ORIGINAL ARTICLE

Restoring Cystic Fibrosis Transmembrane Conductance Regulator Function Reduces Airway Bacteria and Inflammation in People with Cystic Fibrosis and Chronic Lung Infections

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Abstract

Rationale: Previous work indicates that ivacaftor improves cystic fibrosis transmembrane conductance regulator (CFTR) activity and lung function in people with cystic fibrosis and *G551D-CFTR* mutations but does not reduce density of bacteria or markers of inflammation in the airway. These findings raise the possibility that infection and inflammation may progress independently of CFTR activity once cystic fibrosis lung disease is established.

Objectives: To better understand the relationship between CFTR activity, airway microbiology and inflammation, and lung function in subjects with cystic fibrosis and chronic airway infections.

Methods: We studied 12 subjects with *G551D-CFTR* mutations and chronic airway infections before and after ivacaftor. We measured lung function, sputum bacterial content, and inflammation, and obtained chest computed tomography scans.

Measurements and Main Results: Ivacaftor produced rapid decreases in sputum *Pseudomonas aeruginosa* density that began within 48 hours and continued in the first year of treatment. However, no subject eradicated their infecting *P. aeruginosa* strain, and after the first year *P. aeruginosa* densities rebounded. Sputum total bacterial concentrations also decreased, but less than *P. aeruginosa*. Sputum inflammatory measures decreased significantly in the first week of treatment and continued to decline over 2 years. Computed tomography scans obtained before and 1 year after ivacaftor treatment revealed that ivacaftor decreased airway mucous plugging.

Conclusions: Ivacaftor caused marked reductions in sputum *P. aeruginosa* density and airway inflammation and produced modest improvements in radiographic lung disease in subjects with *G551D-CFTR* mutations. However, *P. aeruginosa* airway infection persisted. Thus, measures that control infection may be required to realize the full benefits of CFTR-targeting treatments.

Keywords: cystic fibrosis; *Pseudomonas aeruginosa*; ivacaftor; inflammation

(Received in original form September 28, 2016; accepted in final form February 15, 2017)

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Supported by an investigator-initiated award from Vertex, Inc., National Institutes of Health grants R01AI101307 and K24HL102246 (P.K.S.), European Commission Seventh Framework Program Project 603038 CFMatters (P.K.S.), the Cystic Fibrosis Foundation (K.B.H., P.J., and P.K.S.), and the Burroughs Wellcome Fund (P.K.S.).

Author Contributions: Concept and design, K.B.H., S.L.H., X.W., J.E.B., D.A.S., M.J.W., L.R.H., E.F.M., and P.K.S. Acquisition of data, analysis, and interpretation, K.B.H., S.L.H., C.P., P.J., F.J.A., X.W., M.R., R.M.E., D.J.W., R.J.A., G.C., S.C., B.G., J.L.L., S.C.D., C.G.G., J.E.B., D.A.S., M.J.W., L.R.H., E.F.M., and P.K.S. Drafting the manuscript, K.B.H., S.L.H., M.R., D.A.S., M.J.W., L.R.H., E.F.M., and P.K.S. All authors revised the manuscript critically.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 195, Iss 12, pp 1617–1628, Jun 15, 2017

Originally Published in Press as DOI: 10.1164/rccm.201609-1954OC on February 21, 2017
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: Drugs that increase cystic fibrosis transmembrane conductance regulator (CFTR) activity can improve lung function and health in people with cystic fibrosis. However, it is unclear whether improved CFTR activity affects airway infection and inflammation.

What This Study Adds to the Field: We found that the CFTR-correcting drug ivacaftor markedly reduced sputum *Pseudomonas aeruginosa* concentrations and markers of inflammation during the first treatment year in subjects with chronic *P. aeruginosa* infections. However, no subject eradicated his or her infecting *P. aeruginosa* strain, and after the first year *P. aeruginosa* density rebounded. The finding that chronic infection persists and may rebound after CFTR function is corrected suggests that measures to control infection may be required to realize the full benefits of CFTR-targeting treatments.

Cystic fibrosis (CF) is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR), a membrane anion channel (1). The most important clinical manifestation of CF is lung disease, and respiratory failure is the major cause of death in people with CF (2). CF lung disease is a consequence of a series of events beginning with host defense defects caused by CFTR dysfunction. Impaired host defenses lead to chronic airway infection and inflammation, and eventually to lung injury and respiratory failure (1).

Intensive research has been directed toward developing agents that improve CFTR function, with the hope that correcting the basic defect would reverse disease. The first drug approved, ivacaftor, markedly improves CFTR activity in people with *G551D-CFTR* mutations (3). Indeed sweat chloride measurements (which reflect CFTR activity) approach normal levels almost immediately on treatment (4).

Despite the marked increases in CFTR activity, lung function improves only modestly with ivacaftor (3). Furthermore, the two studies that investigated ivacaftor's effects on the concentration of bacteria and inflammatory markers in airway secretions did not detect improvements in these parameters. The GOAL (G551D Observation-AL) study evaluated 14 subjects and found sputum pathogen abundance and measures of inflammation generally unchanged after ivacaftor (5). A smaller study of three pediatric subjects treated with ivacaftor produced similar results (6).

It is notable that an epidemiologic analysis of patient registry records found a decreased prevalence of *Pseudomonas aeruginosa*–positive cultures in subjects in the year after ivacaftor initiation (7). However, most of the decreased prevalence occurred in subjects who were not chronically infected with *P. aeruginosa*, and reductions in *P. aeruginosa*–positive cultures were not associated with improved lung function (7).

Several mechanisms could explain why reduced concentrations of bacteria and inflammatory markers were not detected after ivacaftor treatment. One possibility is that infection and inflammation progress independently of CFTR function after disease is established. For example, the presence of bronchiectasis, submucosal gland hypertrophy, and epithelial injury could sustain infection and inflammation even after CFTR function is normalized. It is also possible that changes in microbiologic and inflammatory parameters would be apparent if different airway sampling methods or assays were used, or if subjects had been studied at different disease stages.

Understanding the relationship between CFTR activity, infection, and inflammation is important because it could improve knowledge of the pathogenesis of chronic CF lung disease. In addition, if infection and inflammation become uncoupled from CFTR activity in established disease, drugs targeting CFTR may need to be initiated very early in life, or used in combination with agents that suppress infection and inflammation. Here we studied airway microbiology, inflammation, and clinical parameters in adult subjects with *G551D-CFTR* mutations and chronic airway infections before and after initiation of ivacaftor to better understand the new state of disease after CFTR function is restored. Some of the results of these studies have been previously reported in the form of an abstract (8).

Methods

Study Design and Description of Subjects

We prospectively studied 12 subjects with CF and one or more *G551D-CFTR* alleles (Table 1). Eight of the 12 subjects were chronically infected with *P. aeruginosa*, two were chronically infected with *Burkholderia* species, and two were chronically infected with *Staphylococcus aureus* (*see* Table E1 in the online supplement). The subjects' median age was 29.5 years (range, 22–57), and 9 of 12 subjects were female. FEV_1 ranged from 34% to 101% predicted, with a mean $FEV_{1\%}$ predicted of 64.2 (Table 1).

Clinical and Sputum Measurements

Sweat chloride levels and lung function were measured using standard methods. Chest computed tomography (CT) scans were performed before and approximately 1 year after starting ivacaftor, and scored by an experienced thoracic radiologist. Quantitative culture, polymerase chain reaction (PCR), 16S rRNA gene sequencing, and measurements of inflammatory markers were performed on spontaneously expectorated sputum. Multilocus sequence typing (MLST) and pulsed field gel electrophoresis (PFGE) fingerprinting were performed on cultured *P. aeruginosa*.

Statistical Analyses

Paired Student's *t* tests were used, and two-sided *P* values reported. Repeated measures analysis of variance was used to test culture, PCR, sequencing, and inflammatory marker data. Piecewise, mixed-effect linear regression models (9) were used to test FEV_1, bacterial, and diversity index slope changes. Correlation analyses were performed using Spearman correlation coefficient. SAS version 9.4 (SAS Institute, Cary, NC) was used for all analyses. Methods are described in detail in the METHODS section of the online supplement.

Table 1. Subject Demographics and Sputum Culture Data

Subject	Genotype: *G551D/*	Preivacaftor FEV_1 [*L (% Predicted)*]	Sputum Culture Results			
			Pa	Sa	B	Fungi
1	*ΔF508*	2.80 (79%)	3+			
2	*ΔF508*	2.42 (72%)	3+	2+		
3	*ΔF508*	2.46 (79%)		3+		1+
4	*ΔF508*	1.11 (40%)	3+			
5	*ΔF508*	1.49 (51%)	3+	1+		
6	*3659delC*	1.25 (39%)	3+			2+
7	*ΔF508*	2.54 (88%)	3+			
8	*P67L*	0.82 (34%)		1+		3+
9	*ΔF508*	2.16 (77%)	3+			1+
10	*G551D*	3.99 (101%)	3+			
11	*ΔF508*	1.60 (39%)		2+	3+*	
12	*R117H*	2.76 (72%)			3+†	2+

Definition of abbreviations: B = *Burkholderia*; Pa = *Pseudomonas aeruginosa*; Sa = *Staphylococcus aureus*.
Fungi (and mold) refers to *Candida* and *Aspergillus*. Numeric scoring refers to values assigned by the clinical microbiology laboratory (low = 1+ to high = 3+).
**Burkholderia multivorans*.
†*Burkholderia cepacia*.

Results

Ivacaftor Produced Rapid Improvements in Sweat Chloride and FEV_1

We measured sweat chloride concentrations and lung function to verify that our cohort experienced typical responses to ivacaftor. Sweat chloride concentrations were markedly reduced after 2 days of treatment (Figure 1A), indicating improved CFTR function. Lung function (FEV_1) also improved after 2 days (mean increase, 0.29 L; 95% confidence interval [CI], 0.14–0.45; $P = 0.0016$), with continued improvements on Day 7 (additional increase from Day 2 of 0.17 L; 95% CI, 0.0002–0.33; $P = 0.07$) (Figure 1B), and Day 400 (additional increase from Day 7 of 0.16 L; 95% CI, −0.03 to 0.34; $P = 0.09$) (Figure 1C).

We also analyzed all clinically obtained FEV_1 values; adjusted the values for age, sex, and height; and found the rate of change in FEV_1 (the FEV_1 slope) in the 2 years after ivacaftor was significantly improved compared with the 2 years before (0.004 L/mo; 95% CI, −0.0004 to 0.008; vs. −0.007 L/mo; 95% CI, −0.0094 to −0.0053) ($P < 0.001$) (Figure 1D). Thus, ivacaftor produced rapid and sustained improvements in lung function in study subjects (also described in References 10 and 11).

Ivacaftor Markedly Reduced Sputum *P. aeruginosa* in the First Treatment Week

Reducing airway *P. aeruginosa* is a key therapeutic objective in CF (12, 13). Eight of the 12 subjects were chronically infected with *P. aeruginosa* (*see* Table E1), and in these subjects sputum *P. aeruginosa* CFUs began to decrease at Day 2 (Figure 2A). By Day 7, average sputum *P. aeruginosa* CFUs had declined by 10-fold (1.06 $\log_{10}$ CFU; 95% CI, −1.79 to −0.32; $P = 0.012$) (Figure 2A). It was notable that even though subjects' pretreatment sputum *P. aeruginosa* concentrations differed by more than 4 logs (10^5–10^9 CFUs/ml), all subjects demonstrated marked declines in *P. aeruginosa* during the first 7 days of treatment (Figure 2A). We used quantitative PCR (qPCR) measurements of *P. aeruginosa* genome copies to independently investigate ivacaftor-induced changes, and again found significant reductions during the first treatment week (Figure 2C).

P. aeruginosa Density Continued to Decline in the First Treatment Year

We measured long-term changes in sputum *P. aeruginosa* and found continued declines after Day 7. Average *P. aeruginosa* CFU counts declined by 1.67 $\log_{10}$ CFU/ml (95% CI, −2.39 to −0.96; $P = 0.006$) (Figure 2B) and DNA-based measurements declined by 1.19 $\log_{10}$ genome/g (95% CI, −1.80 to −0.59; $P = 0.01$) by Day 210 (Figure 2D). Although some subjects were unable to spontaneously expectorate at some visits, none of the chronically infected subjects became consistently *P. aeruginosa*–culture negative.

P. aeruginosa Counts Rebounded in the Second Year of Treatment

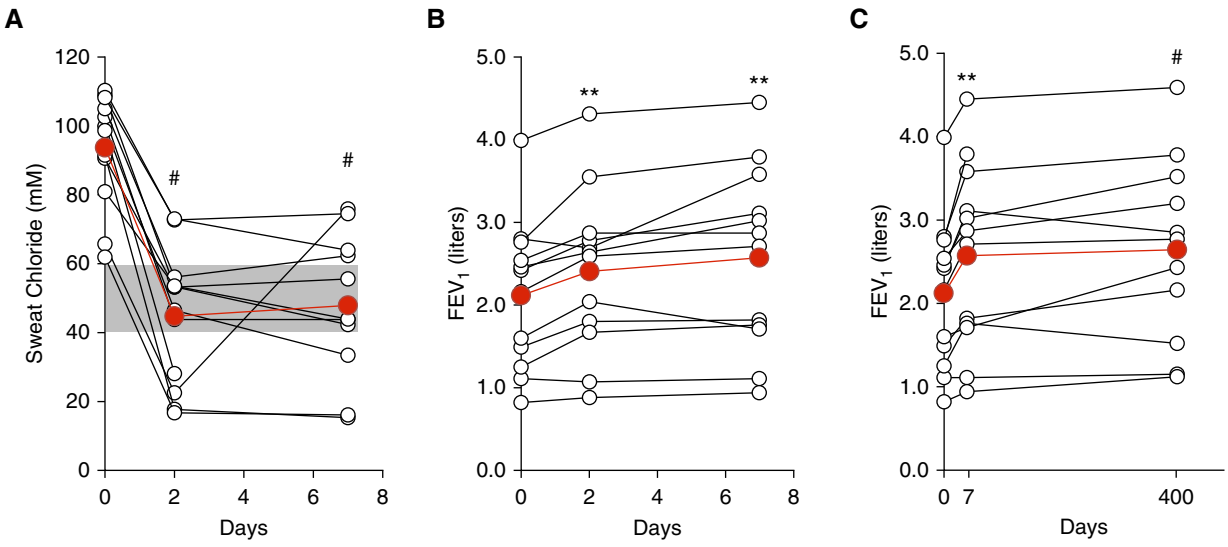
We measured sputum *P. aeruginosa* density for as long as 975 days, and noted that after Day 210, sputum *P. aeruginosa* CFU counts stopped declining and began to increase (Figure 2B). Mean *P. aeruginosa* density decreased with a slope of −0.20 log CFU/mo (95% CI, −0.28 to −0.12) until Day 210 then the slope significantly changed ($P < 0.0001$) to + 0.04 log CFU/mo (95% CI, 0.014–0.069) over the remaining study period (Figure 2E). Increases after Day 210 were noted in six out of seven subjects (Figures 2B) (one *P. aeruginosa*–infected subject was lost to follow-up). The clinical implication of these increases is unclear (*see* Discussion).

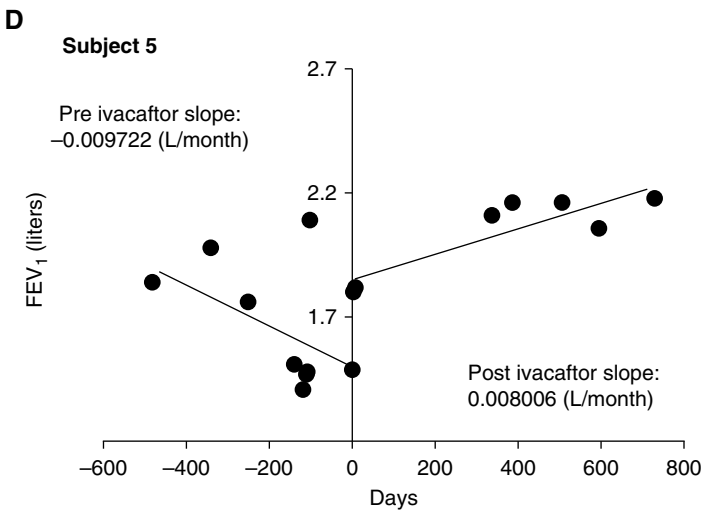
Subjects Remained Persistently Infected with Their Initial *P. aeruginosa* Strain

Continued infection could be explained if the *P. aeruginosa* strains present before treatment persisted, or if preexisting strains were cleared and new infection developed. We used three methods to distinguish between these possibilities using sputum samples obtained before, and at the latest time point available after treatment (typically Day 800 or 975). Two of the methods genotyped *P. aeruginosa* in sputum at a population level, and thus may provide a more comprehensive analysis than studies on individual isolates. We also performed confirmatory studies using a conventional approach on individual isolates.

For the first method, we cultured sputum obtained before and after treatment, picked 96 *P. aeruginosa* colonies from each sample, and pooled and sequenced them. The high-quality reads that mapped to the seven established *P. aeruginosa* MLST gene loci were used to generate a consensus sequence that represented the most prominent allele in each population. The consensus sequence was then assigned an MLST type using the *P. aeruginosa* MLST database (14).

As shown in Figure 3A, the same consensus MLST type was identified before





Subject	FEV_1 slope pre-	FEV_1 slope post-
1	–0.000402	0.002286
2	–0.009313	–0.001042
3	–0.008135	0.012304
4	–0.003482	–0.000346
5	–0.009722	0.008006
6	–0.009724	–0.007877
7	–0.005255	0.008286
8	–0.005741	–0.001197
9	–0.005381	–0.000135
10	–0.010154	0.009563
11	–0.010451	0.012578
12	–0.009932	0.005213
Mean	–0.007308	0.003970

Figure 1. Ivacaftor treatment rapidly improved cystic fibrosis transmembrane conductance regulator activity and lung function. *Black lines* represent responses of individual subjects, and *red lines* represent mean values. (*A*) Ivacaftor reduces sweat chloride levels. The *shaded region* represents borderline values (41–60 mM); higher values are consistent with cystic fibrosis transmembrane conductance regulator dysfunction, and lower values are considered normal. Effects of ivacaftor on FEV_1 during the first week (*B*) and year (*C*) of treatment, with values from the first week removed from *C* for clarity; $^{**}P < 0.005$ and $^{\#}P < 0.0001$ compared with Day 0. (*D*) Representative example (from subject 5) of the change in FEV_1 slope calculated from values obtained over the 2 years before and after ivacaftor. Slopes were estimated via linear regression (*see* Methods). Note that the graph includes 4 years of data, and the *x-axis* is drawn to scale. Thus it appears that the Day 0, 2, and 7 measurements for FEV_1 all fall near the *y-axis*. The table lists preivacaftor and postivacaftor subject-specific FEV_1 slope estimates (L/mo), with negative slopes in *red*, positive slopes in *bright green*, and slopes that were improved but still negative after ivacaftor in *pale green*.

and after treatment in each subject. Two subjects living in the same household were infected by *P. aeruginosa* with the same consensus MLST type, whereas other subjects were infected by different types. Notably, in three cases (subjects 4, 5, and 9) the before and after treatment consensus sequence of one MLST loci (the *acs* gene fragment) mapped to different alleles (*see* Figure E1). However, this ambiguity did not result in different MLST types. Isolates from these subjects were analyzed further (*see below*).

The second method exploited the allelic variation that evolves within clonally related *P. aeruginosa* populations infecting subjects with CF (15–18). We used the high-quality reads from the 96 isolate pools sequenced at early and late time points (described previously) to measure allele variations at every base of all seven MLST loci. We then counted the number of allele frequency changes between early and late isolate pools from each subject, and compared the results to allele frequency differences between *P. aeruginosa* isolate pools from different subjects in the cohort as control subjects.

As shown in Figure 3B and Figure E2, early and late isolate pools from each subject exhibited far fewer allele frequency differences than isolate pools from different subjects ($P < 0.0001$).

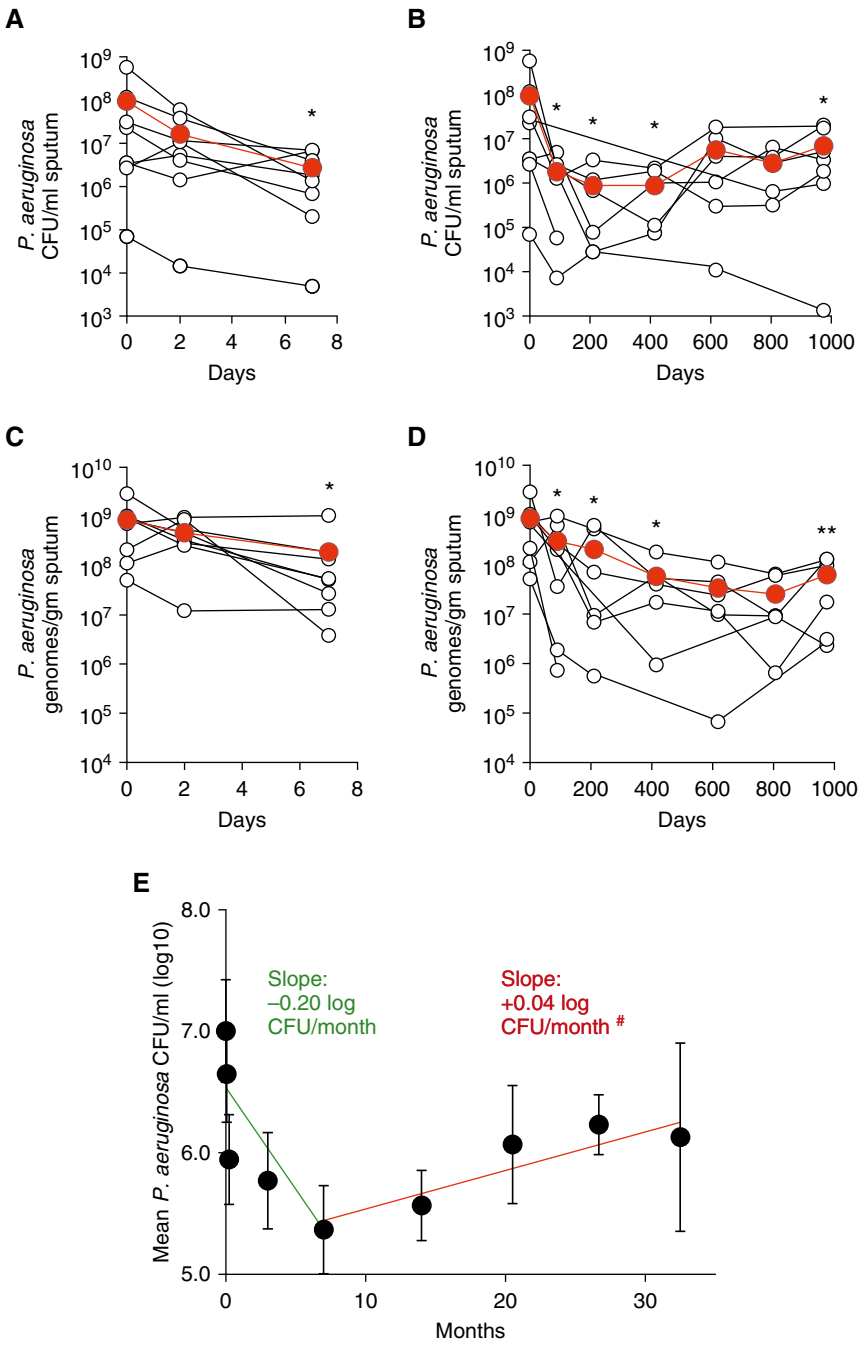


Figure 2. Ivacaftor treatment rapidly reduces sputum *Pseudomonas aeruginosa* density. (*A* and *C*) Changes in the first week. (*B* and *D*) Changes between Day 0 and up to Day 975, with values from the first week removed for clarity. (*A* and *B*) Culture-based measurements of viable *P. aeruginosa* CFU/ml. (*C* and *D*) Quantitative polymerase chain reaction–based measurements of *P. aeruginosa* genome copies; $^{*}P < 0.05$ and $^{**}P < 0.005$ compared with Day 0. (*E*) Mean *P. aeruginosa* CFU/ml increase after 210 days of ivacaftor treatment. CFU values were log transformed and averaged; *error bars* indicate SEM. Piecewise, mixed-effect linear regression models were used to estimate and test $\log_{10}$ CFU slope changes before and after Day 210; $^{\#}P < 0.0001$. CFU = colony-forming units.

Finally, we picked individual *P. aeruginosa* isolates from the sputum of the three subjects for whom the consensus *acs* MLST locus sequence showed ambiguity (subjects 4, 5, and 9), and performed conventional PFGE genotyping. PFGE showed that randomly selected isolates from the early and late time points were indeed clonally related (Figure 3C; *see* Figure E3). Taken together, these analyses indicate that *P. aeruginosa* strains present before treatment persisted after treatment. However, the findings do not exclude the possibility that additional strains (that were not captured in the 96 isolates sampled) were also present in sputum at low relative abundance.

Ivacaftor Changed the Relative Abundance of Sputum Microbiota

We measured the relative abundance of sputum microbiota by amplifying, sequencing, and performing taxonomic classification of bacterial 16S ribosomal RNA gene sequences (16S rDNA), focusing on the subjects chronically infected with *P. aeruginosa* as they were followed long-term. Most of the non–*P. aeruginosa* taxa found in these samples (including *Streptococcus*, *Prevotella*, *Veillonella*, and other taxa) are highly abundant in the oropharynx (19–21), and they have not historically been considered CF pathogens (we call them nonconventional organisms later). Inspection of individual subjects' sputum microbiota profiles suggested that changes in the relative abundance of *P. aeruginosa* were generally accompanied by reciprocal changes in the relative abundance of nonconventional organisms and in the overall diversity of microbiota present (Figure 4).

To explore this further, we calculated indices of microbial diversity, including microbial richness (the number of identified taxa), evenness (how similar the abundances of the taxa were), and the Shannon diversity index (which accounts for both richness and evenness). Average changes in the diversity indices roughly exhibited patterns opposite to those of both *P. aeruginosa* absolute and relative abundance. Average measurements of *P. aeruginosa* absolute (Figure 2E) and relative (Figure 5A) abundance declined during the first year of treatment, and then rebounded, whereas the microbial diversity indices generally increased in the first year, and then decreased (Figures 5B–5D). We estimated the change in microbial diversity indices over time using linear regression analysis, and found that microbial richness and Shannon diversity measures increased in the first year (i.e., showed positive slopes), and then exhibited statistically significant decreases in slopes thereafter ($P < 0.05$) (*see* Table E2).

A

Subject	Day	MLST type
1	0	1394
	975	1394
2	0	1091
	975	1091
4*	0	526
	975	526
5*	0	526
	800	526
6	0	167
	975	167
7	0	395
	90	395
9*	0	1068
	975	1068
10	0	266
	975	266

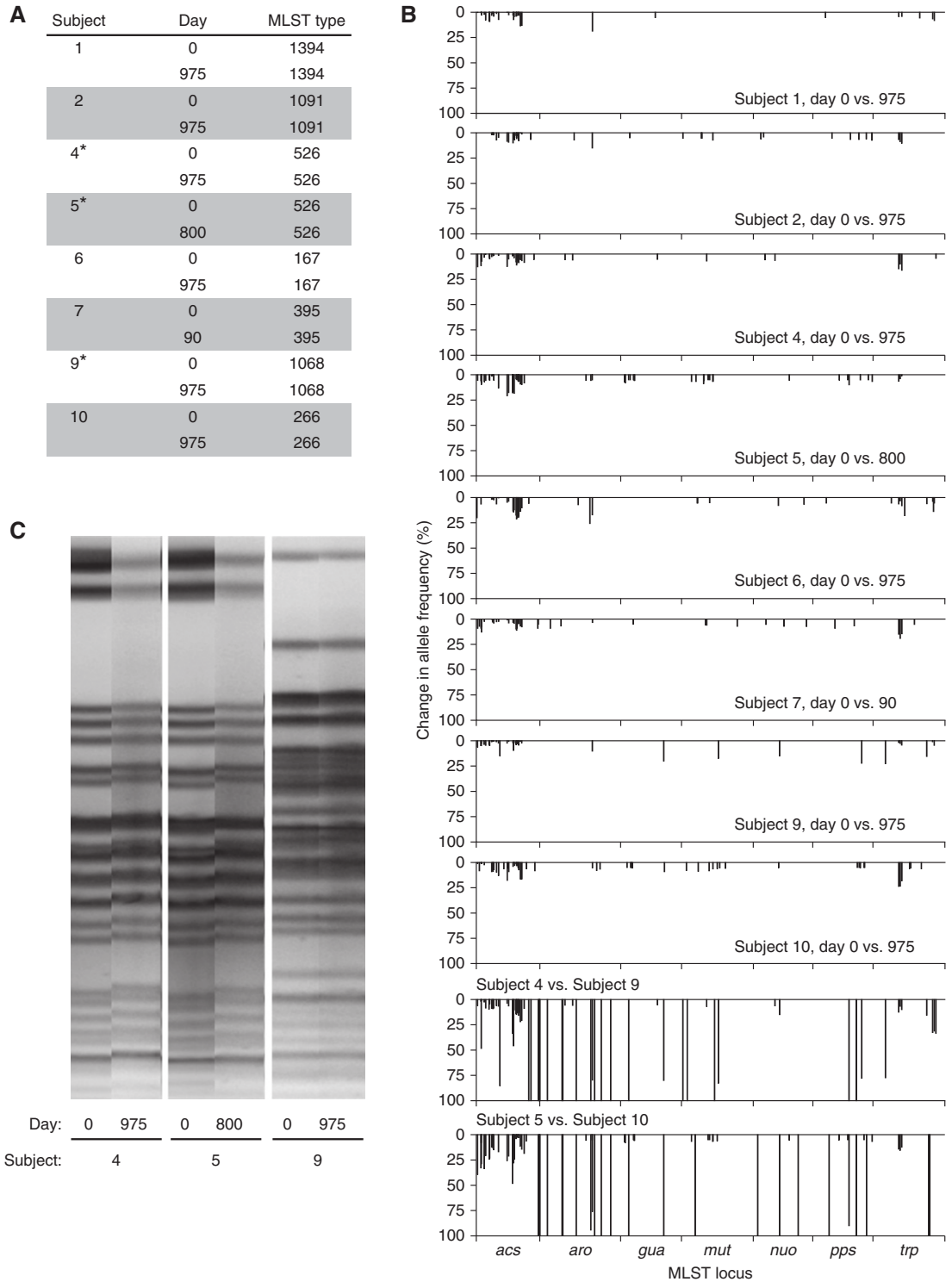


Figure 3. *Pseudomonas aeruginosa* strains infecting subjects persist after treatment. (*A*) Multilocus sequence typing (MLST) analysis of consensus sequences before and after treatment. Ninety-six *P. aeruginosa* isolates collected at the indicated time points were pooled, sequenced, and consensus MLST sequences were generated. The early and late sputum sample of each subject generated the same consensus MLST sequence type. *Subjects in whom the *acs* loci showed ambiguity (*see* Figure E1). (*B*) Changes in MLST allele frequency in *P. aeruginosa* isolate pools collected before and after treatment. *P. aeruginosa* isolate pools from before and after treatment from individual subjects (*top 8 graphs*) showed far fewer differences than pools from different subjects (*bottom 2 graphs*) ($P < 0.0001$). *See* Figure E2 for data from other subjects. (*C*) Pulsed-field gel electrophoresis results of randomly selected isolates before (*left side of each column*) and after (*right side of each column*) treatment from subjects 4, 5, and 9 (*see* also Figure E3). All isolates from individual subjects were >95% related (*see* Figure E3).

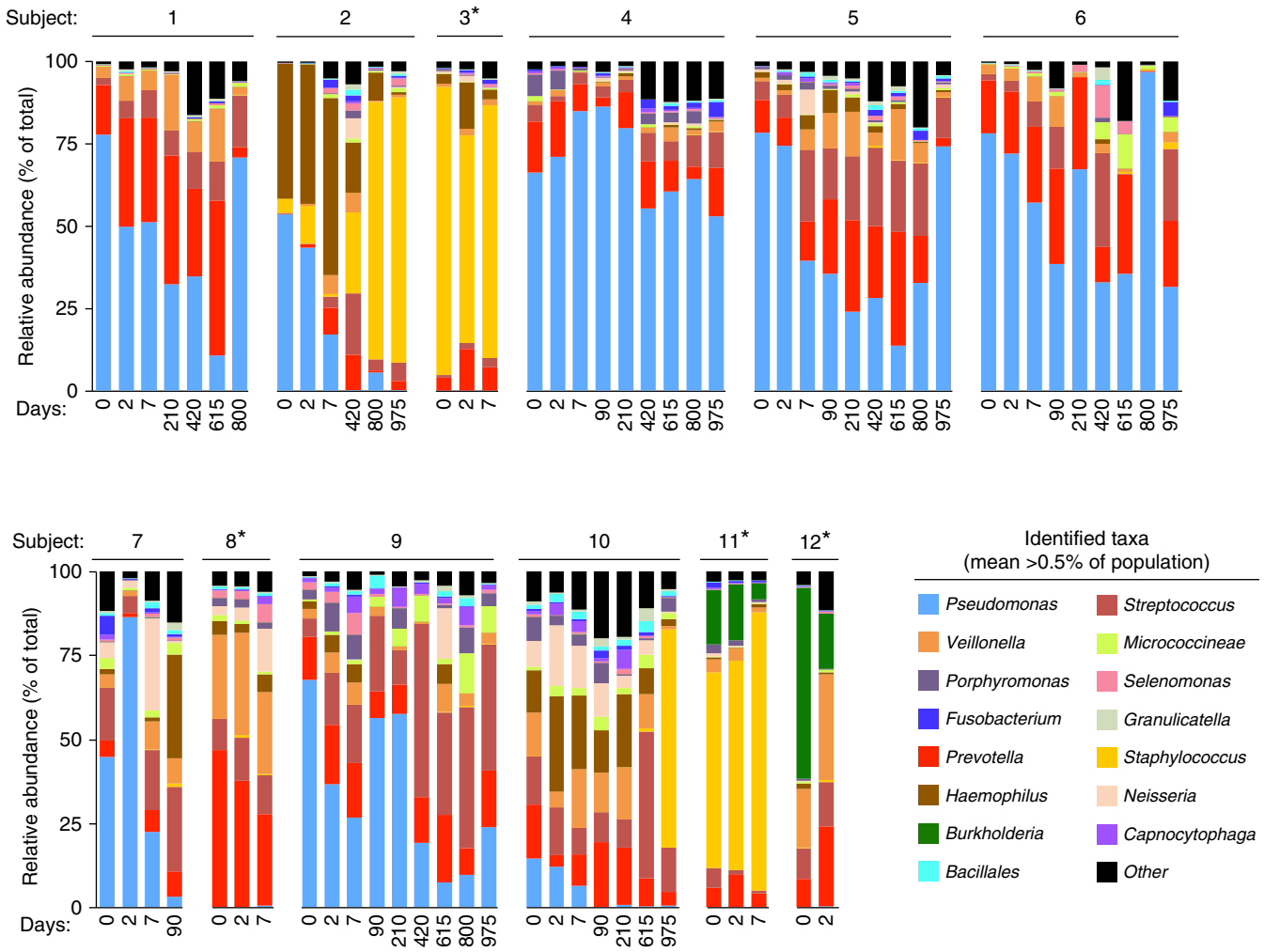


Figure 4. Ivacaftor treatment changes the relative abundance of sputum microbiota. The *colored* segments of each *bar* represent the proportion of 16S rDNA reads mapping to the indicated bacterial taxa. The *key* identifies taxa present at greater than or equal to 0.5% average abundance. Lower-abundance taxa are identified in Table E19. *Subjects that were not chronically infected with *Pseudomonas aeruginosa* based on cultures used for clinical care.

Ivacaftor Did Not Change the Absolute Abundance of Nonconventional Organisms

Previous work measuring airway clearance using radiotracer methods (5), and the rapidity with which *P. aeruginosa* declined (Figure 2), suggest improved mucociliary clearance may be a major therapeutic effect of ivacaftor. It seems likely that mucociliary clearance would remove all species of airway bacteria indiscriminately. Thus we found it perplexing that pattern of change in *P. aeruginosa* abundance was roughly opposite that seen with the microbial diversity indices.

Two explanations for these findings seem plausible. One possibility is that ivacaftor produces opposing effects on the airway abundance of *P. aeruginosa* relative to other organisms. A second possibility is that the reciprocal changes in *P. aeruginosa* abundance and the microbial diversity indices are primarily a consequence of the absolute reductions in *P. aeruginosa* we observed. This is possible because the diversity indices are calculated from relative abundance data. Thus, changes in the absolute abundance of one organism could lead to changes in the relative abundance of others even if the other organisms show no change in absolute abundance. In other words, relative abundance totals must equal 100%.

We used two approaches to explore these possibilities further. First, we used qPCR to directly measure the absolute abundance of *Streptococcus* and *Prevotella*, because they were the most abundant nonconventional organisms in most subjects' initial sputum samples (*Prevotella* was the most abundant nonconventional organism in 9 of 12 subjects and *Streptococcus* in 2 of 12 subjects) (Figure 4). Furthermore, combined relative abundance of *Streptococcus* and *Prevotella* showed roughly the opposite pattern change seen with *P. aeruginosa* (compare Figure E4 and Figure 5A); increasing in the first year, and then decreasing thereafter ($P < 0.05$) (*see* Table E3). However, direct PCR measurements showed that neither *Streptococcus* nor *Prevotella* significantly changed in absolute abundance during the study period (Figures 6A–6D).

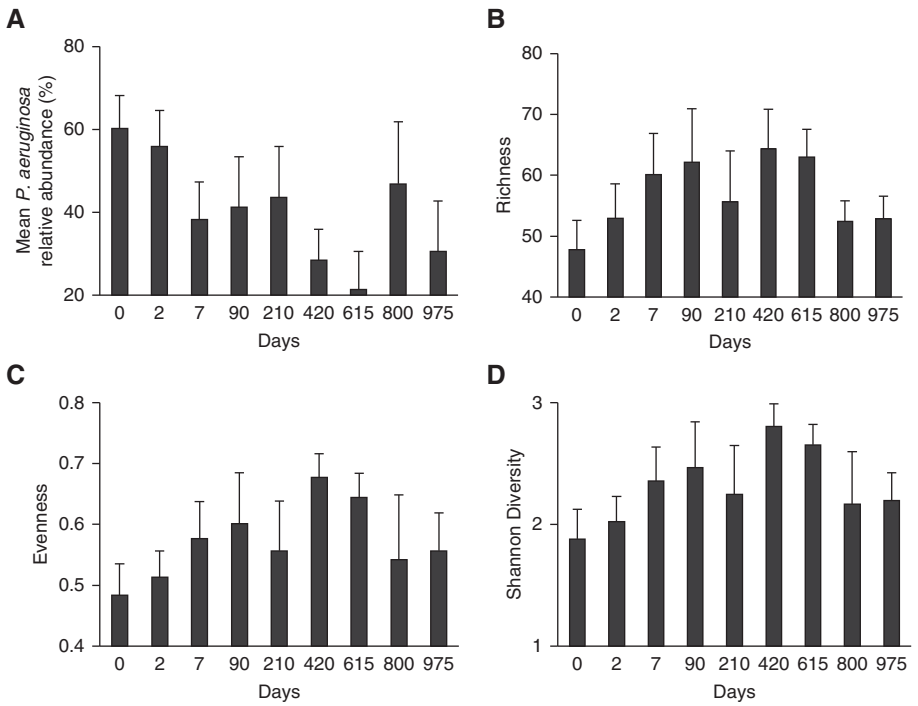


Figure 5. Decreases in *Pseudomonas aeruginosa* abundance are accompanied by increases in microbial diversity. *Graphs* indicate longitudinal changes in subjects with chronic *P. aeruginosa* infections in (*A*) *P. aeruginosa* relative abundance, (*B*) microbial richness, (*C*) microbial evenness, and (*D*) Shannon diversity index. *Bars* represent average values from subjects at each time point; *error bars* indicate SEM. Table E2 shows linear regression analysis indicating that richness and Shannon diversity showed positive slopes in the first year, and significant decreases in slopes thereafter ($P < 0.05$).

Second, we compared changes in the absolute abundance of all bacteria and of *Pseudomonas* species by qPCR. This analysis is informative because if the magnitude of *Pseudomonas* changes exceeded those of total bacteria, changes in the combined relative abundance of non-*Pseudomonas* organisms and microbial diversity could be attributable to *Pseudomonas* declines. Indeed at every time point, *P. aeruginosa* changed to a greater extent than total bacteria (Figure 6E). Taken together, these findings suggest that in the *P. aeruginosa*–infected subjects, the observed changes in the combined relative abundance of non–*P. aeruginosa* organisms and in diversity indices were primarily a consequence of the marked decline in the absolute abundance of sputum *P. aeruginosa* (*see* Discussion).

Microbiologic Effects of Ivacaftor on Subjects without Chronic *P. aeruginosa* Infections

Sputum samples from the four non–*Pseudomonas*-infected subjects were primarily collected during the first study week. Although the number of subjects was very small, and none of the microbiologic changes achieved statistical significance, some interesting changes were noted. For example, as for the *P. aeruginosa*–infected subjects, total sputum bacterial density (measured by qPCR) seemed to decline in all four non–*Pseudomonas*-infected subjects during the first week of treatment (*see* Figure E5A) (P = nonsignificant). Furthermore, the relative abundance of *Burkholderia* species seemed to decline in the two subjects with chronic *Burkholderia* infections (P = nonsignificant) (*see* Figure E5B). The two subjects with *Staphylococcus*-dominated infections showed no decreases in *Staphylococcus* relative abundance (*see* Figure E5B). Additional work is needed to determine if the changes identified in these subjects represent reproducible findings.

Ivacaftor Caused Sustained Reduction in Airway Inflammation

We measured sputum neutrophil elastase, IL-8, and IL-1β using ELISA and found rapid and significant reductions during the first treatment week ($P < 0.05$) (Figures 7A–7C) in the entire cohort (i.e., *P. aeruginosa*–infected and –uninfected subjects considered together). In the *P. aeruginosa*–infected subjects who we followed long term, these three markers continued to decline over the next 2 years (Figures 7A–7C), and all subjects experienced at least a 10-fold decrease by Day 600 ($P < 0.005$).

To independently gauge inflammation, we measured inflammatory proteins in sputum supernatants using mass spectrometry and detected decreases in neutrophil elastase ($P = 0.00003$), arginase-1 ($P = 0.03$), myeloperoxidase ($P = 0.003$), and calprotectin (S100A8 [$P = 0.02$] and S100A9 [$P = 0.03$]) after 1 week of ivacaftor (Figure 7D). We note that we did not apply corrections for multiple comparisons to the analysis of the inflammatory markers (*see* Discussion).

We also separately analyzed inflammatory marker data from the subjects not infected with *P. aeruginosa* to investigate whether ivacaftor decreased inflammation in these subjects. Although the number of subjects was very small, we found that IL-1β decreased significantly at 1 week (*see* Figure E6). Additional work is needed to determine if ivacaftor treatment consistently reduces inflammation in non–*P. aeruginosa* infected subjects.

Ivacaftor Improved Chest CT Scans

We measured radiographic changes in lung disease using Brody scoring (22) of inspiratory and expiratory chest CT scans before and 1 year after treatment. Brody scores measure bronchiectasis, mucous plugging, peribronchial thickening, air trapping, and parenchymal damage; higher scores indicate more disease.

Seven of the 12 subjects presented at 1 year to undergo follow-up CT scans. The subjects who presented for CT scans exhibited similar demographic and disease characteristics as those who did not (*see* Table E4). Although the findings should be interpreted with caution because of the small sample size, we found total Brody scores decreased in four subjects, unchanged in two, and increased in one subject, with the average change trending toward improvement (Figure 8A) ($P = 0.12$). Because some Brody score parameters may be reversible, whereas others may not, we examined individual parameters and found significant improvements in mucous plugging ($P = 0.012$) and a trend toward

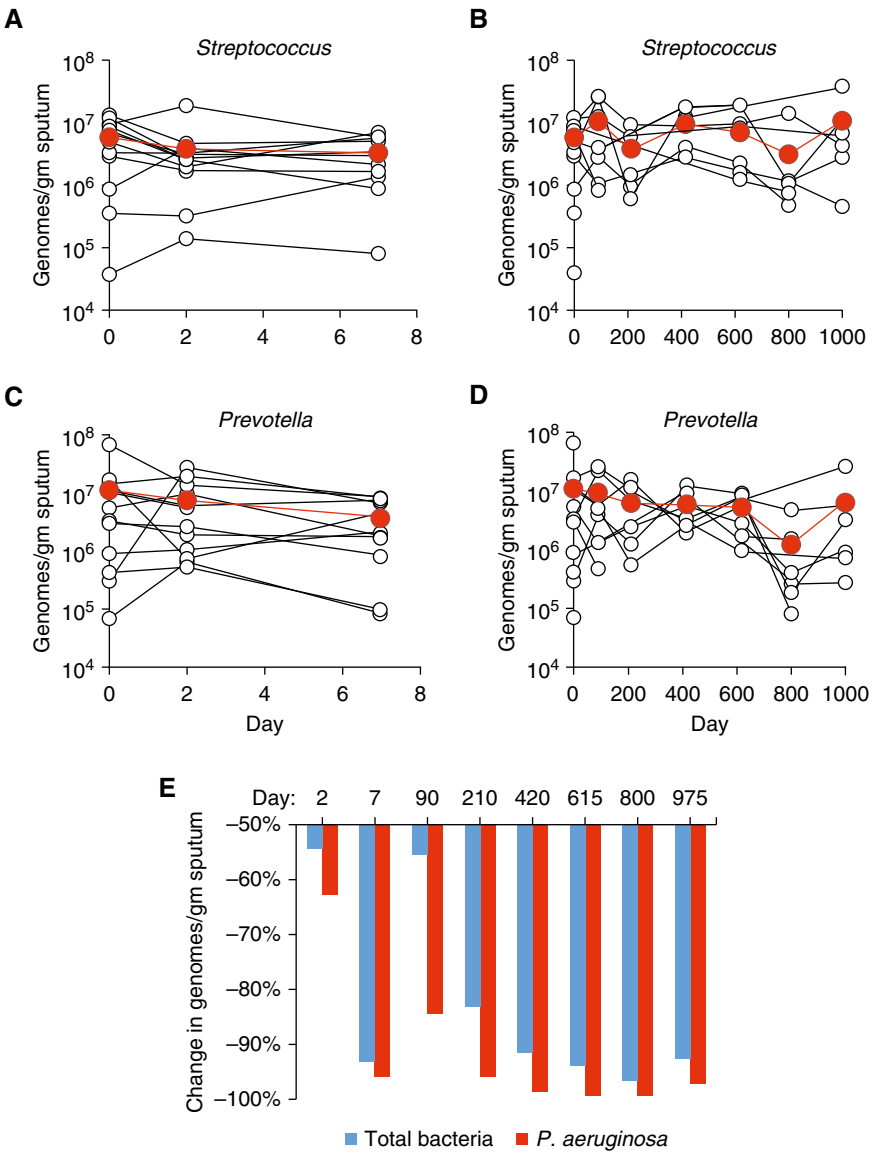


Figure 6. Ivacaftor does not reduce the absolute sputum abundance of *Streptococcus* and *Prevotella*. Quantitative polymerase chain reaction measurements of *Streptococcus* and *Prevotella* species in sputum (includes all available samples for subjects with chronic *Pseudomonas aeruginosa* infections, and Day 0–7 samples for subjects without chronic *P. aeruginosa* infections). (*A* and *C*) Changes in the first week. (*B* and *D*) Changes between Day 0 and up to Day 975, with values from the first week removed for clarity. No statistically significant changes were detected. (*E*) Comparison of the extent of decline of total bacterial and *P. aeruginosa* 16S rDNA in sputum after ivacaftor treatment as represented by percent change in mean values relative to Day 0.

improvements in peribronchial thickening ($P = 0.091$) (Figures 8B–8D; *see* Table E5).

Correlations between Measured Parameters

Although the small size of this study was not conducive to multiple comparisons corrections, we performed exploratory analyses to identify correlations between measured parameters. Tables showing correlations are provided in the online supplement (*see* Tables E6–E15). Additional work is needed to formally test the observed associations.

Discussion

In contrast to prior studies (5, 6), we found that pharmacologic restoration of CFTR activity produced rapid and marked decreases in the sputum abundance of bacteria, particularly the key CF pathogen *P. aeruginosa*, and reduced airway inflammation. Sputum *P. aeruginosa* CFUs decreased by more than 60-fold in the first week of treatment, and reductions were sustained for approximately 7 months. Key inflammatory markers showed approximately fivefold reductions in the first week, with further declines over time. These changes were accompanied by long-term improvements in the rate of lung function decline (FEV_1 slope), and improvements in some radiographic measurements of lung disease. However, restoring CFTR function did not eradicate chronic *P. aeruginosa* infection, and *P. aeruginosa* counts rebounded significantly in the second year of treatment. Next we discuss the limitations, strengths, and implications of our findings.

Study Limitations and Strengths

This study has several limitations. First, it is possible that some parameters may have changed because of off-target drug effects. Most relevant to our work is ivacaftor's reported antimicrobial activity. It is possible that the early declines and late increases in *P. aeruginosa* could reflect initial sensitivity and subsequent resistance to the direct antimicrobial effects of ivacaftor. However, ivacaftor is relatively impotent against *P. aeruginosa in vitro* (23), and seems unlikely that clinically achievable concentrations would produce sustained approximately 100-to-1,000-fold reductions in *P. aeruginosa* density.

Second, we did not have a control group, so responses could be a consequence of study participation rather than treatment. We think this unlikely, because control groups in other CF studies have not experienced improvements in infection, inflammation, or lung function in the range of those found here (3, 24). Third, we were not able to measure the effect of ivacaftor on clinical events, such as exacerbations or long-term antibiotic use, and we did not collect treatment adherence data. Fourth, we note that the DNA-based measurements used do not distinguish live from dead bacteria. Finally, the small study size limited our ability to detect subtle changes or associations. In particular, correlations between parameters were not corrected

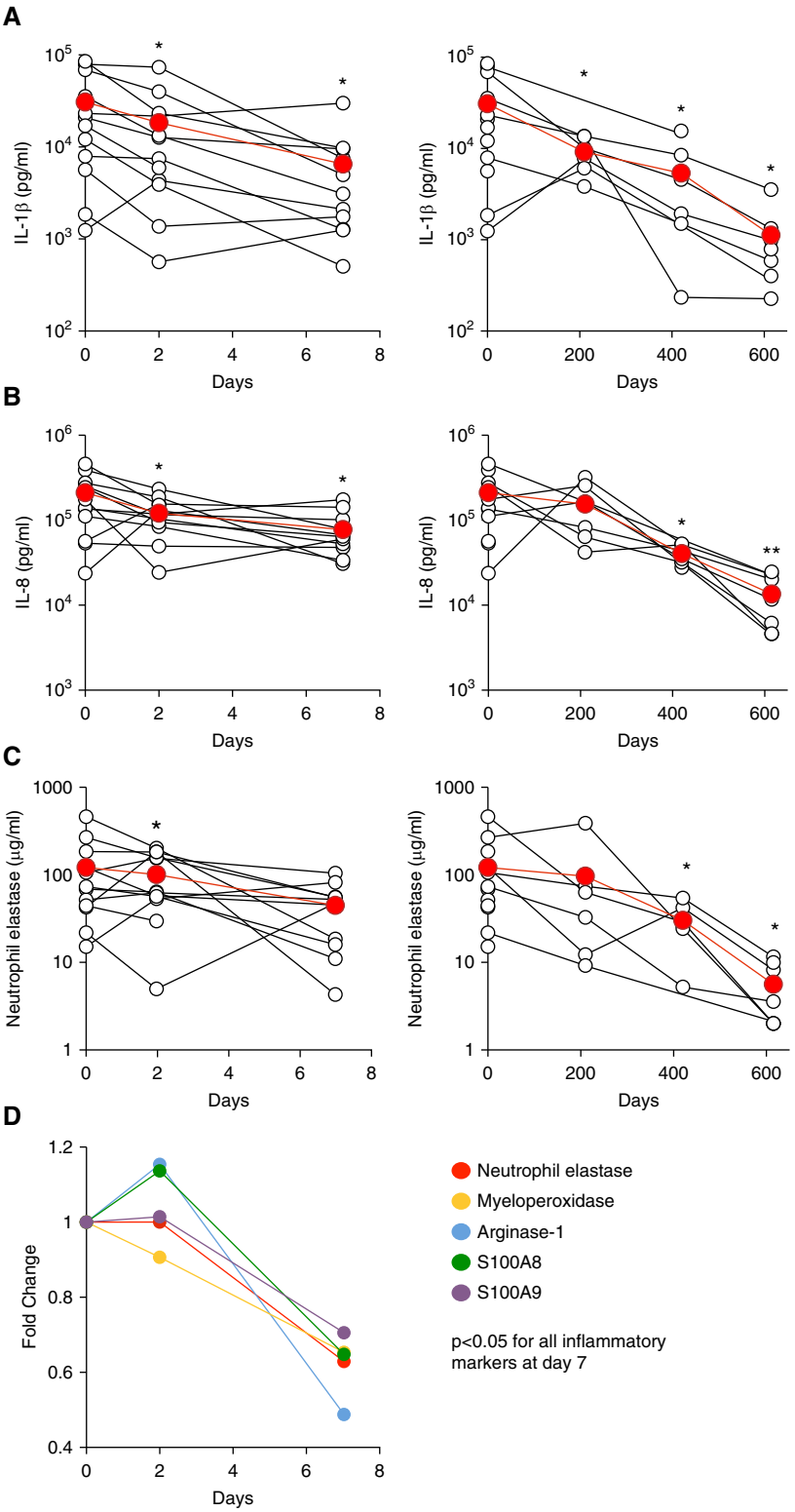


Figure 7. Ivacaftor treatment reduces airway inflammation. ELISA measurements of sputum inflammatory markers show decreases in (*A*) IL-1β, (*B*) IL-8, and (*C*) neutrophil elastase. *Left panels* show changes in the first week; *right panels* show changes between Day 0 and up to Day 600 with values from the first week removed for clarity; $^{*}P < 0.05$; $^{**}P < 0.005$ versus Day 0 values. (*D*) Change in the relative abundance of inflammatory markers in sputum supernatants during the first week, as determined by mass spectrometry. Decreases in all markers at Day 7 were significant with $P < 0.05$.

for multiple comparisons, and we consider those analyses exploratory.

Our study also had strengths that reduced variability, improved our ability to detect changes, and increased confidence in the results. The high prevalence of *G551D-CFTR* mutations in Ireland enabled us to study subjects at one center. Subjects initiated treatment during the same week, so visits were synchronized, and specimens were collected and processed by the same personnel in one laboratory. Each subject served as their own control and was studied at very early and late time points, from 48 hours to almost 1,000 days after treatment. Because of these factors and the magnitude of the responses, many changes reached statistical significance despite the small sample size.

We also note that key conclusions are supported by independent measurements. The finding that ivacaftor produced marked reductions in sputum *P. aeruginosa* during the first year was supported by culture and DNA-based measurements. The finding that ivacaftor produced marked reductions in airway inflammation was supported both by ELISA and proteomics-based measurements. Our interpretation that the observed changes in microbial diversity indices were likely a consequence of *P. aeruginosa* reductions was also supported by two findings. First, direct PCR assays showed no significant changes in the absolute abundance of the two dominant non-*Pseudomonas* taxa detected in subjects' sputum (*Prevotella* and *Streptococcus*). Second, we found that absolute abundance of *P. aeruginosa* changed to greater extent than total bacteria. Thus, the reciprocal changes noted in non–*P. aeruginosa* organisms and microbial diversity are likely to be a consequence of changes in *P. aeruginosa*.

Explanations for Discordance with Previous Studies

Our findings differ from prior studies (5, 6) that did not detect reductions in the density of sputum bacteria, airway pathogens, or airway inflammation after ivacaftor. We speculate that differences in study design and subjects' infection characteristics are responsible. For example, although our study and GOAL (5) studied infection and inflammation

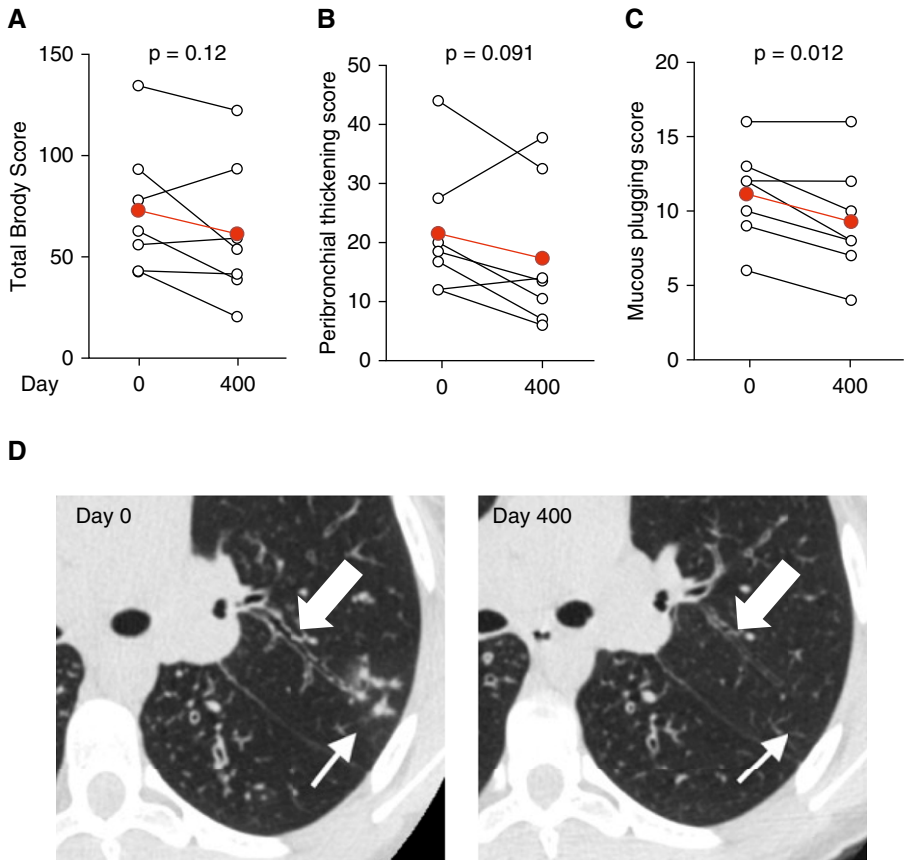


Figure 8. Ivacaftor treatment improves chest computed tomography (CT). Brody scoring of chest CT scans from subjects 1, 3, 4, 5, 6, 9, and 11 obtained before and 1 year after ivacaftor treatment demonstrated a trend toward decreases in the total Brody score (*A*) and the peribronchial thickening subscore (*B*). Significant decreases in mucous plugging (*C*) were observed. (*D*) Representative images showing decrease in mucous plugging (*thin arrow*) and peribronchial thickening (*thick arrow*) in the chest CT scans obtained before (*left*) and 1 year after (*right*) ivacaftor treatment in the same subject.

in similar numbers of subjects, fewer GOAL subjects had chronic *P. aeruginosa* infections (40 vs. 66%). None of the three subjects in the study by Bernarde and coworkers (6) was chronically infected with *P. aeruginosa*. The prevalence of chronic *P. aeruginosa* infections could be an important factor, because changes in *P. aeruginosa* density were the most robust microbiologic finding in our study, and seemed to drive other microbiologic changes we observed, including changes in the combined relative abundance of non-*Pseudomonas* organisms, and changes in microbial diversity indices.

Technical differences may have also affected results. For example, GOAL studied induced sputum, whereas we studied spontaneously expectorated samples. We also fractionated sputum into pellets and supernatants immediately to measure inflammation, whereas GOAL froze sputum. Thawing-induced cell lysis could release cytokines and obscure changes. Finally, GOAL involved multiple study sites, which could affect measurements.

Why Did *P. aeruginosa* and Other Bacteria Show Reciprocal Changes?

Measurements on the *P. aeruginosa*–infected subjects suggest that changes in the relative abundance of non-*Pseudomonas* organisms are likely a consequence of absolute changes in *P. aeruginosa*, rather than being caused by absolute changes in abundance of other bacteria.

These findings could be explained if ivacaftor somehow preferentially affected airway *P. aeruginosa*, and had relatively minor effects on other organisms. Another possibility relates to the fact that expectorated sputum contains secretions from the lower airways and saliva, and perhaps other sources. This is important because many nonconventional organisms detected in CF sputum (including *Prevotella*, *Streptococcus*, and *Veillonella* species) are highly abundant in saliva, and salivary bacterial concentrations rival those found in sputum (19–21). Thus, if ivacaftor changed *P. aeruginosa* density in lower airway secretions, and the extent of salivary contamination was unchanged, the relative abundance of nonconventional organisms in sputum and microbial diversity indices could exhibit reciprocal changes as a secondary effect.

It was notable that that total bacterial abundance seemed to decrease in the subjects who were not chronically infected with *P. aeruginosa*, as it did in *P. aeruginosa*–infected subjects. However, the number of subjects not chronically infected with *P. aeruginosa* was very small, data were only available for the first treatment week, and changes in these subjects did not reach statistical significance. If the trends observed in the non–*P. aeruginosa* infected subjects reflect real changes, the total bacterial abundance reductions could have been driven by declines in the conventional pathogens present (e.g., *Burkholderia* and *Staphylococcus* species). It is also possible that ivacaftor reduced nonconventional organisms in these subjects. Additional work is needed to understand the differential responses of conventional and nonconventional organisms to ivacaftor.

Will Treatment-induced Declines in Pathogen Burden Be Sustained?

The significant increase in sputum *P. aeruginosa* CFUs we observed during the second treatment year is concerning. It is possible that improved health causes reduced adherence to ivacaftor or other treatments (25), and unfortunately treatment adherence data were not collected. A more worrisome possibility is suggested by studies showing that *P. aeruginosa* genetically diversifies during

CF infections (15–18). Therefore, *P. aeruginosa* genetic variants could arise that are capable of surviving or even thriving after CFTR is restored. If adapted *P. aeruginosa* variants do rebound, sustained health improvements may require the combined use of CFTR-restoring and antiinfective treatments. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the Cystic Fibrosis Foundation–supported Genomics Core Facility at the University of Washington for consultation and the Cystic Fibrosis Foundation-Therapeutics Center for Biochemical Markers at the University of Colorado for measurements of inflammatory markers in sputum.

References

1. Welsh MJ, Ramsey BW, Accurso F, Cutting GR. Cystic fibrosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Vogelstein B, editors. The metabolic and molecular basis of inherited diseases. New York: McGraw-Hill; 2001. pp. 5121–5189.
2. Gibson RL, Burns JL, Ramsey BW. Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 2003;168:918–951.
3. Ramsey BW, Davies J, McElvaney NG, Tullis E, Bell SC, Dřevínek P, Griese M, McKone EF, Wainwright CE, Konstan MW, *et al*.; VX08-770-102 Study Group. A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *N Engl J Med* 2011;365:1663–1672.
4. Accurso FJ, Rowe SM, Clancy JP, Boyle MP, Dunitz JM, Durie PR, Sagel SD, Hornick DB, Konstan MW, Donaldson SH, *et al*. Effect of VX-770 in persons with cystic fibrosis and the G551D-CFTR mutation. *N Engl J Med* 2010;363:1991–2003.
5. Rowe SM, Heltshe SL, Gonska T, Donaldson SH, Borowitz D, Gelfond D, Sagel SD, Khan U, Mayer-Hamblett N, Van Dalfsen JM, *et al*.; GOAL Investigators of the Cystic Fibrosis Foundation Therapeutics Development Network. Clinical mechanism of the cystic fibrosis transmembrane conductance regulator potentiator ivacaftor in G551D-mediated cystic fibrosis. *Am J Respir Crit Care Med* 2014;190: 175–184.
6. Bernarde C, Keravec M, Mounier J, Gouriou S, Rault G, Férec C, Barbier G, Héry-Arnaud G. Impact of the CFTR-potentiator ivacaftor on airway microbiota in cystic fibrosis patients carrying a G551D mutation. *PLoS One* 2015;10:e0124124.
7. Heltshe SL, Mayer-Hamblett N, Burns JL, Khan U, Baines A, Ramsey BW, Rowe SM; GOAL (the G551D Observation-AL) Investigators of the Cystic Fibrosis Foundation Therapeutics Development Network. *Pseudomonas aeruginosa* in cystic fibrosis patients with G551D-CFTR treated with ivacaftor. *Clin Infect Dis* 2015;60:703–712.
8. Hisert KB, Pope C, Heltshe SL, Cooke G, Jorth P, Grogan B, Launspach JL, Gallagher C, Welsh M, Accurso F, *et al*. Long term follow up of CF patients with G551D mutations and chronic *Pseudomonas* infections treated with ivacaftor. *Pediatr Pulmonol* 2016;51:299.
9. Laird NM, Ware JH. Random-effects models for longitudinal data. *Biometrics* 1982;38:963–974.
10. Adam RJ, Hisert KB, Dodd JD, Grogan B, Launspach JL, Barnes JK, Gallagher CG, Sieren JP, Gross TJ, Fischer AJ, *et al*. Acute administration of ivacaftor to people with cystic fibrosis and a G551D-CFTR mutation reveals smooth muscle abnormalities. *JCI Insight* 2016;1:e86183.
11. Hisert KB, Schoenfelt KQ, Cooke G, Grogan B, Launspach JL, Gallagher CG, Donnelly SC, Welsh MJ, Singh PK, McKone EF, *et al*. Ivacaftor-induced proteomic changes suggest monocyte defects may contribute to the pathogenesis of cystic fibrosis. *Am J Respir Cell Mol Biol* 2016;54:594–597.
12. Mogayzel PJ Jr, Naureckas ET, Robinson KA, Brady C, Guill M, Lahiri T, Lubsch L, Matsui J, Oermann CM, Ratjen F, *et al*.; Cystic Fibrosis Foundation Pulmonary Clinical Practice Guidelines Committee. Cystic Fibrosis Foundation pulmonary guideline. pharmacologic approaches to prevention and eradication of initial *Pseudomonas aeruginosa* infection. *Ann Am Thorac Soc* 2014;11: 1640–1650.
13. Mogayzel PJ Jr, Naureckas ET, Robinson KA, Mueller G, Hadjiliadis D, Hoag JB, Lubsch L, Hazle L, Sabadosa K, Marshall B; Pulmonary Clinical Practice Guidelines Committee. Cystic fibrosis pulmonary guidelines. Chronic medications for maintenance of lung health. *Am J Respir Crit Care Med* 2013;187:680–689.
14. Jolley KA, Maiden MC. BIGSdb: scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics* 2010;11:595.
15. Ashish A, Paterson S, Mowat E, Fothergill JL, Walshaw MJ, Winstanley C. Extensive diversification is a common feature of *Pseudomonas aeruginosa* populations during respiratory infections in cystic fibrosis. *J Cyst Fibros* 2013;12:790–793.
16. Darch SE, McNally A, Harrison F, Corander J, Barr HL, Paszkiewicz K, Holden S, Fogarty A, Crusz SA, Diggle SP. Recombination is a key driver of genomic and phenotypic diversity in a *Pseudomonas aeruginosa* population during cystic fibrosis infection. *Sci Rep* 2015; 5:7649.
17. Jorth P, Staudinger BJ, Wu X, Hisert KB, Hayden H, Garudathri J, Harding CL, Radey MC, Rezayat A, Bautista G, *et al*. Regional isolation drives bacterial diversification within cystic fibrosis lungs. *Cell Host Microbe* 2015;18:307–319.
18. Williams D, Evans B, Haldenby S, Walshaw MJ, Brockhurst MA, Winstanley C, Paterson S. Divergent, coexisting *Pseudomonas aeruginosa* lineages in chronic cystic fibrosis lung infections. *Am J Respir Crit Care Med* 2015;191:775–785.
19. Salminen A, Kopra KA, Hyvärinen K, Paju S, Mäntylä P, Buhlin K, Nieminen MS, Sinisalo J, Pussinen PJ. Quantitative PCR analysis of salivary pathogen burden in periodontitis. *Front Cell Infect Microbiol* 2015;5:69.
20. Zemanick ET, Wagner BD, Robertson CE, Stevens MJ, Szefler SJ, Accurso FJ, Sagel SD, Harris JK. Assessment of airway microbiota and inflammation in cystic fibrosis using multiple sampling methods. *Ann Am Thorac Soc* 2015;12:221–229.
21. Evaldson G, Heimdahl A, Kager L, Nord CE. The normal human anaerobic microflora. *Scand J Infect Dis Suppl* 1982;35:9–15.
22. Brody AS, Sucharew H, Campbell JD, Millard SP, Molina PL, Klein JS, Quan J. Computed tomography correlates with pulmonary exacerbations in children with cystic fibrosis. *Am J Respir Crit Care Med* 2005;172:1128–1132.
23. Reznikov LR, Abou Alaiwa MH, Dohrn CL, Gansemer ND, Diekema DJ, Stoltz DA, Welsh MJ. Antibacterial properties of the CFTR potentiator ivacaftor. *J Cyst Fibros* 2014;13:515–519.
24. Ramsey BW, Pepe MS, Quan JM, Otto KL, Montgomery AB, Williams-Warren J, Vasiljev-K M, Borowitz D, Bowman CM, Marshall BC, *et al*.; Cystic Fibrosis Inhaled Tobramycin Study Group. Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. *N Engl J Med* 1999;340:23–30.
25. Siracusa CM, Ryan J, Burns L, Wang Y, Zhang N, Clancy JP, Drotar D. Electronic monitoring reveals highly variable adherence patterns in patients prescribed ivacaftor. *J Cyst Fibros* 2015;14:621–626.