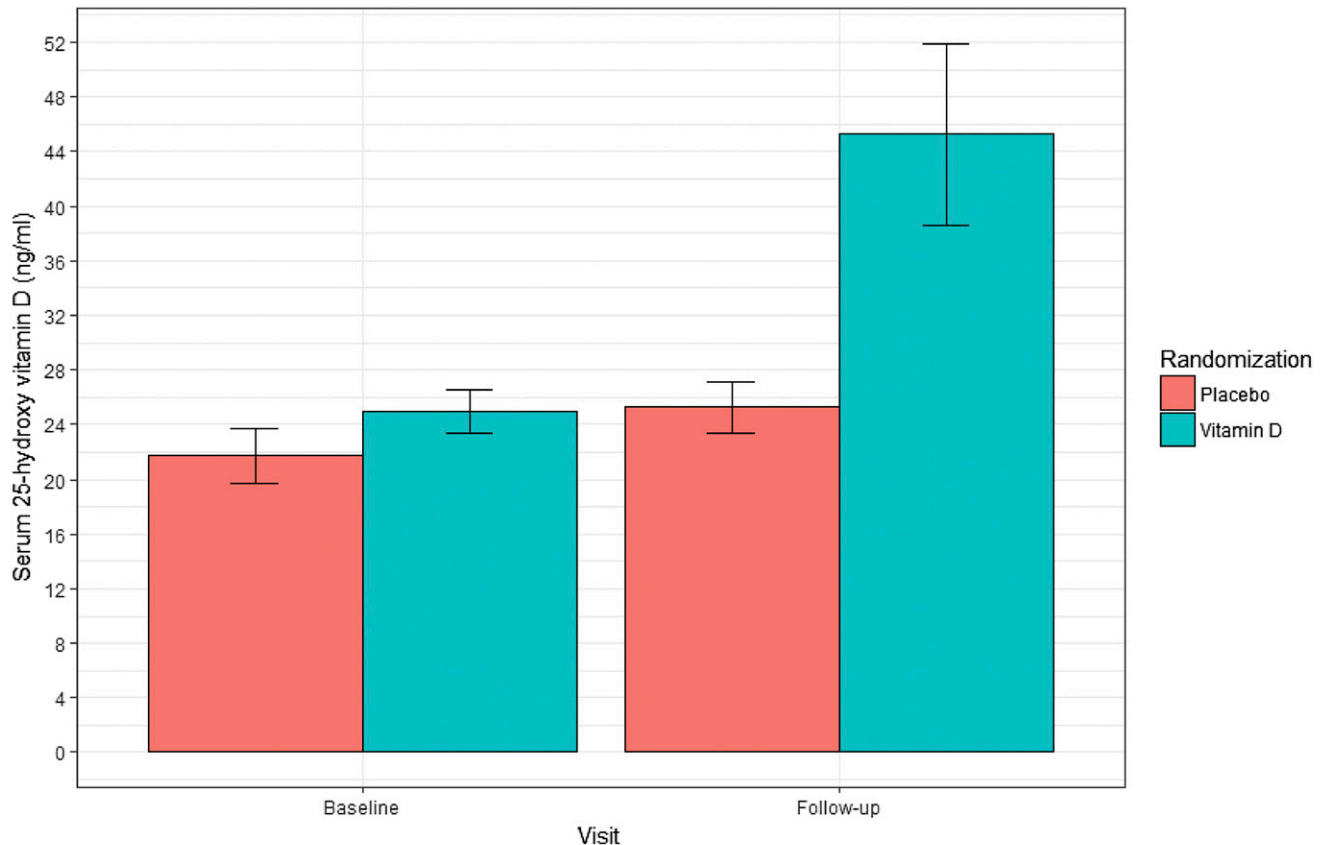

Figure 2. Mean serum 25(OH)D in response to weekly vitamin D_3 treatment or placebo in adults with CF and vitamin D insufficiency. Means with standard error bars of serum 25(OH)D concentrations between the placebo group (red; n = 10) and treatment group (blue; n = 10) at baseline and final visit at 12 weeks. Subjects who were randomized to oral vitamin D_3 50,000 IU had higher serum 25(OH)D concentrations compared with the subjects randomized to placebo [$P = 0.02$ ($\beta = 16.81$)], and this remained significant when adjusted by baseline serum 25(OH)D ($\beta = 17.08$, $P = 0.03$).

measured by FEV1%) postintervention of adult CF patients receiving 50,000 IU vitamin D_3 weekly vs placebo. When adjusted for age, sex, race, CF genetic mutations, and smoking, there continued to be no significant difference in the two groups.

Gut and airway microbiota composition of individuals with CF based on vitamin D status before intervention

Gut microbiota composition analysis of the subjects with CF revealed substantial alterations between subjects with vitamin D sufficiency and vitamin D insufficiency. Taxa belonging to the class *Gammaproteobacteria* were substantially enriched in subjects with vitamin D insufficiency compared with subjects with vitamin D sufficiency; LDA score = 4.96 by LEfSE, whereas *Bacteroidia* class was enriched in subjects with vitamin D sufficiency patients (Fig. 3A and 3B). The analysis of upper-airway microbiota composition showed that initial serum 25(OH)D concentration level correlated with sputum microbiota alterations with differential clustering based on vitamin D status (Supplemental Fig. 1A). Several taxa were enriched in the sputum samples of subjects with vitamin D insufficiency compared with samples from subjects with vitamin D sufficiency at baseline. Of particular interest, the sputum samples of subjects with vitamin D insufficiency were enriched in members of the genus *Bacteroides* (Supplemental Fig. 1A and 1B).

Changes in gut microbiota in response to vitamin D supplementation in vitamin D-insufficient subjects with CF

Subjects who were randomized to once weekly 50,000 IU of oral vitamin D_3 had significantly increased serum 25(OH)D concentrations compared with subjects randomized to placebo at the end of the study period (Fig. 2). This increase in serum 25(OH)D levels in the vitamin D_3 intervention group concentration was associated with a shift in the gut microbiota with differential clustering at the end of 12 weeks of intervention compared with the placebo group (Fig. 4A and 4B). Permanova = 0.723 for Fig. 4A, and permanova = 0.024 for Fig. 4B, indicating statistically significant clustering postintervention. To determine microbial species driving the difference between the two groups, we performed LEfSE (Supplemental Fig. 2A). The change in species abundance following 12 weeks

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

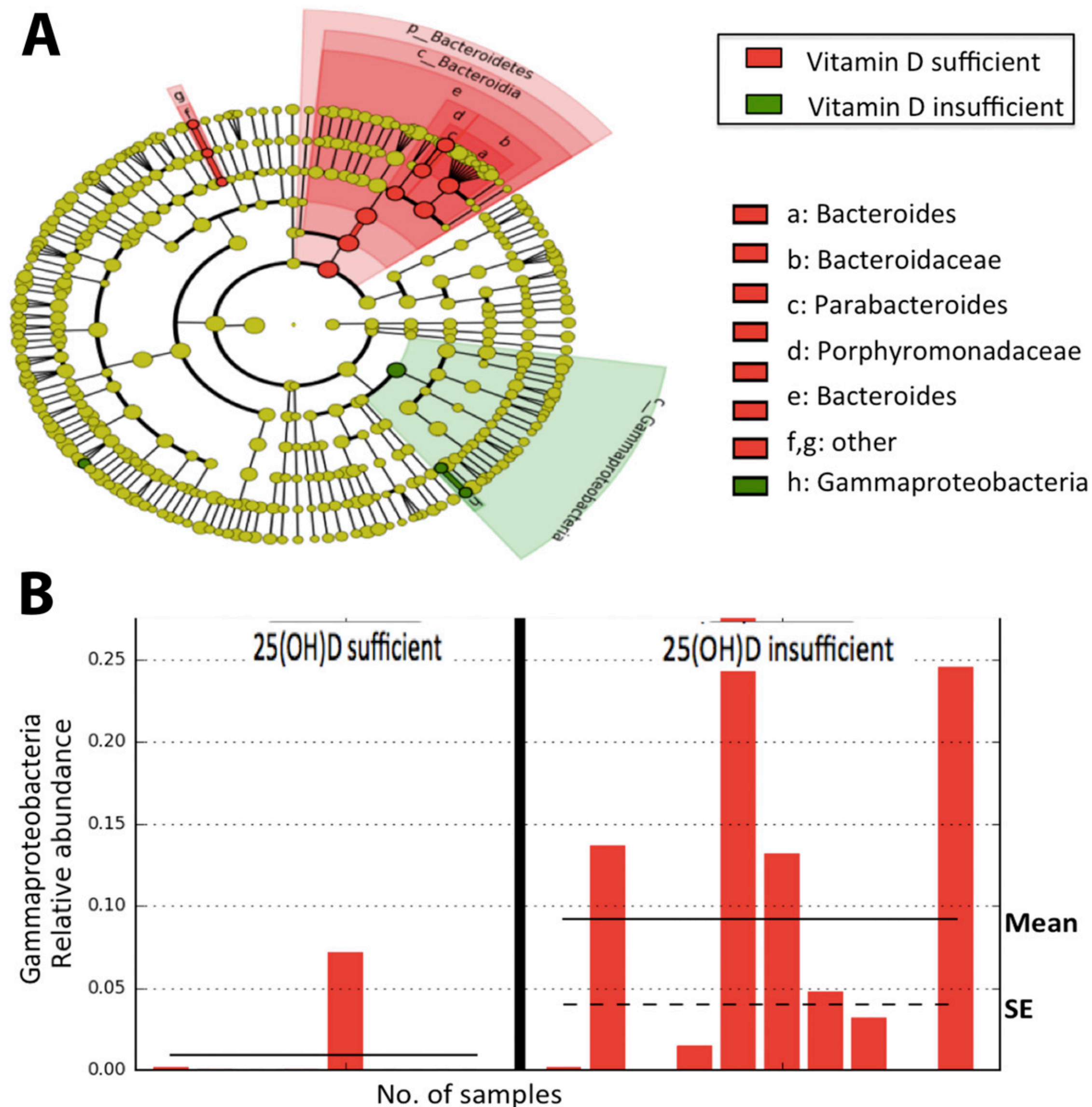

Figure 3. Differentially abundant taxa, according to vitamin D status of the subject at baseline. (A) LEfSE showing genera and species that were significantly altered in the stool samples of vitamin D-sufficient subjects (red) compared with vitamin D-insufficient subjects (green) at baseline. (B) Potentially pathogenic taxa belonging to the class *Gammaproteobacteria* were significantly more abundant in the stool samples of vitamin D-insufficient subjects compared with vitamin D-sufficient subjects at baseline (LDA score = 4.96).

of vitamin D or placebo groups presented in Fig. 5 demonstrate that *Lactococcus* was substantially increased, whereas *Veillonella* and *Erysipelotrichaceae* were substantially decreased after 12 weeks of vitamin D_3 supplementation (Fig. 5).

Changes in airway microbiota in response to vitamin D supplementation in vitamin D-insufficient subjects with CF

There was differential clustering of microbiota in subjects who were randomized to once weekly 50,000 IU of oral vitamin D_3 IU compared with subjects randomized to placebo at the end of the study period (Supplemental Fig. 2A). The genera and species with significantly different abundance between the subjects with vitamin D insufficiency receiving placebo vs 50,000 IU vitamin D_3 are presented in Supplemental Fig. 2B.

Safety and adverse events

The maximum 25(OH)D concentration in an individual subject at 12 weeks was 72 ng/mL and 31 ng/mL in vitamin D and placebo groups, respectively. There were no reported symptoms of vitamin D toxicity, as assessed by the patient questionnaire at the final study visit. There were also no clinical signs of hypercalcemia.

Discussion

Prior limited data suggest that airway and gut microbiota in CF are disrupted as a result of multiple factors, including

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

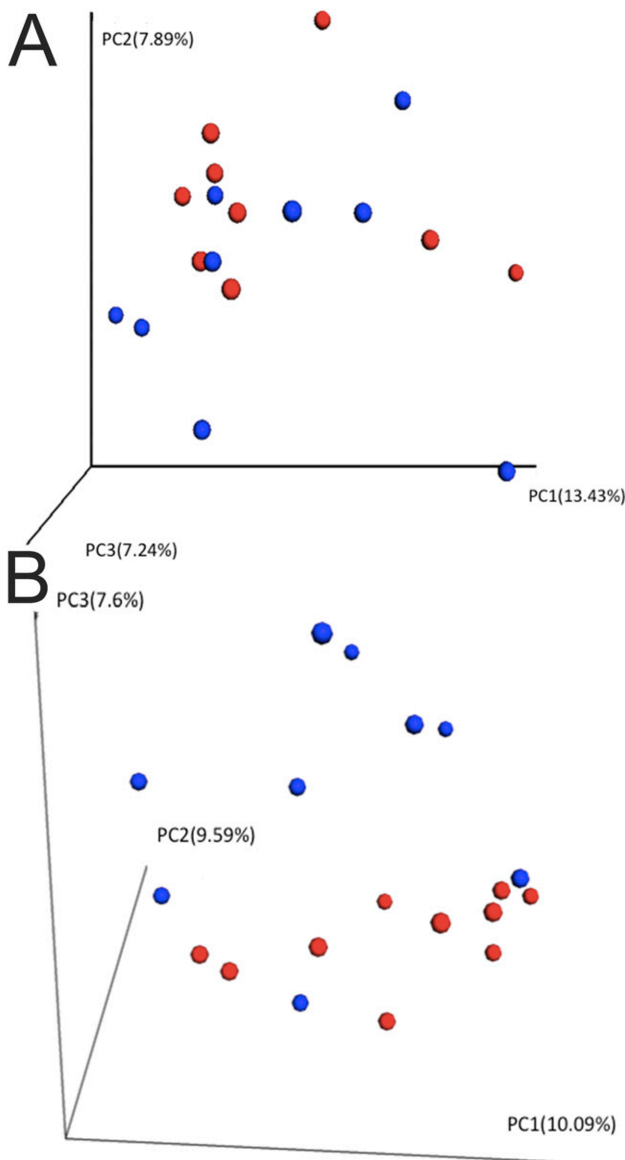

Figure 4. Principal coordinate (PC) analysis based on unweighted UniFrac distance matrices generated with stool 16S rRNA gene sequencing. Stool samples from adults with CF and vitamin D insufficiency [25(OH)D < 30 ng/mL] receiving placebo (red) or receiving vitamin D_3 (blue) were analyzed at (A) baseline and (B) the end of 12 weeks of intervention of weekly 50,000 IU of vitamin D_3.

chronic inflammation in the respiratory and digestive tracts, malabsorption, and recurrent lung infection, and effects as a result of systemic and local antibiotics (33–35). Such dysbiosis may play a role in CF morbidity with an impact on nutrition and pulmonary outcomes (9, 10, 36).

We found that the gut microbiota of CF subjects was altered based on vitamin D status at baseline, and taxa belonging to the class *Gammaproteobacteria* were significantly enriched in the vitamin D-insufficient group compared with the vitamin D-sufficient group. *Gammaproteobacteria* are gram-negative bacteria comprising several pathogenic bacterial species, including *Salmonella enterica*, *Yersinia pestis*, *Vibrio cholera*, *Escherichia coli*, and *Pseudomonas aeruginosa*, the latter being of particular interest given its role in CF pulmonary infection (37). Similar observations were made by Bashir *et al.* (19), who found a decrease in the relative abundance of *Gammaproteobacteria*, particularly *P. aeruginosa* and *Escherichia/Shigella* species in the upper gastrointestinal tract following high-dose vitamin D_3 supplementation in healthy volunteers. In patients with chronic HIV infection, *Gammaproteobacteria* is enriched within the gut microbiota, and its relative abundance is positively associated with plasma interleukin-1β levels, an important mediator of the inflammatory response, suggesting that *Gammaproteobacteria* may have proinflammatory effects (38). Our finding of potentially pathogenic species, such as *Gammaproteobacteria* being enriched in vitamin D deficiency, support a role for vitamin D in ameliorating the chronic inflammation present in the CF gut.

Moreover, our study was designed to explore the effects of a high-dose vitamin D_3 bolus regimen on airway and gut microbiota in adult CF patients matched for age, sex, body mass index (BMI), and lung function. We found that treatment with vitamin D_3 influenced gut microbiota with differential clustering based on treatment assignment and a shift toward a potentially beneficial gut microbiota composition, whereas placebo did not lead to microbiota alterations. In addition, we found that homozygous ΔF508 mutation was significantly more prevalent in those subjects with vitamin D insufficiency, which is in contrast with the findings of Vanstone *et al.* (39), who found that CFTR mutations did not impact the serum 25(OH)D level.

In a cross-sectional study, looking specifically at the gut microbiota in children with CF, a lower abundance and temporal stability of *Bifidobacterium* species was demonstrated in patients with CF compared with their healthy siblings (5). Members of the *Bifidobacterium* species are considered as a marker of intestinal health and are commonly used in probiotic supplements. Likewise, in a study on adults with CF and stable lung disease, Burke *et al.* (6) observed a decrease in gut microbial diversity and suppression of potentially beneficial bacteria—*Bifidobacterium* and *Akkermansia* in CF patients compared with non-CF controls. Moreover, they found that when stratified by percent-predicted FEV1, individuals with CF with severe lung dysfunction (% predicted $FEV_1 \leq 40\%$) exhibited significantly reduced gut microbial diversity compared with patients with CF with mild or moderate dysfunction (6). In a longitudinal study examining gut microbial communities in infants with CF, Madan *et al.* (35) found statistically significant changes in the gut microbiota before the onset of the first pulmonary exacerbation and chronic *P. aeruginosa* colonization. These studies suggest that there is role

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

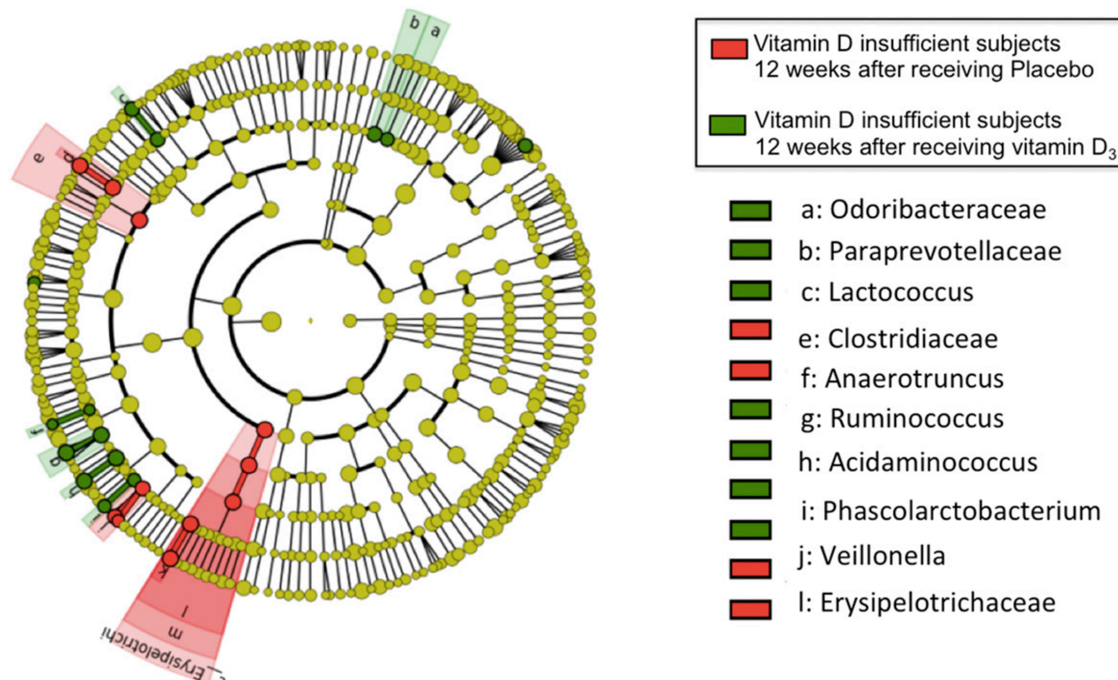

Figure 5. Differentially abundant taxa in vitamin D-insufficient, randomized subjects based on treatment assignment. LEfSE showing genera and species substantially, differentially abundant in the stool samples from adults with CF and vitamin D insufficiency [25(OH)D < 30 ng/mL] after receiving placebo (red) or after receiving 50,000 IU of vitamin D_3 (green).

for gut dysbiosis in CF to impact pulmonary outcomes. In contrast, we did not find any association between microbiota alterations and pulmonary function, as measured by FEV1%.

We found shifts in specific bacterial taxa in the gut microbiota from vitamin D-insufficient subjects receiving D_3 compared with the vitamin D-insufficient subjects receiving placebo at the end of 12 weeks of intervention. Of note, members of the genus *Lactococcus*, which have been positively associated with gut health, were enriched after 3 months of vitamin D_3 treatment compared with the baseline. Moreover, *Veillonella* at the genus level and *Erysipelotrichaceae* at the family level are potentially pathogenic bacteria and were found enriched in the vitamin D-insufficient patients receiving placebo compared with those receiving weekly 50,000 IU vitamin D_3. Specific taxa within *Erysipelotrichaceae* have been correlated to metabolic disorders and inflammation (38, 40). *Veillonella* species have been reported as a rare cause of serious infections, including bacteremia, meningitis, and pleuropulmonary infection (41). In a recent cross-sectional study looking at fecal microbiota based on vitamin D status in healthy subjects, Luthold *et al.* (20) also found *Veillonella* species to be relatively more abundant in individuals with the lowest intake and concentration of vitamin D compared with those with the highest intake and concentration of vitamin D. In our study, treatment with vitamin D_3 compared with placebo appeared to drive changes in both airway and gut bacterial communities with differential clustering compared with baseline based on treatment assignment. These findings suggest a potential role for vitamin D in altering the balance of symbionts to pathobionts and influencing the predominance of specific bacterial taxa, such as *Gammaproteobacteria*, which have a role in pulmonary outcomes in CF.

Analysis of airway microbiota showed that members of the genus *Bacteroides* were enriched at baseline in subjects with CF and vitamin D insufficiency. *Bacteroides* are anaerobic bacteria that are commensals in the gut and generally have a beneficial relationship with the host. However, they can cause substantial pathology, including bacteremia and abscess formation outside of the gastrointestinal tract (42). Given potential gut-barrier dysfunction in CF, it is possible that this finding is related to translocation of microbes from the gut lumen to the systemic circulation (43). There were many differences in the taxa based on treatment assignment in the placebo vs high-dose vitamin D_3 supplementation groups in the subjects with vitamin D insufficiency, as observed by LEfSe analysis. Of interest, *Staphylococcus aureus* and *Staphylococcus epidermidis* species were most significantly enriched in the placebo group with *S. aureus*, which has been implicated in pulmonary infection in CF and associated with a substantial inflammatory response in patients with CF (44). We also found that bacteria belonging to the genera *Corynebacterium* were significantly more abundant in the placebo group, consistent with a previous study showing *Corynebacterium* to be more abundant in the CF airway

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

and as a cause of respiratory infections in children with CF (7, 45).

Microbial dysbiosis is a hallmark of the CF gut underlying to chronic mucosal inflammation. It is reasonable to postulate that vitamin D may mitigate the dysbiosis seen in the CF through its effect on intestinal mucosal inflammation (46). This theory is supported by the work of Morin *et al.* (47), who studied the effects of vitamin D_3 and its metabolites in CFTR knockdown intestinal epithelial cells. They observed that $1,25(OH)_2D_3$ leads to an inhibition of interleukin-8 and reduces cytokine-induced nuclear factor κB nuclear translocation, thus resulting in a suppression of inflammatory mediators (47). Vitamin D has also been proposed to decrease intestinal inflammation through the inhibition of inflammation-induced epithelial cell apoptosis and reducing cytokine-induced nuclear factor κB nuclear translocation, thus resulting in a suppression of inflammatory mediators (48). Given these effects of vitamin D on the immune response, vitamin D deficiency creates an environment that favors the predominance of potentially pathogenic, pathobiont bacteria, such as *Gammaproteobacteria*, which were significantly enriched in the vitamin D-insufficient group compared with the vitamin D-sufficient group in our CF population. Thus, we propose that repletion of vitamin D insufficiency in CF patients may result in a shift toward potentially beneficial microbial communities by decreasing the competitive advantage of pathogenic bacteria.

Although our study cohort was relatively small, we were able to characterize the gut microbiota in individuals with CF, based on baseline vitamin D status, and show a substantial impact of vitamin D treatment on the gut microbiota in CF. Whereas our study was not designed to decipher cause-effect inter-relationships, our findings suggest a role for vitamin D in mitigating dysbiosis in CF by the enrichment or depletion of specific taxa. As this study was conducted in those with vitamin D insufficiency, it is uncertain whether vitamin D would continue to have a beneficial effect on the microbiota in patients who are considered vitamin D replete by current definitions.

The data from this study suggest a possible role for vitamin D in modulating the gut microbiota in CF. Vitamin D deficiency may predispose to a predominance of gram-negative bacteria of the class *Gammaproteobacteria* in the gut and *Bacteroides* in the airway microbiota. We found that the repletion of vitamin D insufficiency in CF patients may result in a shift toward commensal microbial communities in the gut, such as those belonging to the group *Lactococcus*. As this was an exploratory study, long-term studies are needed to confirm our findings and determine if they affect clinical outcomes in CF.

Acknowledgments

Financial Support: This work was supported by a grant from the National Center for Advancing Translational Sciences of the US National Institutes of Health under Awards UL1TR000454, K01 DK102851, DK09970771, and K24 DK096574 and in part by the Cystic Fibrosis Foundation by Center Grant (to V.T.). B.C. is supported by a Crohn's & Colitis Foundation of America Career Development Award. The content is solely the views of the authors and does not necessarily represent the official views of the US National Institutes of Health.

Clinical Trial Information: ClinicalTrials.gov no. NCT02589444 (registered 20 October 2015).

Correspondence and Reprint Requests: Vin Tangpricha, MD, PhD, Division of Endocrinology, Metabolism and Lipids, Department of Medicine, Emory University School of Medicine, 101 Woodruff Circle NE, WRMB 1301, Atlanta, Georgia 30322. E-mail: vin.tangpricha@emory.edu.

Disclosure Summary: The authors have nothing to disclose.

References

1. Higgins CF. Cystic fibrosis transmembrane conductance regulator (CFTR). *Br Med Bull.* 1992;**48**(4):754–765.
2. Collawn JF, Matalon S. CFTR and lung homeostasis. *Am J Physiol Lung Cell Mol Physiol.* 2014;**307**(12):L917–L923.
3. Couper RT, Corey M, Moore DJ, Fisher LJ, Forstner GG, Durie PR. Decline of exocrine pancreatic function in cystic fibrosis patients with pancreatic sufficiency. *Pediatr Res.* 1992;**32**(2):179–182.
4. Duytschaever G, Huys G, Bekaert M, Boulanger L, De Boeck K, Vandamme P. Cross-sectional and longitudinal comparisons of the predominant fecal microbiota compositions of a group of pediatric patients with cystic fibrosis and their healthy siblings. *Appl Environ Microbiol.* 2011;**77**(22):8015–8024.
5. Duytschaever G, Huys G, Bekaert M, Boulanger L, De Boeck K, Vandamme P. Dysbiosis of bifidobacteria and Clostridium cluster XIVa in the cystic fibrosis fecal microbiota. *J Cyst Fibros.* 2013;**12**(3):206–215.
6. Burke DG, Fouhy F, Harrison MJ, Rea MC, Cotter PD, O'Sullivan O, Stanton C, Hill C, Shanahan F, Plant BJ, Ross RP. The altered gut microbiota in adults with cystic fibrosis [published erratum appears is BMC Microbiol. 2017;17(1):102]. *BMC Microbiol.* 2017;**17**(1):58.
7. Renwick J, McNally P, John B, DeSantis T, Linnane B, Murphy P; SHIELD CF. The microbial community of the cystic fibrosis airway is disrupted in early life. *PLoS One.* 2014;**9**(12):e109798.
8. Zemanick ET, Sagel SD, Harris JK. The airway microbiome in cystic fibrosis and implications for treatment. *Curr Opin Pediatr.* 2011;**23**(3):319–324.
9. Coburn B, Wang PW, Diaz Caballero J, Clark ST, Brahma V, Donaldson S, Zhang Y, Surendra A, Gong Y, Elizabeth Tullis D, Yau YC, Waters VJ, Hwang DM, Guttman DS. Lung microbiota across age and disease stage in cystic fibrosis. *Sci Rep.* 2015;**5**(1):10241.
10. Hoen AG, Li J, Moulton LA, O'Toole GA, Housman ML, Koestler DC, Guill MF, Moore JH, Hibberd PL, Morrison HG, Sogin ML, Karagas MR, Madan JC. Associations between gut microbial colonization in early life and respiratory outcomes in cystic fibrosis. *J Pediatr.* 2015;**167**(1):138–147.e131-133.
11. Chesdachai S, Tangpricha V. Treatment of vitamin D deficiency in cystic fibrosis. *J Steroid Biochem Mol Biol.* 2016;**164**:36–39.
12. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, Tavera-Mendoza L, Lin R, Hanrahan JW, Mader S, White JH. Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression [published erratum appears

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025

in J Immunol. 2004;173(10):following 6489]. *J Immunol.* 2004; **173**(5):2909–2912.

13. Liu PT, Stenger S, Tang DH, Modlin RL. Cutting edge: vitamin D-mediated human antimicrobial activity against Mycobacterium tuberculosis is dependent on the induction of cathelicidin. *J Immunol.* 2007;**179**(4):2060–2063.
14. Zhu Y, Mahon BD, Froicu M, Cantorna MT. Calcium and 1 alpha,25-dihydroxyvitamin D3 target the TNF-alpha pathway to suppress experimental inflammatory bowel disease. *Eur J Immunol.* 2005;**35**(1):217–224.
15. Jin D, Wu S, Zhang YG, Lu R, Xia Y, Dong H, Sun J. Lack of vitamin D receptor causes dysbiosis and changes the functions of the murine intestinal microbiome. *Clin Ther.* 2015;**37**(5):996–1009.e7.
16. Sun J. VDR/vitamin D receptor regulates autophagic activity through ATG16L1. *Autophagy.* 2016;**12**(6):1057–1058.
17. Kong J, Zhang Z, Musch MW, Ning G, Sun J, Hart J, Bissonnette M, Li YC. Novel role of the vitamin D receptor in maintaining the integrity of the intestinal mucosal barrier. *Am J Physiol Gastrointest Liver Physiol.* 2008;**294**(1):G208–G216.
18. Li YC, Chen Y, Du J. Critical roles of intestinal epithelial vitamin D receptor signaling in controlling gut mucosal inflammation. *J Steroid Biochem Mol Biol.* 2015;**148**:179–183.
19. Bashir M, Prietl B, Tauschmann M, Mautner SI, Kump PK, Treiber G, Wurm P, Gorkiewicz G, Hogenauer C, Pieber TR. Effects of high doses of vitamin D on mucosa-associated gut microbiome vary between regions of the human gastrointestinal tract. *Eur J Nutr.* 2016;**55**(4)2060–2063.
20. Luthold RV, Fernandes GR, Franco-de-Moraes AC, Folchetti LG, Ferreira SR. Gut microbiota interactions with the immunomodulatory role of vitamin D in normal individuals. *Metabolism.* 2017;**69**:76–86.
21. Tangpricha V, Kelly A, Stephenson A, Maguiness K, Enders J, Robinson KA, Marshall BC, Borowitz D; Cystic Fibrosis Foundation Vitamin D Evidence-Based Review Committee. An update on the screening, diagnosis, management, and treatment of vitamin D deficiency in individuals with cystic fibrosis: evidence-based recommendations from the Cystic Fibrosis Foundation. *J Clin Endocrinol Metab.* 2012;**97**(4):1082–1093.
22. Song SJ, Amir A, Metcalf JL, Amato KR, Xu ZZ, Humphrey G, Knight R. Preservation methods differ in fecal microbiome stability, affecting suitability for field studies. *mSystems.* 2016;**1**(3):e00021-16.
23. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 2012;**6**(8):1621–1624.
24. Gilbert JA, Meyer F, Jansson J, Gordon J, Pace N, Tiedje J, Ley R, Fierer N, Field D, Kyrpides N, Glöckner FO, Klenk HP, Wommack KE, Glass E, Docherty K, Gallery R, Stevens R, Knight R. The Earth Microbiome Project: Meeting report of the "1 EMP meeting on sample selection and acquisition" at Argonne National Laboratory October 6 2010. *Stand Genomic Sci.* 2010;**3**(3):249–253.
25. Apprill A, McNally S, Parsons R, Weber L. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat Microb Ecol.* 2015;**75**:129–137.
26. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* 2010;**7**(5):335–336.
27. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics.* 2010;**26**(19):2460–2461.
28. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R, Hugenholtz P. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J.* 2012;**6**(3): 610–618.
29. Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol Biol Evol.* 2009;**26**(7):1641–1650.
30. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol.* 2005; **71**(12):8228–8235.
31. Lozupone C, Hamady M, Knight R. UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics.* 2006;**7**:371.
32. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C. Metagenomic biomarker discovery and explanation. *Genome Biol.* 2011;**12**(6):R60.
33. Scanlan PD, Buckling A, Kong W, Wild Y, Lynch SV, Harrison F. Gut dysbiosis in cystic fibrosis. *J Cyst Fibr.* 2012;**11**(5):454–455.
34. Cox MJ, Allgaier M, Taylor B, Baek MS, Huang YJ, Daly RA, Karaoz U, Andersen GL, Brown R, Fujimura KE, Wu B, Tran D, Koff J, Kleinhenz ME, Nielson D, Brodie EL, Lynch SV. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. *PLoS One.* 2010;**5**(6):e11044.
35. Madan JC, Koestler DC, Stanton BA, Davidson L, Moulton LA, Housman ML, Moore JH, Guill MF, Morrison HG, Sogin ML, Hampton TH, Karagas MR, Palumbo PE, Foster JA, Hibberd PL, O'Toole GA. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: interaction between intestinal and respiratory tracts and impact of nutritional exposures. *MBio.* 2012; **3**(4):e00251-12.
36. Zemanick ET, Harris JK, Wagner BD, Robertson CE, Sagel SD, Stevens MJ, Accurso FJ, Laguna TA. Inflammation and airway microbiota during cystic fibrosis pulmonary exacerbations. *PLoS One.* 2013;**8**(4):e62917.
37. Oliver A, Cantón R, Campo P, Baquero F, Blázquez J. High frequency of hypermutable Pseudomonas aeruginosa in cystic fibrosis lung infection. *Science.* 2000;**288**(5469):1251–1254.
38. Dinh DM, Volpe GE, Duffalo C, Bhalchandra S, Tai AK, Kane AV, Wanke CA, Ward HD. Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. *J Infect Dis.* 2015;**211**(1):19–27.
39. Vanstone MB, Egan ME, Zhang JH, Carpenter TO. Association between serum 25-hydroxyvitamin D level and pulmonary exacerbations in cystic fibrosis. *Pediatr Pulmonol.* 2015;**50**(5):441–446.
40. Kaakoush NO. Insights into the role of Erysipelotrichaceae in the human host. *Front Cell Infect Microbiol.* 2015;**5**:84.
41. Brook I. Veillonella infections in children. *J Clin Microbiol.* 1996; **34**(5):1283–1285.
42. Wexler HM. Bacteroides: the good, the bad, and the nitty-gritty. *Clin Microbiol Rev.* 2007;**20**(4):593–621.
43. Hendriks HJ, van Kreel B, Forget PP. Effects of therapy with lansoprazole on intestinal permeability and inflammation in young cystic fibrosis patients. *J Pediatr Gastroenterol Nutr.* 2001;**33**(3):260–265.
44. Gangell C, Gard S, Douglas T, Park J, de Klerk N, Keil T, Brennan S, Ranganathan S, Robins-Browne R, Sly PD; AREST CF. Inflammatory responses to individual microorganisms in the lungs of children with cystic fibrosis. *Clin Infect Dis.* 2011;**53**(5):425–432.
45. Bittar F, Cassagne C, Bosdure E, Stremler N, Dubus JC, Sarles J, Reynaud-Gaubert M, Raoult D, Rolain JM. Outbreak of Corynebacterium pseudodiphtheriticum infection in cystic fibrosis patients, France. *Emerg Infect Dis.* 2010;**16**(8):1231–1236.
46. Kanhere M, Chassaing B, Gewirtz AT, Tangpricha V. Role of vitamin D on gut microbiota in cystic fibrosis [published online ahead of print November 3, 2016]. *J Steroid Biochem Mol Biol.*
47. Morin G, Orlando V, St-Martin Crites K, Patey N, Mailhot G. Vitamin D attenuates inflammation in CFTR knockdown intestinal epithelial cells but has no effect in cells with intact CFTR. *Am J Physiol Gastrointest Liver Physiol.* 2016;**310**(8):G539–G549.
48. Chen Y, Liu W, Sun T, Huang Y, Wang Y, Deb DK, Yoon D, Kong J, Thadhani R, Li YC. 1,25-Dihydroxyvitamin D promotes negative feedback regulation of TLR signaling via targeting microRNA-155-SOCS1 in macrophages. *J Immunol.* 2013;**190**(7):3687–3695.

Downloaded from https://academic.oup.com/jcem/article/103/2/564/4634321 by guest on 29 April 2025