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Coláiste na hOllscoile Corcaigh

Extended-culture and culture-independent molecular analysis of the airway microbiota in cystic fibrosis following CFTR modulation with ivacaftor.

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Abstract

Background: Treatment with Ivacaftor provides a significant clinical benefit in people with cystic fibrosis (PWCF) with the class III *G551D*-CFTR mutation. This study determined the effect of CFTR modulation with ivacaftor on the lung microbiota in PWCF.

Methods: Using both extended-culture and culture-independent molecular methods, we analysed the lower airway microbiota of 14 PWCF, prior to commencing ivacaftor treatment and at the last available visit within the following year. We determined total bacterial and *Pseudomonas aeruginosa* densities by both culture and qPCR, assessed ecological parameters and community structure and compared these with biomarkers of inflammation and clinical outcomes.

Results: Significant improvement in FEV_1, BMI, sweat chloride and levels of circulating inflammatory biomarkers were observed POST-ivacaftor treatment. Extended-culture demonstrated a higher density of strict anaerobic bacteria ($p=0.024$), richness ($p=1.59*10^{-4}$) and diversity ($p=0.003$) POST-treatment. No significant difference in fold change was observed by qPCR for either total bacterial 16S rRNA copy number or *P. aeruginosa* density for *opr*L copy number with treatment. Culture-independent (MiSeq) analysis revealed a significant increase in richness ($p=0.03$) and a trend towards increased diversity ($p=0.07$). Moreover, improvement in lung function, richness and diversity displayed an inverse correlation with the main markers of inflammation ($p<0.05$).

Conclusions: Following treatment with ivacaftor, significant improvements in clinical parameters were seen. Despite modest changes in overall microbial community composition, there was a shift towards a bacterial ecology associated with less severe

CF lung disease. Furthermore, a significant correlation was observed between richness and diversity and levels of circulating inflammatory markers.

INTRODUCTION

In people with CF (PWCF), CFTR dysfunction in the lower airways results in dehydrated airway secretions and a severe impairment of mucociliary clearance, which results in a cycle of chronic infection and inflammation. Ivacaftor, a first-in-class molecule, enhances CFTR function in class III (gating) and class IV (conductance) mutations [1, 2] by increasing the probability of CFTR-channel opening. The *G551D*-CFTR mutation displays a severe phenotype and is the most commonly detected class III mutation with a worldwide prevalence of 4-5% [3]. However, significant regional variation exists with the prevalence highest in Ireland with the Cork CF Centre at around 23% [4]. Studies focusing on the efficacy of ivacaftor have demonstrated significant improvement in lung function (FEV_1), weight gain, improved respiratory symptoms, reduction in sweat chloride levels and a decrease in frequency of pulmonary exacerbations [1, 4]. The physiochemical nature of the environment in the lungs of PWCF may act as a strong selective force affecting microbiota composition in the lower airways [5] . Ivacaftor increases transportation of chloride ions across the cell membrane resulting in rehydration of the airway surface layer. These changes in the airway microenvironment also alter pH and mucus viscosity and may also have an effect on the resident microbiota. Indeed, a number of studies have reported changes in the lung microbiota following treatment with ivacaftor [6-8]. Furthermore, it has been suggested that ivacaftor may exhibit antimicrobial properties with Payne and colleagues demonstrating activity against Gram-positive microorganisms [9].

Given the relatively high allele frequency of *G551D*-CFTR in our centre, we previously reported the largest single centre study focusing on ivacaftor treatment in this group of PWCF [4]. In the current study, we hypothesised that, in addition to improvement in

clinical status in PWCF with at least one copy of the *G551D*-CFTR mutation, treatment with ivacaftor results in significant changes in lower airway microbial community composition and structure and inflammation.

MATERIALS AND METHODS

Ethics statement and patient recruitment

Ethical approval was obtained from the Clinical Research Ethics Committee of the Cork Teaching Hospitals. PWCF (n=14; median age, 26 years; range: 13-39 years) with at least one copy of the *G551D*-CFTR mutation, attending the Cork Cystic Fibrosis centre from March to December 2013, were followed prospectively, for a mean period of 12 months. This cohort represented a subset of PWCF from our previously published study that were able to provide sufficient sputum at baseline (prior to commencing ivacaftor treatment; [PRE]) and at the last available time-point up to 12 months (post-treatment; [POST]) for microbiota analysis. Clinical data including spirometry, performed in keeping with ERS/ATS guidelines, sweat chloride, BMI and number of courses of IV antibiotics was recorded prospectively before commencing ivacaftor and in the year after treatment.

Sample collection and processing

Expectorated sputum samples were collected during the PRE-treatment visit for the initiation of ivacaftor treatment and at the last POST-treatment visit (i.e. at 9 or 12 months). All 14 patients provided sufficient sputum to allow culture-independent molecular analysis with a further 10 patients providing sufficient sputum to also allow processing by extended-quantitative bacterial culture. Sputum samples were stored at

-80^{o}C and shipped on dry ice to Queen's University Belfast where they were stored at -80^{o}C until processing. Blood samples were also collected from all participants at each clinic visit. A multiplex enzyme linked immunosorbent assay (MesoScale Discovery platform) was used to quantify inflammatory biomarkers in blood: interleukin 6 and 8 (IL-6; IL-8), Tumour Necrosis Factor α (TNF-α), Interferon-γ (IFN-γ)] and C-reactive protein (CRP) (Meso Scale Diagnostics, USA).

Extended bacterial culture

Extended-quantitative bacterial culture of sputum samples and detection and identification of isolated bacteria were performed as previously described [10, 11]. For further description of detailed methods, see Supplementary File S1.

Molecular detection

Genomic DNA (gDNA) was extracted from ~200mg sputum aliquots, along with appropriate controls, by treatment with Sputolysin®, mechanical disruption and processing on an automated nucleic acid purification platform (Roche MagNA Pure).

i. qPCR quantification of total bacterial and *P. aeruginosa* (PA) density

P. aeruginosa and total bacterial density was determined by qPCR using the LightCycler®480 instrument (Roche, CH) using the Probes Master kit. Total bacterial load was determined using a primer/probe targeting the bacterial 16S rRNA marker-gene [12], with *P. aeruginosa* load determined using a primer/probe set targeting the *opr*L gene [13]. For further description of detailed methods, see Supplementary File S1.

ii. Illumina MiSeq sequencing

Molecular characterization of the airway microbiota was performed using the lllumina MiSeq NGS platform (Illumina, USA) targeting the 16S rRNA marker gene as described below. For sequencing, we targeted the V4 region of the bacterial 16S rRNA marker-gene in a two-step library preparation, applying modified universal primers as previously described by Lundberg et al. [14]. In brief, bead-cleaned nucleotide libraries in equimolar concentrations of amplicons (approx. 452 base pairs in size) were sequenced on an Illumina MiSeq sequencing platform using the version-3 (V3) paired-end 600 cycle kit. Following completion of the MiSeq run, the raw sequence data was deposited to the European Nucleotide Archive (ENA) (Study Accession: PRJEB37510). Downstream analysis of raw sequence read, OTU calling (operational taxonomic units) and data analysis was performed in QIIME 1.9.1 (Quantitative Insights Into Microbial Ecology) [15] and R version 3.4.2. (https://www.r-project.org/). Further details regarding sample handling, sequence library preparation, quantification, MiSeq marker-gene sequencing, handling and removal of potential background contamination from technical sequence controls and downstream analysis are provided in Supplementary File S1. The metadata mapping file for MiSeq processing and analysis is provided in Table S1.

Statistical analysis and ecological community measurements

Assessment of the data demonstrated that for most of the included variables, the data did not conform to normal normality (Shapiro-Wilks normality test; $p \leq 0.05$). Hence, nonparametric analyses were performed, which included the Mann-Whitney *U* test, Wilcoxon signed-rank test, Spearman's (r) ranked correlation and Pearson (r) correlation test for count based and clinically relevant data where appropriate. The

analysis of microbial community based (extended culture and molecular based data) and clinical data was conducted in the R environment (https://www.r-project.org). P <0.05 was accepted as statistically significant. Further details regarding specific packages and analysis are provided in Supplementary File S1.

RESULTS

Patient demographic data are summarised in Table 1 with Table S2 summarising total antibiotic usage in the year prior to or after ivacaftor treatment for this cohort. A significant reduction in sweat chloride concentration (p=0.0001) and a significant increase in lung function (FEV_1 % predicted; p=0.001) was observed in the year following ivacaftor treatment (Fig. S1).

Extended bacterial culture

Bacteria were cultured in high numbers from all sputum samples collected PRE- (up to 2.08 x 10^8 CFU/g of sputum) and POST-treatment (up to 1.25 x 10^8 CFU/g of sputum) with ivacaftor. There was no significant difference in the total bacterial density cultured from PRE- (1.32 x 10^7 [3.00 x 10^4 to 8.60 x 10^7] CFU/g of sputum; median [range]) or POST-treatment (8.52 x 10^6 [3.60 x 10^5 to 5.60 x 10^7] CFU/g of sputum; median [range]) samples (Fig. 1A). Aerobic bacteria were cultured in high abundance from all PRE- (up to 2.07 x 10^8 CFU/g of sputum) and POST-treatment (up to 1.08 x 10^8 CFU/g of sputum) samples. There was no significant difference in the median values of total aerobic bacterial density from PRE- (1.23 x 10^7 [3.00 x 10^4 to 8.60 x 10^7] CFU/g of sputum; median [range]) versus POST-treatment (7.49 x 10^6 [3.60 x 10^5 to 4.79 x 10^7 CFU/g of sputum; median [range]) samples (Fig. 1B). Anaerobic bacteria were cultured from 5/10 PRE- (up to 1.40 x 10^6 CFU/g of sputum) and from all 10

POST-treatment samples (up to 1.71 x 10^7 CFU/g of sputum). The total anaerobic bacterial density PRE-treatment (4.31 x 10^4 [0.0 to 2.39 x10^6] CFU/g of sputum; median [range]) was significantly lower than POST-treatment (3.50 x 10^6 [9.26 x 10^3 to 2.96 x 10^7] CFU/g of sputum; median [range]) ($p = 0.024$, Fig. 1C). In total, we cultured 54 different bacterial taxa with no single taxa demonstrating significant difference PRE- and POST-treatment. All 6 patients who were *P. aeruginosa* positive by extended-culture pre-treatment were also *P. aeruginosa* positive POST-treatment with no significant difference in density. Further detailed information regarding total bacterial counts (CFU/g sputum [Log10]) and detected taxa are shown in Table S3. Significant differences were detected between PRE- and POST-treatment samples for both taxonomic richness and community diversity ($p=1.590 \times 10^{-4}$ and $p=0.003$, respectively) (Fig. 1D and 1F, respectively).

qPCR quantification of total bacterial and *P. aeruginosa* density

No significant difference in fold change was observed for either total bacterial 16S rRNA copy number [6.30 x10^8 (PRE) and 4.14 x 10^8 (POST)] or *P. aeruginosa* density for *opr*L copy number [1.65 x 10^7 (PRE) and 1.79 x 10^7 (POST)] following ivacaftor therapy (Table S1). We observed significant concurrence between *P. aeruginosa* TVC's (extended-culture) and *opr*L qPCR values for *P. aeruginosa* (Pearson product-moment correlation coefficient $r = 0.56$; $R^2 = 0.301$; $p = 0.007$; Bonferroni adjusted for multiple comparisons) (Fig. S2A). Furthermore, there was excellent agreement between the relative abundance of *Pseudomonas* spp. from Illumina MiSeq 16S rRNA marker-gene sequencing and *opr*L qPCR values for *P. aeruginosa* (Pearson product-moment correlation coefficient $r=0.88$; $R^2=0.775$; $p=3.91*10^{-10}$; Bonferroni adjusted for multiple comparisons) (Fig. S2B).

Illumina MiSeq sequencing

Following quality filtering and pre-processing of the paired-end sequence reads, each sample was normalised to 58,391 reads. In total, we detected 179 different taxa in the paired samples (PRE-ivacaftor [n=123]; POST-ivacaftor treatment [n=160]). Furthermore, a limited number of taxa (26/179) accounted for 99% of the total sequence read number in our cohort.

At the phylum level, the mean relative abundance of Proteobacteria was reduced by 22% POST-treatment, while mean relative abundance of Firmicutes, Bacteriodetes, Actinobacteria and Fusobacteria increased by 12%, 13%, 57% and 500%, respectively (Fig. 2A). At genera level, there was a significant change in community composition following treatment in samples from 9/14 PWCF, with only two of these individuals receiving IV antibiotics in the previous 8 weeks. In 5/14 PWCF, community composition remained relatively stable following treatment with only marginal changes in the relative abundance of the dominant genera (Fig. 2B); none of these 5 patients received IV antibiotics in the previous 8 weeks.

Comparison of taxonomic richness demonstrated a significant increase in observed taxa richness POST-treatment (Fig. 3A; p=0.031, Mann-Whitney test). Community diversity (Shannon–Wiener index [H']), although trending towards a higher diversity in the POST-treatment samples, did not show a significant difference between visits (Fig. 3B, p=0.069; Mann-Whitney test). Community evenness ($e^{H/S}$) and dominance (D) were similar PRE- and POST-treatment (p=0.085 and p=0.094, respectively; Mann-Whitney test) (Fig. 3C and 3D, respectively).

We observed a high degree of variation in the microbiota between patients (Fig. 2B). Inter- similarities/differences, based on β-diversity computed with the Bray-Curtis dissimilarity measures, demonstrated a significant difference between individual patients (R^2 = 0.70; p=0.006; sample ADONIS; 9999 permutations). In contrast, analysis between PRE- and POST-treatment samples was not shown to be statistically significant (R^2 = 0.01; p=0.653; PERMANOVA; 999 permutations) (Fig. S3A and S3B).

Communities dominated by a high relative abundance of members of *Pseudomonas* spp. were associated with lower taxonomic richness and diversity (Fig. S4 A-C), while communities with relatively higher abundance of members of *Streptococcus* spp. were associated with both higher taxonomic richness and diversity (Fig. S4 A-B and D). There was a general change in the direction of community composition following ivacaftor treatment from communities dominated by *Pseudomonas* spp., *Stenotrophomonas* spp. (classified as a "family of unclassified Xanthomonadaceae") and *Staphylococcus* spp. PRE-treatment towards communities with a higher relative abundance of *Streptococcus* spp. POST-treatment (Fig. S4 G, C, E and F, respectively).

Lower Airway Microbiota and Host Inflammation

For exploratory analysis, calculated the relationship between taxonomic richness and community diversity with the main markers of inflammation (adjusted for repeated measures). Circulating IL-6 and IL-8, blood CRP and TNF-α levels showed a significant inverse relationship with change in community richness (Fig. S5 A-D) (r=-0.472 [p=0.013]; r=-0.472 [p=0.013]; r=-0.434 [p=0.024]; r=-0.445 [p=0.020], respectively). There was also a significant inverse correlation between IL-8 (r=-0.412; p=0.033), TNF-α (r=-0.567; p=0.002) and community diversity; however, IL-6 (r=-

0.262; p=0.187) and CRP (r=-0.150; p=0.455), did not show significant correlation with levels of community diversity (Fig. S5 E-H). No correlation was observed between ecological indices (taxonomic richness and community diversity) and IFN-γ (r=-0.205 [p=0.304]; r=-0.131 [p=0.514]), respectively.

DISCUSSION

This is the first study to simultaneously use extended quantitative culture and culture-independent methods to assess changes in microbial community composition following ivacaftor treatment. Extended culture detected a high number of both aerobic and strict anaerobic bacteria prior to commencing and POST-ivacaftor treatment, with total anaerobic bacterial numbers, community richness and diversity significantly higher following treatment. Culture-independent analysis did not demonstrate a significant effect on airway microbial community composition following ivacaftor treatment, despite significant changes being observed within individual patients. In general, where such changes occurred, the shift was from communities with a higher proportion of *Pseudomonas* spp., *Stenotrophomonas* spp. and *Staphylococcus* spp. towards communities with a higher proportion of *Streptococcus* spp. Following treatment, community structures displayed an increase in taxonomic richness as well as a trend towards higher community diversity. Furthermore, we observed a significant inverse relationship between community richness and markers of inflammation in blood. This shift is in the direction of a bacterial ecology associated with less severe CF lung disease [16].

A key finding from the current study is that, despite significant improvements in many of the clinically relevant parameters, ivacaftor treatment did not result in eradication of *P. aeruginosa* from the airways. This contrasts with the findings of Heltshe and colleagues who reported a significant reduction in *P. aeruginosa* culture positivity one

year after initiation of ivacaftor therapy [17]. However, the results of this earlier study may have been skewed by the inclusion of patients defined as having intermittent *P. aeruginosa* infection prior to initiation of ivacaftor therapy. Our findings are similar to those of Hisert et al. who followed PWCF longitudinally for up to 3 years and reported that none of the 8 patients chronically colonised with *P. aeruginosa* eradicated their infecting strain [18]. We did not demonstrate, by either culture or qPCR, a significant difference in *P. aeruginosa* density in PRE- and POST-treatment samples. In contrast, Hisert et al., who performed more frequent sample analysis, reported a significant reduction in *P. aeruginosa* density in the first week of treatment which was maintained for approximately 7 months (day 210). However, *P. aeruginosa* density increased in 6/7 patients over the remaining study period [18]. As our POST-treatment samples were collected at either 9 or 12 months, it is likely that any initial reduction in *P. aeruginosa* density, if present, would have been reversed by 9 or 12 months and therefore not detected.

Of interest, Peleg and co-workers demonstrated that, although significant changes in microbial community composition occurred following ivacaftor treatment, they were primarily as a result of concurrent antibiotic exposure. PWCF that did not receive intravenous antibiotic treatment only demonstrated modest changes in their microbiota [19]. However, only 2/14 PWCF in our study that displayed a significant shift in microbial community composition had received a course of intravenous antibiotics in the eight weeks preceding collection of the POST-treatment sample. Changes in the lung environment, such as modulation in pH levels of the airway surface liquid (ASL) can have a significant effect on its viscosity [20], stabilisation of mucin binding and local host defence mechanisms [21]. This suggests that changes in the airway environment could play a significant role in shaping community composition as well as

modulating the virulence potential of a number of the main pathogenic taxa as these were seldom eliminated from the community following ivacaftor treatment [22, 23].

No direct association between individual bacterial taxa, community diversity or taxonomic richness and lung function were detected. However, increased taxonomic richness was associated with decreased systemic and airway inflammation. This suggests that an increase in bacterial diversity, richness and abundance of anaerobic bacteria may be linked to improvements in clinical status in PWCF receiving ivacaftor therapy via downregulation of the host's inflammatory response.

The current study has a number of limitations. As this is a single centre study, inferring the results to findings elsewhere may be difficult. However, the current study is the largest single centre study reported to date. Importantly, we used both extended-culture and culture-independent methods to examine the effect of ivacaftor on bacterial communities present in sputum from PWCF in a region that shows the highest prevalence of the *G551D*-CFTR mutation in the world. Our study is also observational in nature and did not include a matched control group of individuals that did not receive ivacaftor. However, the benefits of ivacaftor relative to placebo have been well demonstrated in existing trials, and the observed clinical improvements in the current study are comparable to those previously reported.

CONCLUSIONS

Variation in microbial community composition and the prevalence of pathogenic taxa were highly individual without an overall significant change in either following ivacaftor treatment. Changes in the airway environment and circulating inflammatory cytokine

levels as a consequence of ivacaftor treatment, may result in an indirect effect on the local microbiota and modulate the virulence of pathogenic members of the community.

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Contribution: NJR, FS, JAE, MMM and BJP contributed to study design. GGE, NJR, DM, MT, DM, CMcG, MC, GOC, DMM, OJOC, CAS, MMT, JAE, MMM, JSE, BJP and MMT contributed to data acquisition, analysis and interpretation. All authors contributed to drafting the work and final approval.

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Competing interests: None declared.

Ethics approval: Ethical approval was obtained from the Clinical Research Ethics Committee of the Cork Teaching Hospitals.

Data sharing statement Data deposition: Sequencing data are deposited in the European Nucleotide Archive database (Study Accession: PRJEB37510). Data submitted but unreleased to public until publication.

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415 **Table 1. Patient demographics.**

Number of PWCF	**14**		
Age at Baseline (years; median; range)	**25.3 (26.5; 13-37)**		
Gender (female/male)	**5/9**		
	PRE	**POST**	**p-value**
BMI	**20.90 (16.20-24.80)**	**22.18 (17.30-26.00)**	**0.006**
IL-6 (Log10; mean [range])	**2.81 (2.39-3.76)**	**2.68 (2.43-3.25)**	**0.009**
IL-8 (Log10; mean [range])	**3.36 (2.90-3.81)**	**3.35 (3.01-3.88)**	**0.799**
CRP (Log10; mean [range])	**4.13 (2.98-5.74)**	**4.13 (3.18-5.21)**	**0.076**
TNF-α (Log10; mean [range])	**2.86 (2.57-2.93)**	**2.84 (2.31-3.02)**	**0.683**
IFN-γ (Log10; mean [range])	**2.89 (2.79-3.11)**	**2.85 (2.63-3.02)**	**0.227**
Genotype			
G551D/F508del (12); G551D/G551D (1); G551D/R553x (1)			

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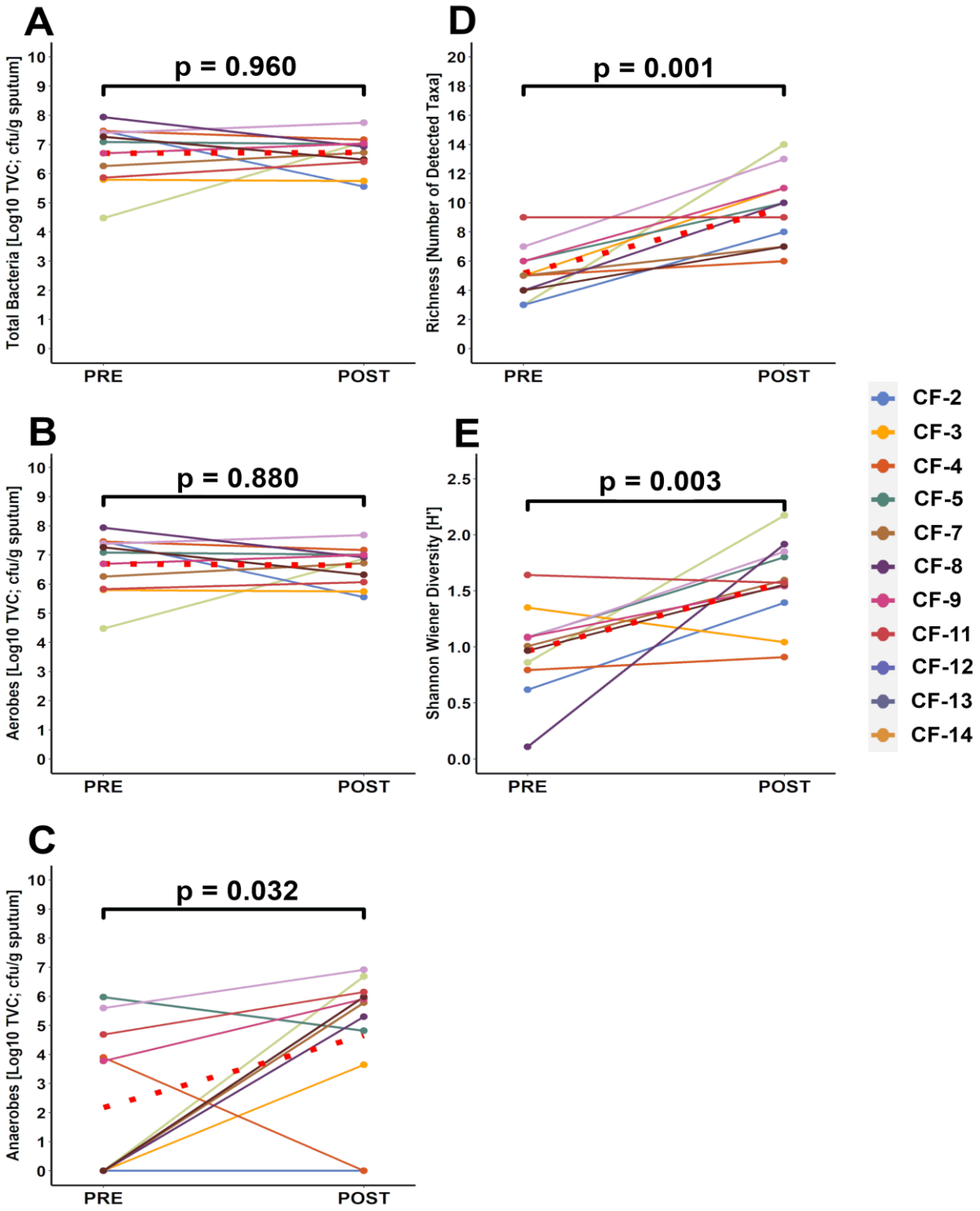

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418 **Fig 1.** Comparison of sputum viable counts and ecological parameters by extended-bacterial
419 culture in sputum samples from PWCF PRE- and POST-treatment with ivacaftor. (A) total
420 bacterial load (B) total bacterial load for aerobic bacteria (C) total bacterial load for anaerobic
421 bacteria (D) taxonomic richness and (E) community diversity (Shannon Wiener Index [H']).
422 Red dotted line demonstrates the change in mean values. P<0.05 denotes statistical
423 significance.

424

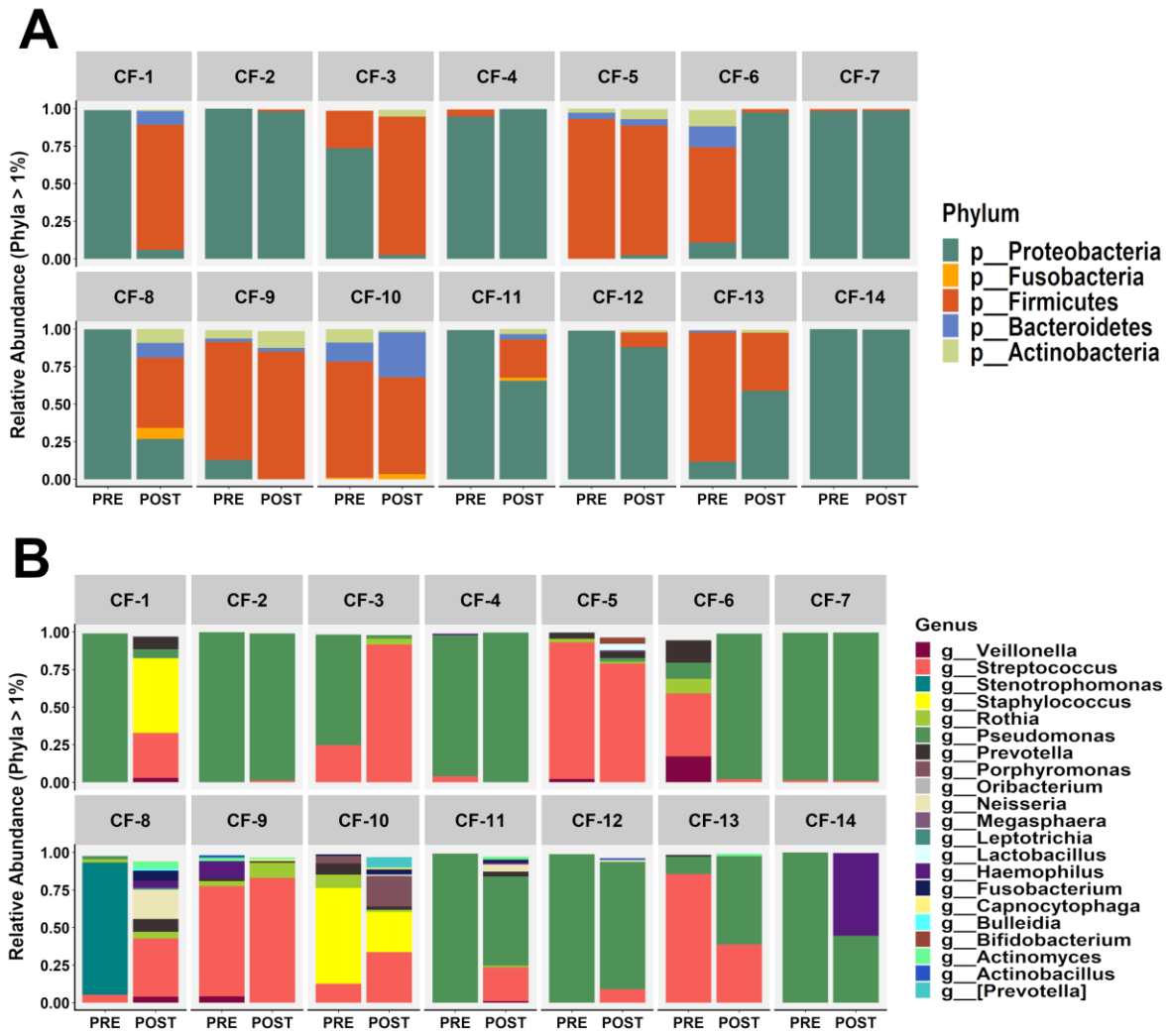

Fig 2. Mean relative abundance (%) of (A) top 5 phyla and (B) all genera in sputum samples from PWCF PRE- and POST-treatment with ivacaftor. Values shown depict percentage relative abundance >1% of the total bacteria detected.

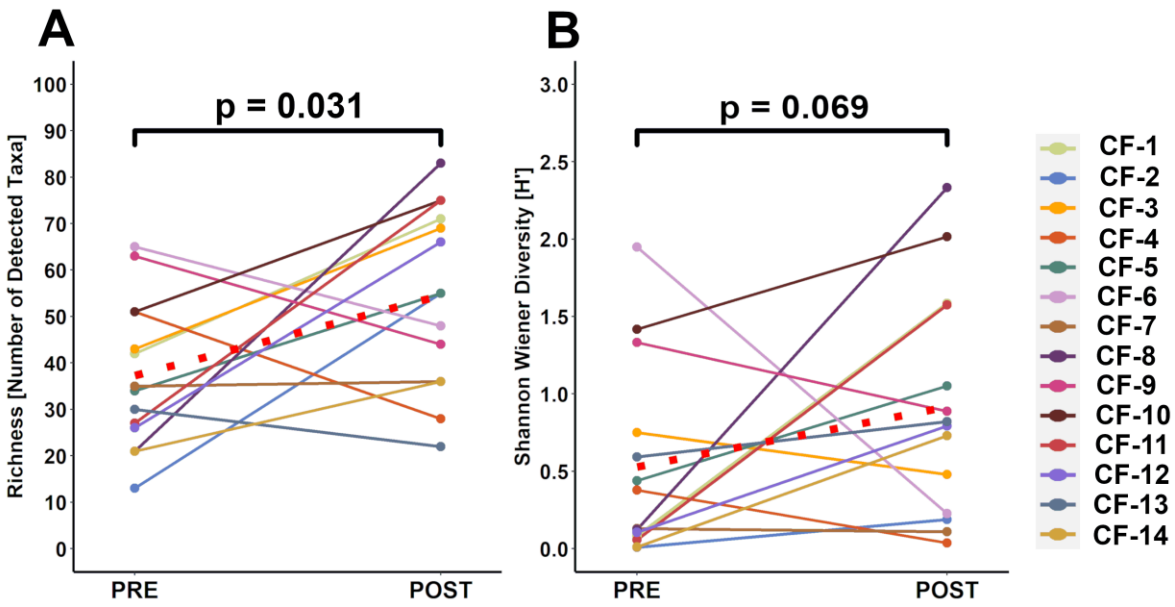

Fig 3. Comparison of ecological parameters by molecular based analysis in sputum samples from PWCF PRE- and POST-treatment with ivacaftor. (A) taxonomic richness (B) community diversity (Shannon Wiener Diversity [H']). Red dotted line demonstrates the change in mean values. P<0.05 denotes statistical significance.

Online Supplement

Extended-culture and culture-independent molecular analysis of the airway microbiota in cystic fibrosis following CFTR modulation with ivacaftor.

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Methods

Extended-quantitative culture

Expectorated sputum samples were collected into a sterile specimen cup, snap frozen and immediately stored at -80°C until being shipped on dry-ice to the Halo Research Group laboratory at Queen's University Belfast were samples were processed according to Standard Operating Procedures. Specimens were transferred to an anaerobic workstation (Don Whitley Scientific, UK) prior to loosening of the lid of the specimen cup. In the anaerobic cabinet, a sterile pipette was used to transfer ~0.5 gram of the sputum sample to a sterile 15 ml centrifuge tube. The sputum samples were homogenized and liquefied by the addition of 10% Sputolysin

(Calbiochem, USA) in a ratio of 1:1 (v/v). The sputum/sputolysin mixture was thoroughly mixed by vortexing for 30 seconds, incubated for 15 minutes at 37°C, under anaerobic conditions, before being vortexed for a further 30 seconds. Next, the sputum/sputolysin mixture was diluted 1:10 in 900 µl of QSRS and serially diluted, 1:10, to a 10^{-5} dilution in QSRS in micro-tubes. An aliquot (100 µl) of each dilution was inoculated onto anaerobic blood agar (ABA), kanamycin-vancomycin laked blood agar (KVLB) and blood chocolate agar (BCA) containing bacitracin and incubated aerobically, anaerobically (10% hydrogen, 10% carbon dioxide and 80% nitrogen) or in 5% CO_2 at 37ºC for 2-5 days. The total viable count (colony forming units per gram of sputum) of all distinct colony morphologies was performed for enumeration of bacteria. Genomic DNA (gDNA) was extracted from bacteria that had been freshly grown on agar plates. For aerobes, DNA was isolated using a DNeasy Blood and Tissue kit (Qiagen, DE) as per manufacturer's instructions. For anaerobes, DNA was isolated using a ZR Fecal DNA MiniPrep kit (Zymo Research, USA) as per manufacturer's instructions. The final concentration of gDNA obtained was determined spectrophotometrically (Absorbance at 260/280nm).

To identify the genera of the cultured taxa, an initial amplification of the 16S rRNA marker-gene was performed using the 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) primer pair [24] resulting in a near full-length 16S rRNA product. For each reaction, 1 µL of genomic DNA template was added to the reaction mixture (25 µL, final volume) containing 12.5 µL of MyTaq™ Red x2 master-mix (Bioline, UK), 11 µL of DEPC treated water (Ambion, USA) and 0.5 µL of each primer (10 µM stock). The amplification step was performed on the Veriti 96-well thermal cycler (Applied Biosystems, USA) and included an initial denaturation step at 95 °C for 3 minutes, followed by 30 cycles of denaturation at 95 °C for 20 seconds, annealing at 50 °C for 15 seconds, elongation at 72 °C for 60 seconds, and a final elongation step at 72 °C for 5 minutes. Following amplification, the resulting product was separated by electrophoresis on a 1.5% agarose gel (Invitrogen, Life

Technologies, USA) and visualised under short-wavelength UV light to ensure the appropriate amplification specificity of the 16S rRNA gene. For 16S rRNA gene sequencing, an additional primer, 926R (5′-CCGTCAATTCCTTTRAGTTT-3), was used [25]. Resulting sequences were quality checked and the MUSCLE algorithm [26] was used to assemble overlapping amplicons into a single contig spanning near full length 16S rRNA gene. Contigs were compared to the NCBI reference genome database (https://www.ncbi.nlm.nih.gov/refseq/) using the Blastn algorithm [27], and also to reference sequences contained within the Ribosomal Database Project (RDP) using the RDP Classifier algorithm [28] enabling relevant taxonomic classification of each cultured bacterium.

Generation and processing of 16S rRNA amplicon sequences for microbiome analysis

Sputum samples extraction of gDNA was performed on the MagNA Pure 96 instrument (Roche Diagnostics Ltd., UK) as follows. A volume of 1.8ml of sterile Phosphate Buffered Saline (PBS) was added to the corresponding sample tube of pre-aliquoted Sputolysin stock to obtain a 10% Sputolysin solution. Library preparation for Illumina MiSeq sequencing of the 16S rRNA marker-gene was preformed as follows according to previously published protocol by Lundberg et al. [29]:

PCR 1: Pre-amplification of 16S rRNA marker gene region is necessary for potentially low biomass template in order to carry enough tagged amplicon through to the final indexing-amplication steps. Perform PCR using ~200 ng of gDNA from each sample. Using non-modified primers targeting positions 515F and 806R within the V4 region of the 16S rRNA marker gene prepare a mastermix solution [5µl 5x Phusion Hifi Buffer, 0.5µl (10 mM) dNTP, 1µl (10µM) V4 primer mix; 0.25µl Phusion HS II polymerase and make to 25µl per reaction using DEPC water] and amplify using the following condition: 98°C for 30 sec (x1) →98°C for 10 sec + 52°C for 30 sec + 72°C for 20 sec (10 cycles)

→72°C for 5 min →hold at 4°C for ∞. Next clean-up the PCR products from PCR 1 using AxyPrep Mag PCR Clean-up kit as follows; vortex magnetic beads well before use to resuspend any magnetic beads that may have settled. Aliquot 15µl of Axygen beads to 10µl of PCR product into a sterile 96 well plate. Mix well by repeated pipetting and incubate at R°T for 5 min. Place the reaction plate onto the IMAG separation device and wait until the liquid goes clear. Remove the clear liquid from the plate and discard. Next add 180µl of 70% EtOH to each well of the reaction plate and incubate for 30 sec at R°T. Remove the 70% EtOH from each well and discard. Repeat the previous step once. Air dry the beads at R°T for no more than 5 min and be careful not to over dry the magnetic beads as this will cause the beads to crack and lead to decreased elution efficiency. Ensure that all the ethanol has been removed from each well. Add 11µl of molecular grade H_2O to each well. Remove reaction plate from the IMAG separation device and mix well by gentle vortexing. Place the reaction plate onto the IMAG separation device for 1 minute to separate the beads from the solution. Transfer 10µl of the cleaned up PCR product to a sterile 96 well plate for the next PCR step.

PCR 2: Reverse Tagging Step using the cleaned product from PCR 1 using equimolar mixture of the reverse frame-shift (FS) primers 808R_f1, 808R_f2, 808R_f3, 808R_f4, 808R_f5, 808R_f6). Primers are combined into a working stock of 0.5 µM. Perform 1 cycle PCR using 10µl of product from PCR 1. Prepare a mastermix solution [5µl 5x Phusion Hifi Buffer, 0.5µl (10 mM) dNTP, 2µl (0.5µM, Reverse_MT_tag Primer mix); 0.25µl Phusion HS II polymerase and 7.25µl DEPC water] and amplify using the following condition: 98°C for 60 sec (x1) →98°C for 10 sec + 50°C for 30 sec + 72°C for 60 sec (1 cycle) → hold at 4°C for ∞. Next clean-up the PCR products from PCR 1

using AxyPrep Mag PCR Clean-up kit as follows; vortex magnetic beads well before use to resuspend any magnetic beads that may have settled. Aliquot 15µl of Axygen beads to 10µl of PCR product into a sterile 96 well plate. Mix well by repeated pipetting and incubate at R°T for 5 min. Place the reaction plate onto the IMAG separation device and wait until the liquid goes clear. Remove the clear liquid from the plate and discard. Next add 180µl of 70% EtOH to each well of the reaction plate and incubate for 30 sec at R°T. Remove the 70% EtOH from each well and discard. Repeat the previous step once. Air dry the beads at R°T for no more than 5 min and be careful not to over dry the magnetic beads as this will cause the beads to crack and lead to decreased elution efficiency. Ensure that all the ethanol has been removed from each well. Add 11µl of DECP water to each well. Remove reaction plate from the IMAG separation device and mix well by gentle vortexing. Place the reaction plate onto the IMAG separation device for 1 minute to separate the beads from the solution. Transfer 10µl of the cleaned up PCR product to a sterile 96 well plate for the next PCR step.

PCR 3: Forward-Tagging Step using the cleaned product from PCR 2 using equimolar mixture of the forward frame-shift (FS) primers 515F_f1, 515F_f2, 515F_f3, 515F_f4, 515F_f5, 515F_f6). Primers are combined into a working stock of 0.5 µM. Perform 1 cycle PCR using 10µl of product from PCR 2. Prepare a mastermix solution [5µl 5x Phusion Hifi Buffer, 0.5µl (10 mM) dNTP, 2µl (0.5µM, Reverse_MT_tag Primer mix); 0.25µl Phusion HS II polymerase and 7.25µl DEPC water] and amplify using the following condition: 98°C for 60 sec (x1) →98°C for 10 sec + 50°C for 30 sec + 72°C for 60 sec (1 cycle) → hold at 4°C for ∞. Next clean-up the PCR products from PCR 3 using AxyPrep Mag PCR Clean-up kit as follows; vortex magnetic beads well before use to resuspend any magnetic beads that may have settled. Aliquot 17.5µl of Axygen

beads to 10µl of PCR product into a sterile 96 well plate. Mix well and incubate at R°T for 5 min. Next place the reaction plate onto the IMAG separation device and wait until the liquid goes clear. Remove the clear liquid from the plate and discard. Add 180µl of 70% EtOH to each well of the reaction plate and incubate for 30 sec at R°T. Remove the 70% EtOH from each well and discard. Repeat previous step once. Air dry the beads at R°T for no more than 5 min, be careful not to over dry the magnetic beads as this will cause the beads to crack and lead to decreased elution efficiency. Ensure that all the ethanol has been removed from each well. Add 16µl of DEPC water to each well and remove the reaction plate from the IMAG separation device and mix well to resuspend the magnetic beads. Next place the reaction plate onto the IMAG separation device for 1 minute to separate the beads from the solution. Transfer 15µl of the cleaned up PCR product to a sterile 96 well plate for the next PCR step.

PCR 4: Nextera-Adapter/Indexing Amplification step by performing a 34 cycle PCR, targeting the V4 region of the 16S rRNA marker gene, using 15µl of the cleaned reverse and forward tagged product from step PCR 3. Each reaction will have the same forward primers and a unique reverse primer which acts as the index (barcode) for each sample. The forward and reverse primers are typically diluted to a working stock of 5µM and can be added separately to each reaction (the forward primer is universal and could be added to any master-mixes instead), or the forward primer can be added to each reverse primer in a working stock in a plate for further use. Prepare a mastermix solution [10µl 5x Phusion Hifi Buffer, 1µl (10 mM) dNTP, 2.5µl forward primer (SEQ_V4_F; AATGATACGGCGACCACCGAGATCTACACGCCTCCCTCGCGCCATCAGAGATG TG); 2.5µl reverse primer (INDEX_R_bc1 to bc96;

CAAGCAGAAGACGGCATACGAGAT XXXXXXXX GTGACTGGAGTTCAGACGTGTGCTC); 0.5µl Phusion HS II polymerase and 7.25µl DEPC water] and amplify using the following condition: 98°C for 30 sec (x1) →98°C for 10 sec + 63°C for 30 sec + 72°C for 30 sec (34 cycle) →hold at 4°C for ∞. Next run 5µl of each reaction on a 1% agarose gel to visually confirm presence of products (~453bp). Clean the PCR products from step PCR 4 with AxyPrep Mag PCR Clean-up kit as follows; vortex magnetic beads well before use to resuspend any magnetic beads that may have settled. Aliquot 35µl of Axygen beads and the entire PCR product into a sterile 96 well plate and mix well and incubate at R°T for 5 min. Next place the reaction plate onto the IMAG separation device and wait until the liquid goes clear. Remove the clear liquid from the plate and discard. Add 180µl of 70% EtOH to each well of the reaction plate and incubate for 30 sec at R°T. Remove the 70% EtOH from each well and discard. Repeat the previous step once. Air dry the beads at R°T for no more than 5 min, avoiding to not over drying the magnetic beads as this will cause the beads to crack and lead to decreased elution efficiency. Next add 50µl of DEPC water to each well and remove reaction plate from the IMAG separation device and mix well. Place the reaction plate back onto the IMAG separation device for 1 minute to separate the beads from the solution. Transfer all of the cleaned up PCR product to a sterile 96 well plate. Next quantify products using Quant-iT™ PicoGreen® dsDNA Assay kit (Life Technologies, UK) in a 96 well plate using 2µl of cleaned product. Pool equimolar amounts from each sample adding no more than 20µl of each reaction to the final pool. Typically, only reactions that failed will need to be added at this volume (the pool will not be equimolar for them). Gel purify the pool by running it on a 1% agarose gel and gel extracting the correct size band (~453bp) using the QIAEX II kit (Qiagen ,UK) according to manufactures instructions, removing as much of the excess agarose gel

as possible. The final sample pool was quantified in triplicate using Quant-iT™ PicoGreen® dsDNA Assay kit (Life Technologies, UK) according to manufacturer's instructions. Samples were stored at -20°C/-80°C until submission for Illumina MiSeq 16S rRNA marker-gene sequencing.

Culture-independent analysis

Samples were joined together and de-multiplexed according to unique barcode sequences using QIIME 1.9.1 [30] pipeline. PhiX internal sequencing control was removed by aligning all sequences against the PhiX genome [31] using the bbduk.sh shell script from the BBTools package (available at https://jgi.doe.gov/data-and-tools/bbtools) where unaligned reads were retained. Sequences were clustered into their representative Operational taxonomic units (OTUs) at 97% sequence identity using the UCLUST algorithm [32] in a *de novo* reference style. A representative sequence from each OTU was chosen based upon abundance within that OTU and taxonomy was assigned using the RDP naïve Bayesian classifier[33] against the QIIME compatible Greengenes 13.8 database [34, 35]. The resulting OTU table (.biom) was converted to a tab-spaced text file to assess the prevalence of OTUs within sputum samples (n=28), positive- (n=4) and negative-controls (n=4), which were included throughout both DNA extraction and library preparation procedures. We compared OTUs occurring in the background of the negative controls to those observed in the clinical samples and subsequently filtered any OTUs that were considered to be contaminants from the dataset prior to further analysis. A number of OTUs were detected in the background of the negative controls; with OTUs accounting for over 90% (OTUs >0.5%) of reads in the four negative controls displayed a low contribution in the clinical samples accounting for 0-0.008% of the total read number. Within the negative controls the most abundant OTUs belonged to taxa such as members of family Comamonadaceae Unclassified, family Oxalobacteraceae Unclassified, family Methylophilaceae Unclassified, *Dechloromonas* spp., *Ralstonia* spp., *Sediminibacterium* spp., family Bradyrhizobiaceae Unclassified, order Elusimicrobiales Unclassified and family Xanthomonadaceae Unclassified. Conversely, the main taxa in clinical samples belonged to members most often associated microbiota observed in the airways of

PWCF, such as *Pseudomonas* spp., *Streptococcus* spp., *Staphylococcus* spp., *Haemophilus* spp., *Porphyromonas* spp., *Prevotella* spp. and *Fusobacterium* spp. Those accounted for over 90% of total read number within clinical samples (range 0.5-58%) compared to 0.5% (range 0-0.4%) in the background of the negative-controls. Therefore, taxa that were most common in the clinical samples were retained for analysis as their presence in the clinical samples was not estimated to be significantly affected by background contamination. Furthermore, OTUs representing potential human sequences, Archaea, Cyanobacteria and unassigned OTUs were filtered out and treated as contaminating sequences prior to downstream analysis. The full list of the unrarefied count data for clinical samples, positive- and negative-controls is shown in Table S4.

Statistical analysis and ecological community measurements

A single R object was created from the .biom formatted OTU table, containing representative sequences and associated clinical metadata using phyloseq [36] for subsequent analysis in R (ver. 3.5.2). Calculations of ecological indices was performed using PAST3 (https://folk.uio.no/ohammer/past/). All statistical analysis was performed in R using the packages phyloseq (ver. 1.26.1), vegan (ver. 2.5-4) [37], dplyr (ver. 0.8.4) [38], ggplot2 (ver. 3.3.0) [39], Hmisc (ver. 4.2-0) [40], reshape (ver. 0.8.8) [41] and rmcorr (ver. 0.3.0) [42]. Furthermore, OTUs representing potential human sequences, Archaea, Cyanobacteria and unassigned OTUs were filtered out and treated as contaminating sequences prior to downstream analysis and all samples were randomly sub-sampled to 58,391 reads for diversity and dissimilarity calculations.

Alpha-diversity (within group) indices, such as community richness (S), diversity (Shannon-Wiener index; H'), evenness ($e^{H/S}$) and dominance (D) were compared between groups using the Wilcoxon-Rank sum test (2 groups). Beta-diversity (between groups) was assessed using distance-based metrics (Euclidean-distance) on centered log-ratio (CLR) transformed count

data and presented as a principle coordinates plot (PCoA) showing variance explained for the first two components. Differences between groups were evaluated by multivariate-permutational analysis (PERMANOVA). Correlation between variables was made by calculating the spearman correlation coefficient, both before and after adjusting for repeated measures for PRE- and POST-ivacaftor samples. Where appropriate, p-values were adjusted for multiple testing using the Benjamini-Hochberg (BH) method for false-discovery rate.

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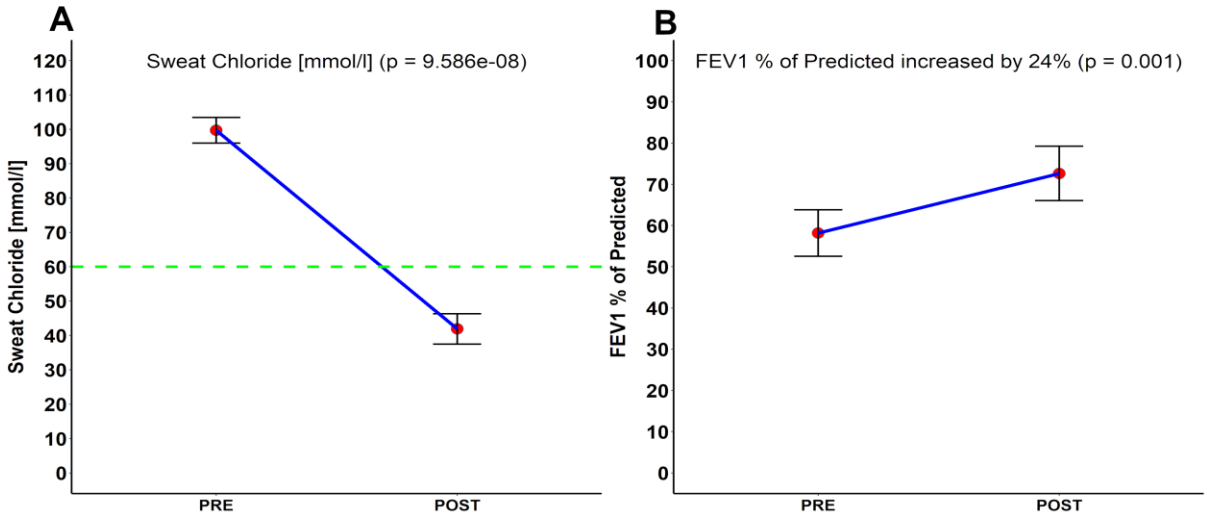
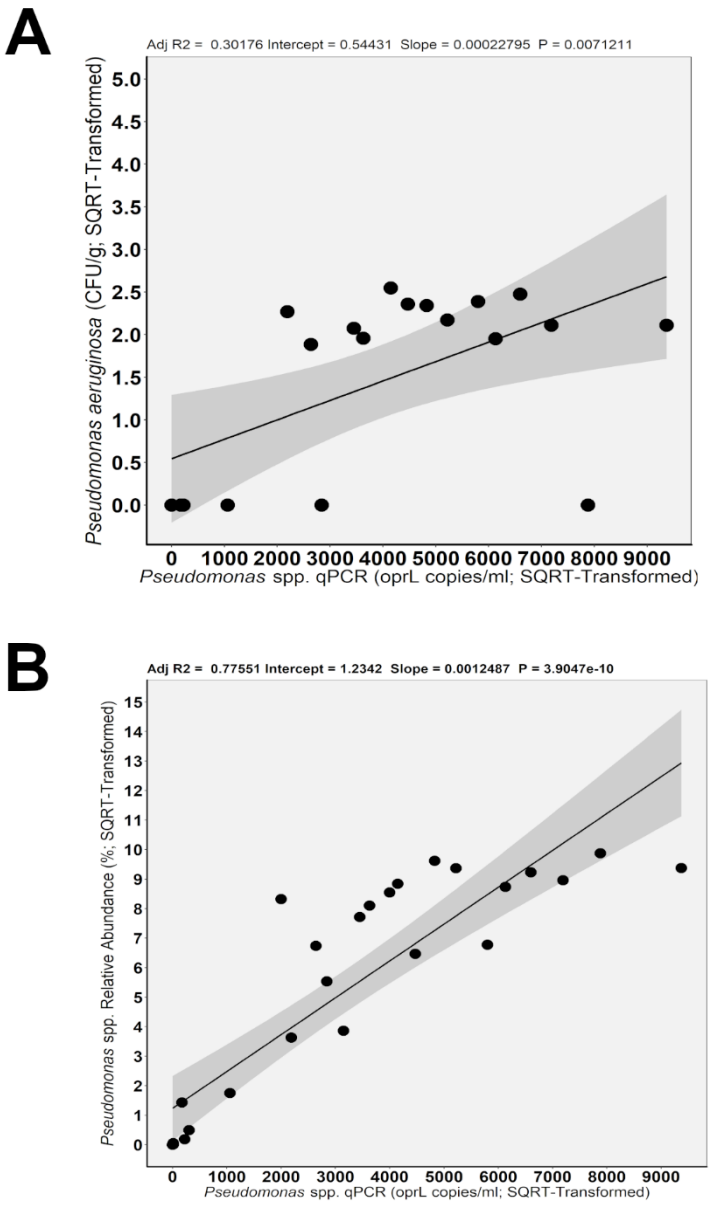


Fig S1. Changes in (A) sweat chloride levels and (B) lung function following ivacaftor treatment. Red dots demonstrate mean levels and whiskers standard deviation around the mean. The green dotted line displays the threshold of 60 mmol/l which is indicative of cystic fibrosis diagnosis. Wilcoxon signed-rank test, P<0.05 denotes statistical significance**.**



816

817 **Fig S2.** Pearson's correlation coefficient demonstrating the relationship between (A) the total
818 viable counts (CFU/g sputum) of *Pseudomonas aeruginosa* (from culture-dependent analysis)
819 with *P. aeruginosa opr*L copy number (copies/ml) as quantified by qPCR. Pearson's
820 correlation coefficient r = 0.556 (R^2 = 0.301; p = 0.007) and (B) between the relative abundance
821 (%) of *Pseudomonas* spp. (from Illumina MiSeq 16S rRNA marker-gene sequencing) with *P.*
822 *aeruginosa opr*L copy number (copies/ml) as quantified by qPCR. Pearson's correlation
823 coefficient r = 0.885 (R^2 = 0.776; p = $3.91*10^{-10}$).

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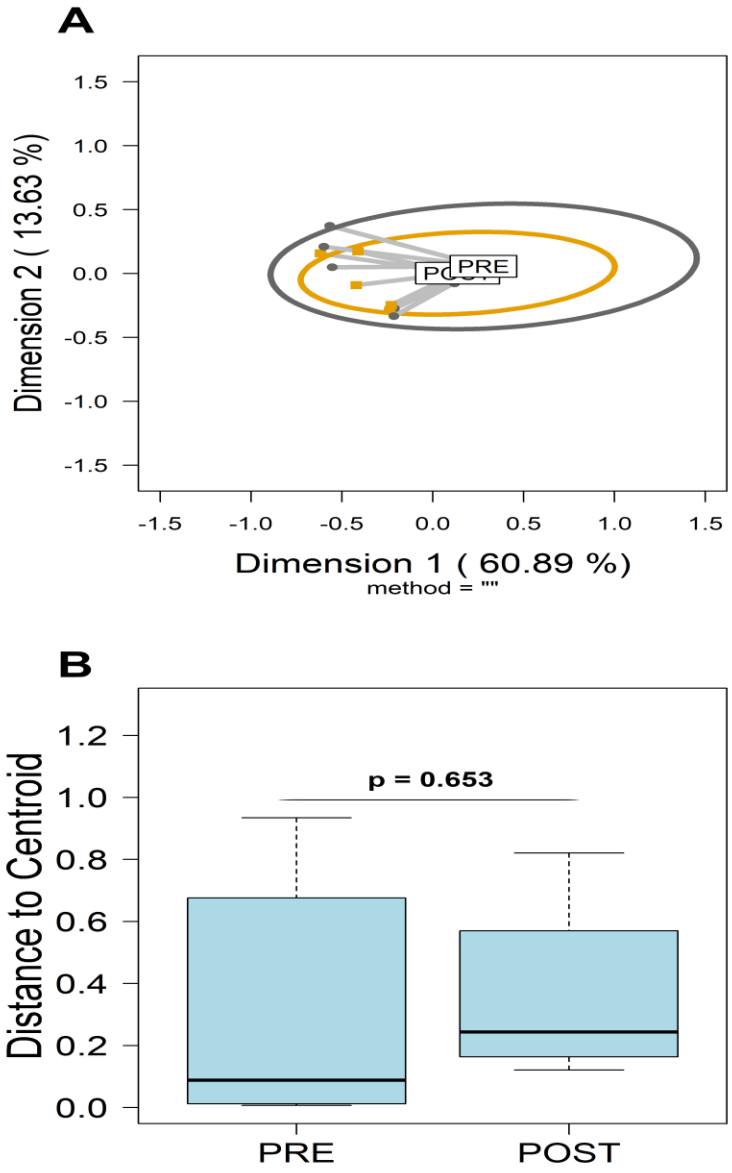

826

827 **Fig S3.** Differences in the compositional variance calculated using the Bray-Curtis distance
828 measure and the "betadisper" function from the vegan package (2.4-6) in R, followed by
829 significance testing using a permutation test. The permutation tests assess significant
830 differences of median distance to centroid. PRE- and POST-ivacaftor visits: (A) distances to
831 the centroids on the first two Principle Co-ordinates Analysis (PCoA) axes (90% confidence
832 interval) and (B) distribution of variance. *P*<0.05 denotes statistical significance with 999
833 permutations.

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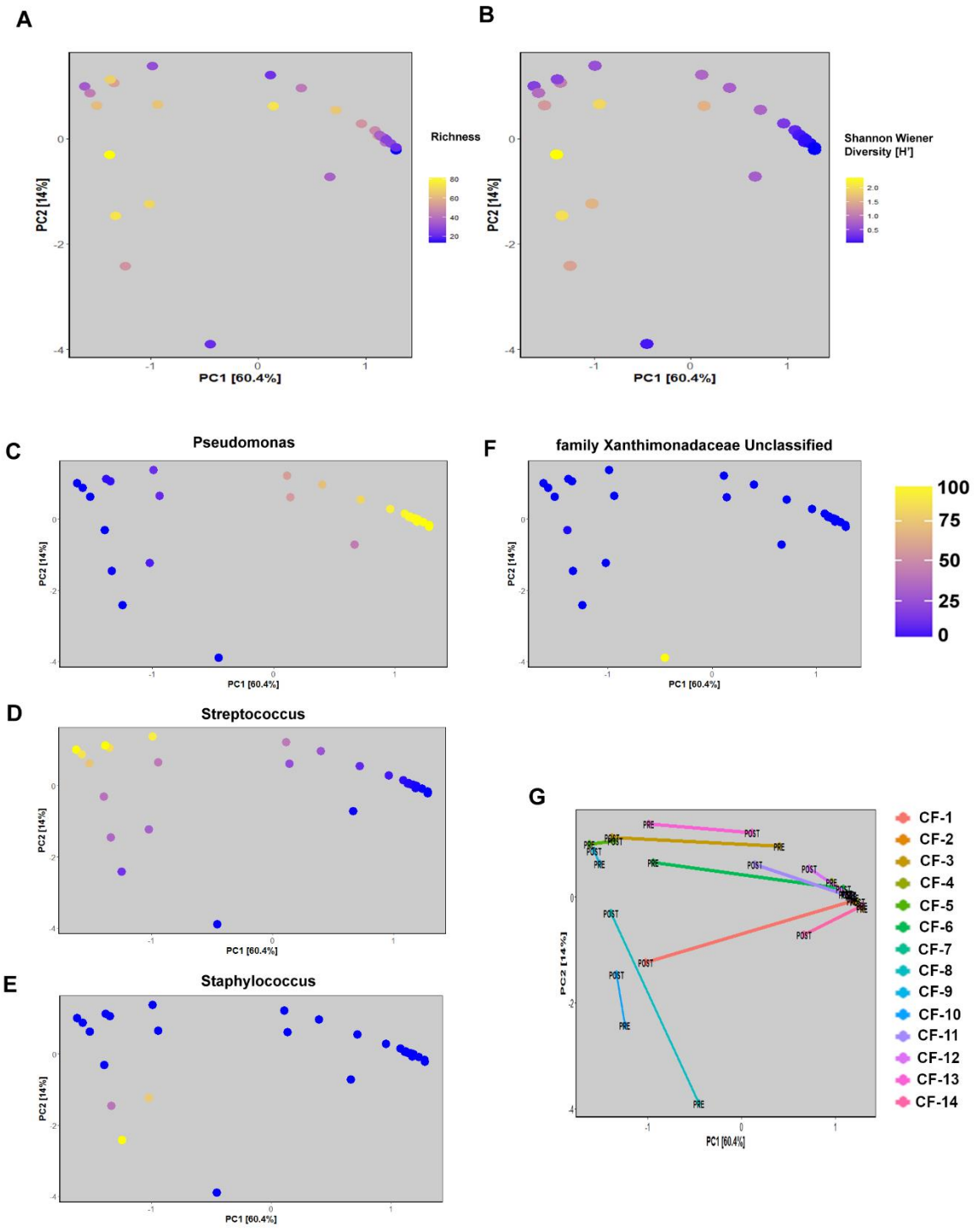

836

837 **Fig S4.** Principal Component Analysis (PCA) for the two main ecological community
838 estimators: (A) Richness and (B) Shannon Wiener Diversity [H']. PCA analysis for the four
839 main genera based on relative abundance (% normalised counts): (C) *Pseudomonas* spp.,
840 (D) *Streptococcus* spp., (E) *Staphylococcus* spp. and (F) unclassified *Xanthomonadaceae.*
841 *(G) Direction of community changes from PRE- to POST-ivacaftor treatment.* PCA axis 1
842 accounts for 60.4% of explained variation and the PCA axis 2 accounts for 14.0% of the
843 variation explained.

844

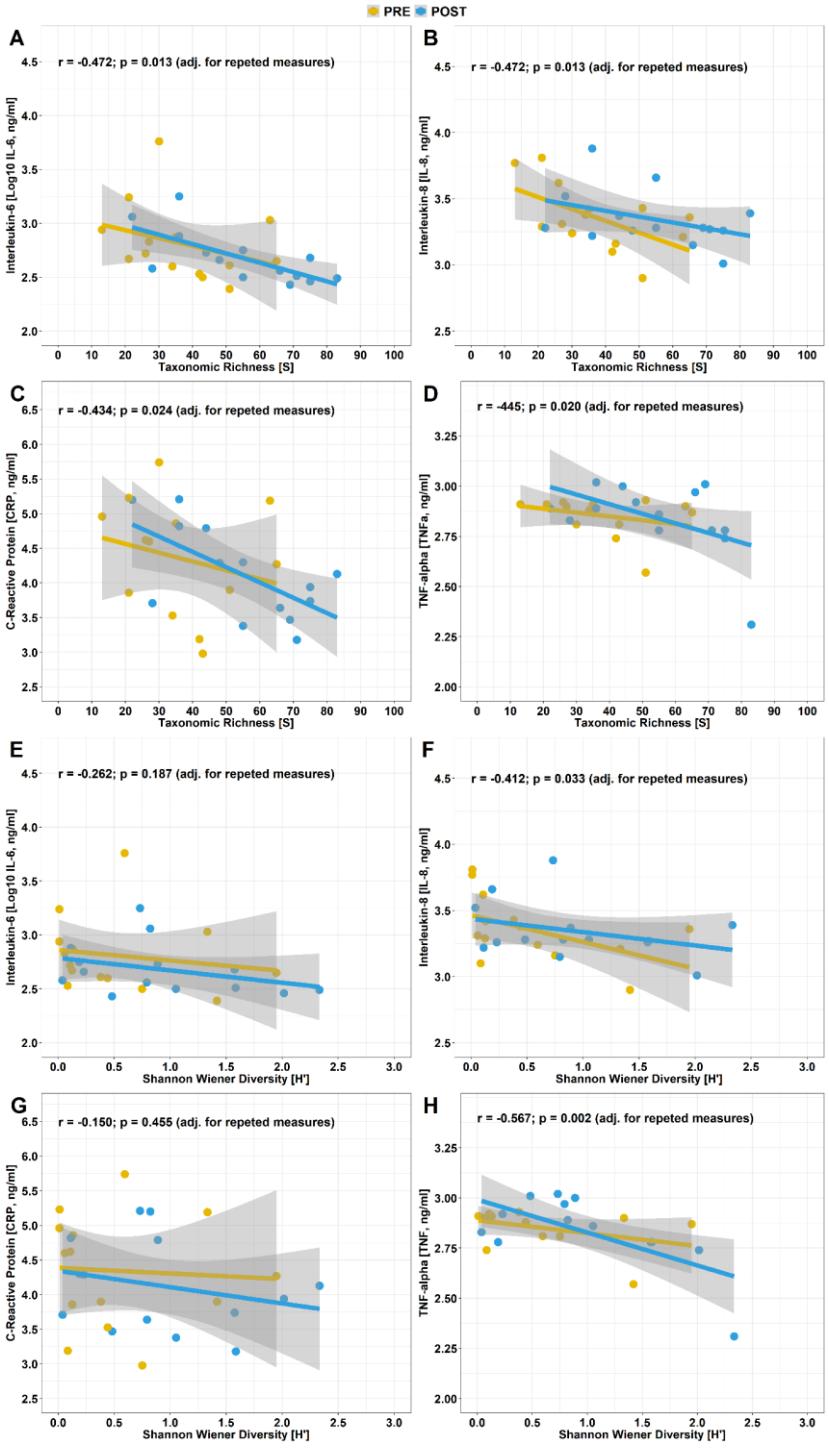

Fig S5. Correlation (r) between taxonomic richness (A-D) and Shannon–Wiener diversity [H'] (E-H) and markers of inflammation (IL-6, IL-8, C-Reactive Protein [CRP] and TNF-α) within the whole meta-community (n=14). Correlation was adjusted for repeated measures (r) taking into account the matched PRE- and POST-ivacaftor samples from the same individual using the rmcorr package (0.3.0) in R. Line indicates linear relationship between variables and the Spearman's correlation coefficient (r); P<0.05 denotes statistical significance; shaded area denotes 95% confidence limits.

Table S2. Number of course of oral (PO) and intravenous (IV) antibiotics in the year before and after ivacaftor.

Patient	1 year before	1 year after
CF-1	2 PO 0 IV	0 PO 0 IV
CF-2	2 IV 6 PO	0 IV 4 PO
CF-3	0 IV 2 PO	0 IV 0 PO
CF-4	0 IV 2 PO	0 IV 2 PO
CF-5	0 IV 0 PO	0 IV 1 PO
CF-6	0 IV 2 PO	0 IV 0 PO
CF-7	5 IV 2 PO	1 IV 2 PO
CF-8	1 IV 0 PO	0 IV 0 PO
CF-9	0 IV 4 PO	0 IV 0 PO
CF-10*	2 IV 1 PO	1 IV 2 PO
CF-11	0 IV 2 PO	0 IV 1 PO
CF-12	1 IV 3 PO	0 IV 0 PO
CF-13*	1 IV 2 PO	2 IV 2 PO
CF-14	1 IV 2 PO	0 IV 0 PO

***patients received antibiotics within 8 weeks of post ivacaftor sputum sample