Assessment of stability and fluctuations of cultured lower airway bacterial communities in people with cystic fibrosis

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ABSTRACT

Background: Routine clinical culture detects a subset of the cystic fibrosis (CF) airways microbiota based on culture-independent (molecular) methods. This study aimed to determine how extended sputum culture of viable bacteria changes over time in relation to clinical status and predicts exacerbations.
Methods: Sputa from patients at a baseline stable and up to three subsequent time-points were analysed by extended-quantitative culture; aerobe/anaerobe densities, ecological indexes and community structure were assessed together with clinical outcomes.
Results: Eighty patients were prospectively recruited. Sputa were successfully collected and cultured at 199/267 (74.5%) study visits. Eighty-two sputa from 25 patients comprised a complete sample-set for longitudinal analyses. Bacterial density, ecological indexes and clinical outcomes were unchanged in 18 patients with three sequential stable visits. Conversely, in 7 patients who had an exacerbation, total bacterial and aerobe densities differed over four study visits ($P < .001$) with this difference particularly apparent between the baseline visit and completion of acute antibiotic treatment where a decrease in density was observed. Bacterial communities were more similar within than between patients but stable patients had the least variation in community structure over time. Using logistic regression in a further analysis, baseline features in 37 patients without compared to 15 patients with a subsequent exacerbation showed that clinical measures rather than bacterial density or ecological indexes were independent predictors of an exacerbation.
Conclusions: Greater fluctuation in the viable bacterial community during treatment of an exacerbation than between stable visits was observed. Extended-quantitative culture did not provide prognostic information of a future exacerbation.

1. Introduction

Chronic infection of the lower airways is the major cause of morbidity and reduced survival in people with cystic fibrosis (CF) [1]. Clinical microbiological laboratories routinely determine the presence of recognised CF pathogens in respiratory secretions using a combination of nonselective and selective agars [2]. Information from routine culture is used by physicians to guide treatment decisions. However, studies using culture-independent

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analyses, which provide more in-depth analysis of the CF airway bacterial community composition and structure, have shown that reduced community diversity and increased dominance by a recognised pathogen, is associated with increasing age, greater antibiotic use, lower lung function and disease progression [3–5]. Both clinical microbiological and culture-independent techniques have been used to determine whether changes in the bacterial density or community underpin the aetiology of pulmonary exacerbations or are associated with their onset or future risk [6–8].

Not all bacteria, especially so-called "commensal" respiratory flora, are cultured using standard clinical culture protocols. In contrast, culture-independent studies may include genomic DNA from non-viable cells, which can impact analyses of the bacterial community. Extended-quantitative culture is an alternative method to assess respiratory secretions, similar in methodology to routine culture, but more comprehensively targeting the growth and quantity of bacteria, including facultative and obligate anaerobes. This method, therefore, can be used to assess the bacterial community analogous to culture-independent methods from an ecological perspective but with the added benefit of only detecting viable bacteria [9–12].

In this study, extended-quantitative culture of prospectively collected sputum was used to investigate the lower airway bacterial community of people with CF, an approach in which the abundance of individual cultured taxa is assessed as a proportion of the total number of colony-forming units obtained for each sputum sample. We hypothesised that extended-quantitative culture could (1) demonstrate conserved bacterial density and communities in clinically stable disease; (2) detect temporal changes in bacterial density and communities during a pulmonary exacerbation; and (3) predict a future pulmonary exacerbation.

2. Methods

2.1. Participants

Patients diagnosed with CF [13] were recruited during routine outpatient appointments at the paediatric and adult CF Centres of the Belfast Health and Social Care Trust (Royal Belfast Hospital for Sick Children and Belfast City Hospital). Written informed consent/assent was obtained. The study was approved by the Office for Research Ethics Committees Northern Ireland (10/NIR01/41) and co-sponsored by the Belfast Health and Social Care Trust and Queen's University Belfast (10067SE-OPMS). Cross-sectional studies involving patients recruited at study enrolment are published [12,14–16].

2.2. Study visits and target sample collection

Spontaneously expectorated sputum was collected at study enrolment ('Baseline') when patients were clinically stable which was defined as no requirement for intravenous or additional inhaled/oral antibiotics for respiratory symptoms in the prior 4-weeks. Up to two further consecutive stable samples ('S2' and 'S3') were collected from patients who remained clinically stable (no requirement for intravenous antibiotics) for the study duration when they attended outpatient appointments. For patients who subsequently presented with an exacerbation [17], sputum was collected at initiation of ('PEx1'; 24 h before to a maximum of 48 h after the first dose of intravenous antibiotics) and at completion of ('PEx2'; 24 h before to a maximum of 48 h after last dose of intravenous antibiotics) treatment [9]. Another sample was collected post-PEx2 when the patient had recovered and was clinically stable at outpatient review ('Follow-up').

2.3. Extended-quantitative culture

Comprehensive details are provided in the supplementary file. Briefly, samples were immediately placed into an anaerobic pouch (AnaeroGen^{TM} COMPACT, Oxoid Limited, Hampshire, UK) and processed within an anaerobic cabinet. Quantitative culture was performed and the total viable count (TVC; colony forming units per gram of sputum [CFU/g]) of all distinct colony morphologies were enumerated and identified to the genus-level using near full-length 16S rRNA sequencing.

2.4. Clinical characteristics

Age, gender, body mass index (BMI), spirometry with percent predicted calculated using reference ranges for all ages [18,19], co-morbidities and qualitative routine culture results were obtained. No patients had a history of non-tuberculous mycobacteria (NTM) infection at enrolment to the study nor became NTM-positive during the study period. Treatments received in the previous month or additional medications to treat an exacerbation were recorded. No patients received cystic fibrosis transmembrane conductance regulator (CFTR) modulators during the study period. An age-appropriate version of the Cystic Fibrosis Questionnaire-Revised (CFQ-R) was used to report outcomes at Baseline [20]. CFTR function was recorded from the genotype [21].

2.5. Analyses

Analyses were generated using IBM SPSS (v22) and the R environment (http://www.r-project.org). $P < .05$ was considered statistically significant. Ecological indexes commonly used to characterise community composition and structure in various natural environments, including human disease, were calculated: richness (number of counted taxa); dominance; evenness; community diversity (Shannon-Wiener index [H′] combining community richness and evenness). Bacterial community structures were compared using the Bray-Curtis quantitative index of dissimilarity. Continuous variables were analysed using a Student's *t*-test, Mann-Whitney *U* test, repeated measures analysis of variance (ANOVA) or Friedman's test. Categorical variables were compared using Pearson's chi-square/Fisher's exact test. Logistic regression was performed to identify predictors of a future pulmonary exacerbation within 4-months of Baseline [22]. This time-frame reflects the median time between outpatient appointments, when clinically stable. Further details are provided in the supplementary file.

3. Results

3.1. Study overview: visits and patients

A total of 267 study visits occurred in 80 patients. Sputa ($n = 199$; 74.5% of study visits) were successfully collected and cultured at least once from all patients. For the remaining study visits ($n = 68$), there was an insufficient volume of sputum for extended-quantitative culture ($n = 53$) or the patient was unable to expectorate ($n = 15$). The majority of these visits ($n = 49/68$; 72.1%) were when patients were clinically stable.

Characteristics of children (6–18 years, $n = 10$) and adults ($\geq$18 years, $n = 70$) at study enrolment are provided in Table 1. Fig. 1 shows how patients were selected to address the three study hypotheses. Thirty-five patients were treated for $\geq$1 exacerbation. Intravenous antibiotics were administered to all patients with additional oral antibiotics prescribed to treat nine exacerbations. Dual intravenous antibiotic therapy with tobramycin

Table 1
Overview of characteristics of children and adults at study enrolment.

Variable	6–18 years ($n=10$)	≥18 years ($n=70$)	*P*-value
Age, mean (±sd)	13.2 (2.9)	31.9 (12.4)	NA
Gender; female, number (%)	3 (30.0)	29 (41.4)	.7
FEV_1% predicted, mean (±sd)[a]	81.4 (11.7)	65.1 (20.5)	.02
BMI (kg/m^2), mean (±sd)[a]	19.3 (2.8)	23.5 (4.1)	.002
Diagnoses, number (%)			
Pancreatic insufficient	7 (70.0)	61 (87.1)	.2
Cystic fibrosis-related diabetes	0 (0)	9 (12.9)	.6
Liver disease	0 (0)	3 (4.3)	1.0
CFTR function, number (%)			
Minimal	7 (80.0)	42 (60.0)	
Residual	0 (0)	22 (31.4)	.03
Non-classified	3 (3.0)	6 (8.6)	
CFQ-R (scores from 0 to 100), median (range)[a]			
Respiratory domain score	61.2 (8.3–83.3)	66.7 (0.0–88.9)[c]	.3
Emotional domain score	73.4 (41.7–100.0)	80.0 (0.0–100.0)[c]	.6
Routine bacterial culture[b], number positive (%)			
Pseudomonas aeruginosa	4 (40.0)	35 (51.5)	.7
MSSA	6 (60.0)	17 (25.0)	.06
MRSA	2 (20.0)	3 (4.4)	.1
Haemophilus influenzae	1 (10.0)	0 (0)	.1
Stenotrophomonas maltophilia	3 (30.0)	5 (7.4)	.06
Achromobacter spp.	0 (0)	2 (2.9)	1.0
Burkholderia cepacia complex	0 (0)	9 (13.2)	.6
Chronic treatments prescribed, number (%)			
Oral azithromycin	2 (20.0)	45 (64.3)	.01
Inhaled antibiotics	4 (40.0)	41 (58.6)	.3
Oral flucloxacillin	6 (60.0)	2 (2.9)	<.001
DNase	9 (90.0)	47 (67.1)	.3
Hypertonic saline	1 (10.0)	14 (20.0)	.7
Insulin	0 (0)	6 (8.6)	1.0
Antacid	1 (10.0)	31 (44.3)	.05

FEV_1% predicted, forced expiratory volume in the first second percent predicted; BMI, body mass index, CFTR, cystic fibrosis transmembrane conductance regulator (Definitions of CFTR function: residual function, harbouring ≥1 allele with Class IV-V mutations; minimal function, harbouring two alleles with Class I-III mutations; non-classified, harbouring two alleles with mutations of unknown function); CFQ-R, Cystic Fibrosis Questionnaire revised (a higher score indicates a higher patient-reported quality of life with regard to respiratory and emotional status); MSSA, methicillin-susceptible *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; NA, non-applicable.

[a] Two people were excluded as they were recruited on the same day that treatment of a pulmonary exacerbation was subsequently started.

[b] Data not available for two adult patients; [c]Two adults did not complete the CFQ-R at study enrolment.

and piperacillin/tazobactam ($n=8$) was most common. The median (range) length of time between PEx1 and PEx2 was 0.5 (0.2–2) months. The Follow-up sample was collected a median (range) of 2 (1-10) months post-PEx2.

3.2. Detection of bacteria

Bacteria were cultured in 199 sputum samples and taxa were classed confidently to the genus-level. Consensus of species identity could not be reached using different reference sequence databases for some taxa, such as a number of streptococci, which have a high sequence similarity. This precluded analysis to the species-level. Aerobic ($n=37$) and obligate anaerobic ($n=23$) genera were identified with 10/60 (16.7%) genera found in >25% of samples (Table S1). Multiple genera were found in all samples with a median (range) of 7 (2–12) organisms. In >50% of positive samples, the majority of detected genera were present at $\geq 1.0 \times 10^4$ CFU/g.

3.3. Identification of cohorts to address hypotheses 1 & 2

Eighty-two sputa from 25 patients comprised a complete sample-set for longitudinal analyses. These patients had similar clinical characteristics to those who were excluded ($n=55$) from these analyses (Table S2).

There were 18 patients who provided three consecutive sputum samples that were sufficient for extended-quantitative culture (Baseline, S2, S3; Fig. 1) and remained clinically stable during the study. The median (range) time between first and third samples in this "clinically stable disease" cohort was ~8 (6-16) months. There were also seven patients who provided four consecutive sputum samples sufficient for extended-quantitative culture before, during and after an exacerbation (Baseline, PEx1, PEx2, Follow-up; Fig. 1). This "pulmonary exacerbation cohort" was treated with intravenous antibiotics with additional oral antibiotics prescribed for two patients (Fig. S1).

Baseline characteristics were similar between the cohorts (Table S3). Mean [±sd] lung function was higher in the clinically stable disease cohort (70.1 [23.2] % predicted) compared to the pulmonary exacerbation cohort (51.0 [19.9] % predicted) but this was not statistically significant ($P=.06$) (Table S3). No statistically significant changes were detected longitudinally for FEV_1% predicted or BMI in those with stable disease (Table 2). Lung function (but not BMI) fluctuated within the pulmonary exacerbation cohort. At PEx1 there was a mean (±sd) decrease of 12.3 (15.6) % in lung function compared with Baseline. At PEx2 and Follow-up, there was a mean (±sd) increase of 14.4 (22.7) % and 8.5 (21.4) % compared to Baseline, respectively. This was not statistically significant ($P=.07$; Table 2).

Most patients were chronically infected with ≥1 recognised pathogen by routine culture ($n=19/25$, 76.0%) (Table S3) [23]. The clinically stable disease cohort had lower rates of chronic *Pseudomonas aeruginosa* infection ($n=10/18$; 55.6%) compared to the pulmonary exacerbation cohort ($n=6/7$, 85.7%) (Fig. 2A and Fig. S1). *Pseudomonas*, however, was not always the dominant taxa isolated in patients with *P. aeruginosa* infection. For example, in patient B002, *Pseudomonas* TVC comprised only 0.03–0.12% of the community (Fig. 2A).

3.4. Hypothesis 1: bacterial communities in clinically stable disease

To address our first hypothesis that extended-quantitative culture could demonstrate conserved bacterial density and communities in clinically stable disease, bacterial communities were analysed in the clinically stable disease cohort (Fig. 1).

No statistical differences in TVCs or ecological indexes (Table 2), in sputum were observed in longitudinal stable subject samples.

Although the occurrence and relative abundance of bacterial genera fluctuated longitudinally and differed between patients (Fig. 2A), principle components analysis (PCA) indicated that within-patient bacterial communities were very similar in most patients as shown by the closeness of the points on the plot (e.g. B026, B063, B156) (Fig. 2B(i)). However, four patients (B018, B050, B067, B099) had one of three sputum community structures that clustered more closely with other patient samples than to their other two, based on bifurcation of major branches (Fig. 2C). Shifts in sputum community structure occurred at each study visit for only one patient (B098) (Fig. 2C). Overall, significant similarities within-patients were observed which explained ~70% of the variation ($R^2=0.70$; $P=.001$; Analysis of variance using distance matrices

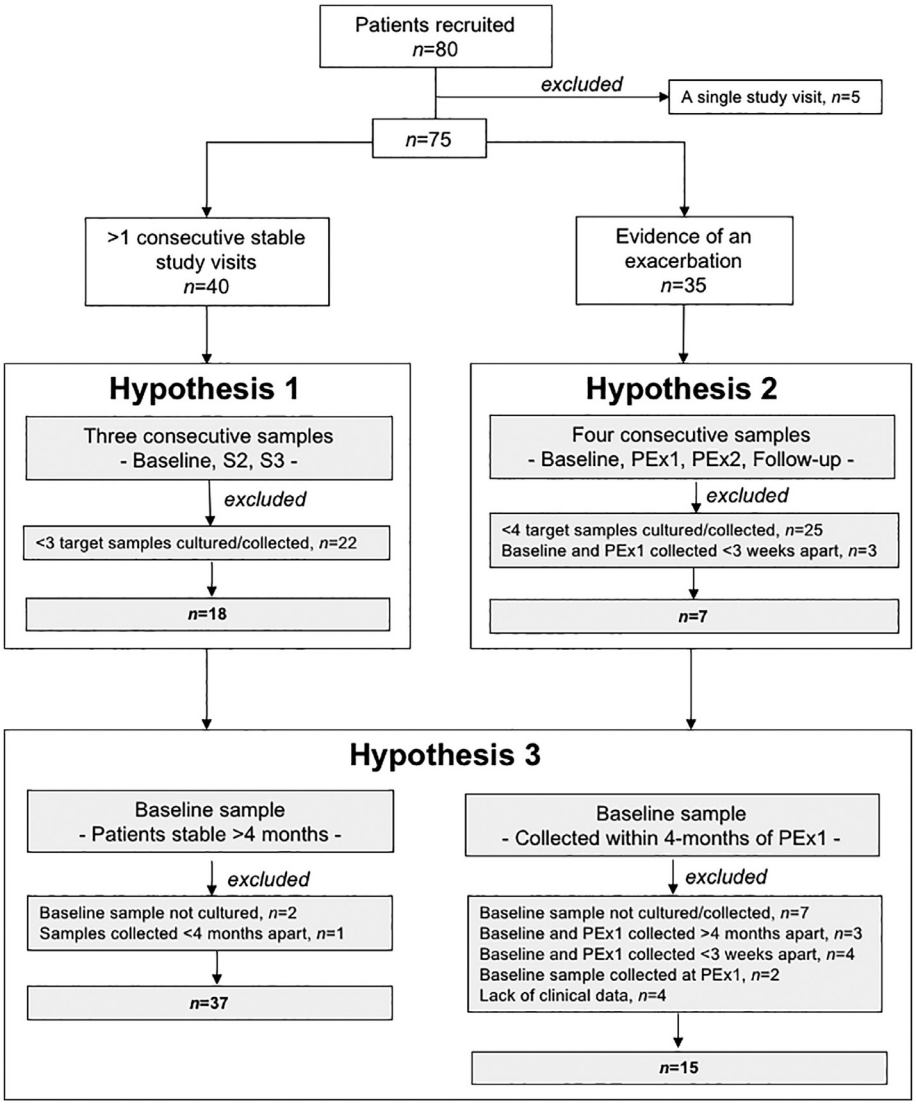

Fig. 1. Patient stratification to test three study hypotheses: extended-quantitative culture could (1) demonstrate conserved bacterial density and communities in clinically stable disease (2) detect temporal changes in bacterial density and communities during a pulmonary exacerbation and (3) predict a future pulmonary exacerbation. *Description of samples:* Baseline, sputum collected when clinically stable; S2, sputum collected at second clinically stable visit; S3, sputum collected at third clinically stable visit; PEx1, sputum collected at the initiation of treatment of a pulmonary exacerbation; PEx2, sputum collected at completion of treatment of a pulmonary exacerbation; Follow-up, sputum collected when clinically stable post-exacerbation.

permutational multivariate [adonis]; 999 permutations). Moreover, bacterial community structures between study visits (Fig. 2B (ii)) were more variable compared to within-patient samples (Fig. 2B (i)), demonstrated by greater distance between points on the former plot. However, a permutation test using the Bray-Curtis dissimilarity measures for homogeneity of multivariate dispersion, showed no significant difference ($P = .481$; 999 permutations) (Fig. S2A (i) and (ii)).

3.5. Hypothesis 2: bacterial communities during pulmonary exacerbations

To address our second hypothesis that extended-quantitative culture could detect temporal changes in bacterial density and communities during a pulmonary exacerbation, the pulmonary exacerbation cohort was analysed (Fig. 1 and Fig. S1).

Total bacterial density ($P < .001$) and density of aerobes ($P < .001$), but not obligate anaerobes ($P = .1$), differed significantly over time (Table 2). Post-hoc analysis showed that statistical differences in TVCs of total bacteria were not detected between Baseline vs PEx1 ($P = .8$) but were detected for Baseline vs PEx2 ($P = .004$) and PEx1 vs PEx2 ($P = .007$) whilst differences in aerobic TVCs occurred between Baseline vs PEx2 ($P = .02$) (Table S4). TVCs of 6/10 most prevalent aerobic (*Streptococcus*, *Rothia*, *Actinomyces*, *Pseudomonas*, *Gemella*) and obligate anaerobic (*Prevotella*) genera decreased sequentially between Baseline and PEx2; however, TVCs had increased again by Follow-up (Fig. S3). There were no statistically significant fluctuations in ecological indexes (Table 2).

PCA demonstrated that bacterial community structures were variable within-patients(Fig. 3A(i)) and a within-patient shift in sputum community structure occurred for all individuals for at least one study visit based on divergence of major branches (Fig. 3B). It was noted that some patients experienced a substantial shift in their bacterial community structure between Baseline and PEx1 (B014, B019, B023, B034, B203) whilst communities in other patients remained relatively unchanged (B004, B008) (Fig. 3B). However, significant similarities within-patients were still observed, which explained ~34% of the variation ($R^2 = 0.34$; $P = .008$; adonis;

Table 2
Longitudinal change in the geometric mean bacterial total viable count and ecological indexes in sputum and clinical parameters of the clinically stable disease ($n = 18$) and pulmonary exacerbation ($n = 7$) cohorts to address hypotheses 1 & 2.

	Clinically stable disease cohort			*P*-value	Pulmonary exacerbation cohort				*P*-value
	Baseline	S2	S3		Baseline	PEx1	PEx2	Follow-up	
CFU/g, geometric mean (95% CI)									
Total	5.2×10^7 (2.0×10^7–1.3×10^8)	5.6×10^7 (2.8×10^7–1.2×10^8)	4.4×10^7 (2.5×10^7–7.8×10^7)	.7	2.0×10^8 (7.8×10^7–5.5×10^8)	7.1×10^7 (1.3×10^7–4.0×10^8)	2.5×10^6 (7.1×10^5–9.1×10^6)	3.7×10^7 (9.8×10^6–1.4×10^8)	<.001*
Aerobe	4.6×10^7 (1.9×10^7–1.1×10^8)	5.4×10^7 (2.6×10^7–1.1×10^8)	3.4×10^7 (1.9×10^7–6.3×10^7)	.4	1.9×10^8 (7.1×10^7–5.0×10^8)	6.8×10^7 (1.2×10^7–3.9×10^8)	1.0×10^6 (1.0×10^5–1.0×10^7)	3.4×10^7 (8.5×10^6–1.3×10^8)	<0.001*
Obligate anaerobe	2.7×10^4 (8.7×10^2–8.3×10^5)	2.0×10^5 (1.1×10^4–3.9×10^6)	2.0×10^5 (9.5×10^3–4.1×10^6)	.3	5.5×10^5 (1.7×10^3–1.8×10^8)	1.2×10^6 (3.2×10^5–4.7×10^6)	1.4×10^3 (2.3×10^0–8.7×10^5)	1.1×10^6 (1.5×10^5–8.7×10^6)	.1
Ecological indexes, median (range)									
Richness	7 (3–12)	8 (4–12)	8 (2–10)	.5	7 (3–9)	6 (5–8)	5 (2–7)	7 (5–8)	.2
Dominance	0.5 (0.2–1.0)	0.4 (0.2–0.9)	0.4 (0.2–1.0)	.7	0.5 (0.4–1.0)	0.5 (0.3–0.8)	0.6 (0.3–1.0)	0.5 (0.3–0.9)	1.0
Diversity	0.9 (0.09–1.7)	1.1 (0.3–1.7)	1.2 (0.07–1.9)	.6	1.0 (0.08–1.1)	0.9 (0.5–1.5)	0.8 (0.02–1.3)	1.0 (0.3–1.4)	.7
Evenness	0.4 (0.2–0.7)	0.4 (0.1–0.6)	0.5 (0.1–0.7)	.3	0.3 (0.2–0.5)	0.4 (0.3–0.7)	0.5 (0.2–0.7)	0.5 (0.3–0.5)	.07
Clinical parameter, mean (±sd)									
FEV_1% predicted	70.1 (23.2)	68.7 (21.1)	70.8 (22.9)	.5	51.0 (19.9)	45.0 (20.1)	59.4 (26.6)	56.9 (26.2)	.07
BMI, kg/m^2	23.0 (2.6)	23.0 (2.4)	23.3 (3.1)	.5	22.0 (3.0)	21.7 (3.1)	21.7 (3.0)	22.0 (3.0)	.1

CFU/g, colony-forming units per gram of sputum; CI, confidence interval; FEV_1% predicted, forced expiratory volume in the first second percent predicted; BMI, body mass index.
Description of samples: Baseline, sputum collected when clinically stable; S2, sputum collected at second clinically stable visit; S3, sputum collected at third clinically stable visit; PEx1, sputum collected at the initiation of treatment of a pulmonary exacerbation; PEx2, sputum collected at completion of treatment of a pulmonary exacerbation; Follow-up, sputum collected when clinically stable post exacerbation.
BMI comparison: clinically stable disease cohort, $n = 17$; pulmonary exacerbation cohort, $n = 5$.
* Statistical difference between timepoints identified using a repeated measures ANOVA with post-hoc analysis showing that statistical differences in TVCs of total bacteria were detected between Baseline and PEx2 (P = .004) and PEx1 and PEx2 (P = .007) whilst differences in aerobic TVCs occurred between Baseline and PEx2 (P = .02) (all post-hoc results shown in Table S4).

999 permutations). PCA indicated that bacterial community structures were variable between study visits (Fig. 3A(ii)) and a permutation test using the Bray-Curtis dissimilarity measures for homogeneity of multivariate dispersion showed a significant dissimilarity in community structures over time, with the largest proportion of the variance driven by changes in the community composition at PEx2 ($P = .004$; 999 permutations) (Fig. S2B (i) and (ii)).

3.6. Hypothesis 3: prediction of a pulmonary exacerbation

To address our third hypothesis that extended-quantitative culture could predict a future exacerbation, data were analysed from 52 eligible patients (Fig. 1). Patients were stratified depending on whether they had an exacerbation within 4-months of Baseline ($n = 15$) or remained clinically stable ($n = 37$).

In univariable logistic regressions (Table S5), higher lung function (odds ratio [OR], 0.95; 95% confidence interval [CI], 0.91–0.98; $P = .004$) and BMI (OR, 0.80; 95% CI, 0.65–0.99; $P = .04$) were significantly associated with reduced odds of a future exacerbation. Female gender (OR, 3.09; 95% CI, 0.89–10.74; $P = .08$) and chronic azithromycin treatment (OR, 4.22; 95% CI, 1.02–17.47; $P = .05$) were associated with an elevated, but non-significant, odds of a future exacerbation whilst a better CFQ-R respiratory symptom score was associated with a reduced, but non-significant, odds (OR, 0.96; 95% CI, 0.92–1.01; $P = .09$). After controlling for all these factors in a multivariable logistic regression, lung function ($P = .01$), BMI ($P = .02$) and respiratory symptom score ($P = .03$) remained statistically significant (Table S5). The future likelihood of an exacerbation reduced as lung function (OR, 0.91; 95% CI, 0.85–0.98), BMI (OR, 0.61; 95% CI, 0.40–0.93) or CFR-Q respiratory score (OR, 0.92; 95% CI, 0.85–0.99) increased. None of the extended-quantitative culture variables (TVCs and ecological indexes) were independent predictors of an exacerbation in this cohort.

4. Discussion

This study describes extended-quantitative culture analysis of CF lower airway communities during both clinically stable disease and pulmonary exacerbation and begins to address if extended-quantitative culture provides further information with respect to clinical outcomes. A key study strength is that patients were followed prospectively and were stratified according to their clinical course during the study period. Bacterial community structures were more similar within than between patients and those with stable disease had more conserved bacterial communities longitudinally compared to those who had an exacerbation. Most other studies investigating the lower airway dynamics are primarily based on molecular analysis of samples [5–9]. Our findings complement those studies and confirmed that a complex bacterial community exists. However, a further strength of our study is that we have provided key information on the viability and density of the bacteria identified, which cannot be accurately determined using next-generation sequencing analyses. Despite the recovery and quantification of a large number of potentially relevant taxa and assessment of the viable lower airway ecology, the extended-quantitative culture variables did not predict an exacerbation which might limit the potential prognostic applicability of the method to clinical practice.

Most patients who remained clinically stable throughout the sampling period were chronically infected with a CF pathogen and demonstrated conserved bacterial densities and ecological indexes. However, fluctuations in the community structure were found in five patients (Fig. 2C) without any change in clinical status or intravenous antibiotic treatment; three of these patients had different CF pathogens detected at one time-point by routine culture (B018, B067, B098). The latter finding supports clinical guidelines which recommend that CF respiratory samples are cultured at every clinic visit to identify new pathogens as early as possible [24].

Prior culture-independent studies found limited within-patient changes in the bacterial load and/or diversity measures between stability and during treatment of an exacerbation while others reported that significant changes occurred [3,5,6]. In our study, bacterial community composition and treatment regimens for exacerbations were individual but a temporal reduction in the bacterial density was found not at the inception of treatment of the exacerbation (PEx1) but during antimicrobial therapies for exacerbation (PEx2) before returning to pre-treatment levels at review. Most of the variation was, therefore, likely to be driven by treatment. A similar pattern was observed for the density of some of the most common genera (Fig. S3) identified suggesting resilience

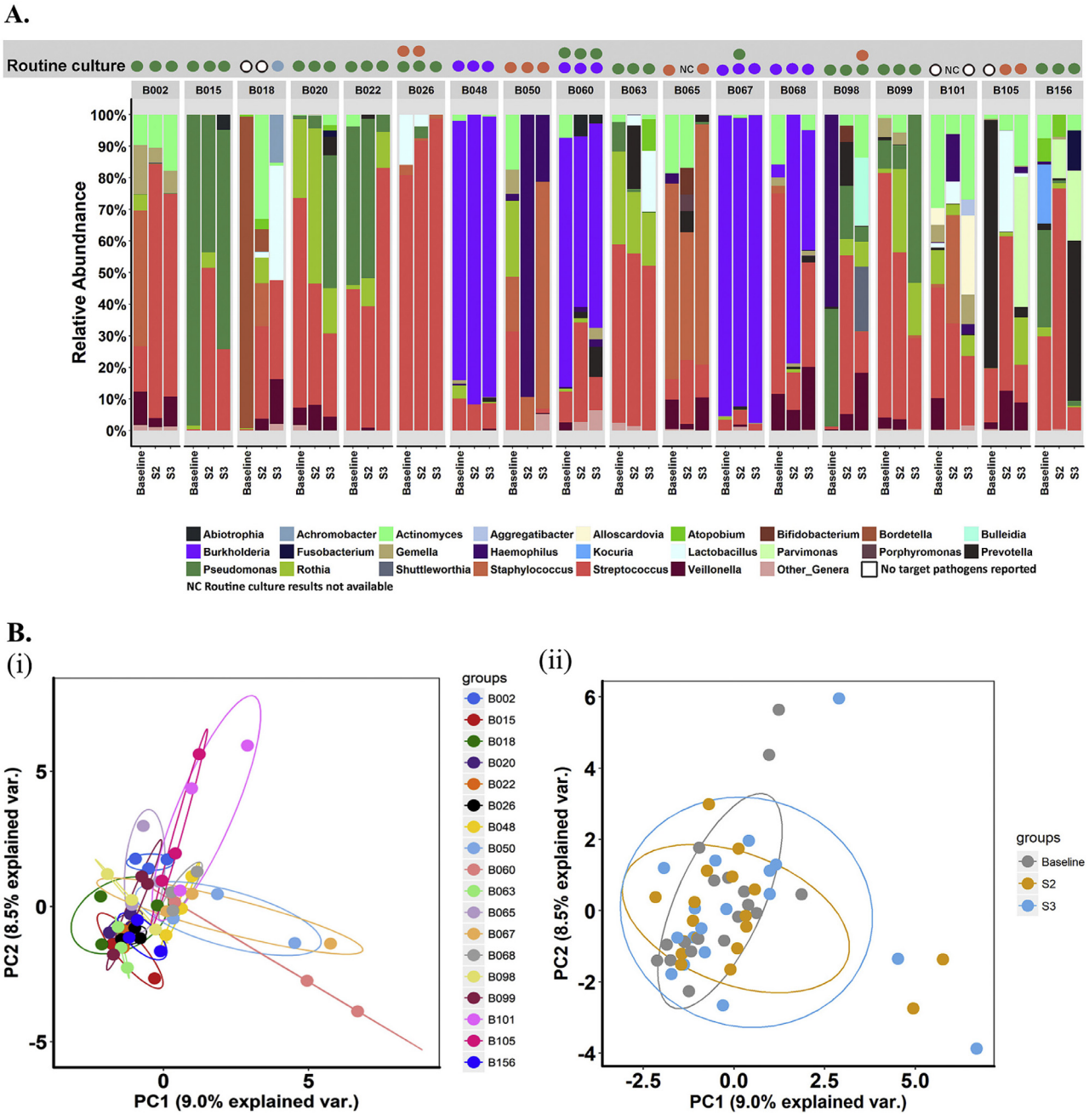

Fig. 2. Clinically stable disease cohort ($n = 18$; hypothesis 1). A. The temporal variation in the relative abundance of genera detected (based on CFU/g) within each sample is shown. Other genera detected (found in a relative abundance of <5% in all samples): *Aestuariimicrobium*, *Bacillus*, *Brevibacterium*, *Campylobacter*, *Capnocytophaga*, *Cardiobacterium*, *Dermacoccus*, *Enterococcus*, *Escherichia*, *Granulicatella*, *Leptotrichia*, *Moraxella*, *Neisseria*, *Peptoniphilus*, *Propionibacterium*, *Scardovia*. B. Principal components analysis (i) individual patients, with different coloured points (samples) indicating the study visit or patient, respectively and (ii) Baseline, S2, and S3 samples. The first principle component (PC1) accounts for most of the data variability. The proximity of the points on the plot indicates the similarity between bacterial communities with more similar communities being closer together. Elipses indicate 95% confidence intervals. PC2, second principle component. C. Dendogram displaying the realtionship between the community structure (bacterial membership [CFU/g]) of sputum samples according to the Bray-Curtis quantitative index of dissimilarity. Major clusters of individual community structures are shown by different coloured boxes (labelled 1–5). Bifurcation of clusters indicates the Bray-curtis dissimilarity score with scores closer to 1 indicating increasing dissimilarity. *Description of samples:* Baseline, sputum collected when clinically stable; S2, sputum collected at second clinically stable visit; S3, sputum collected at third clinically stable visit.

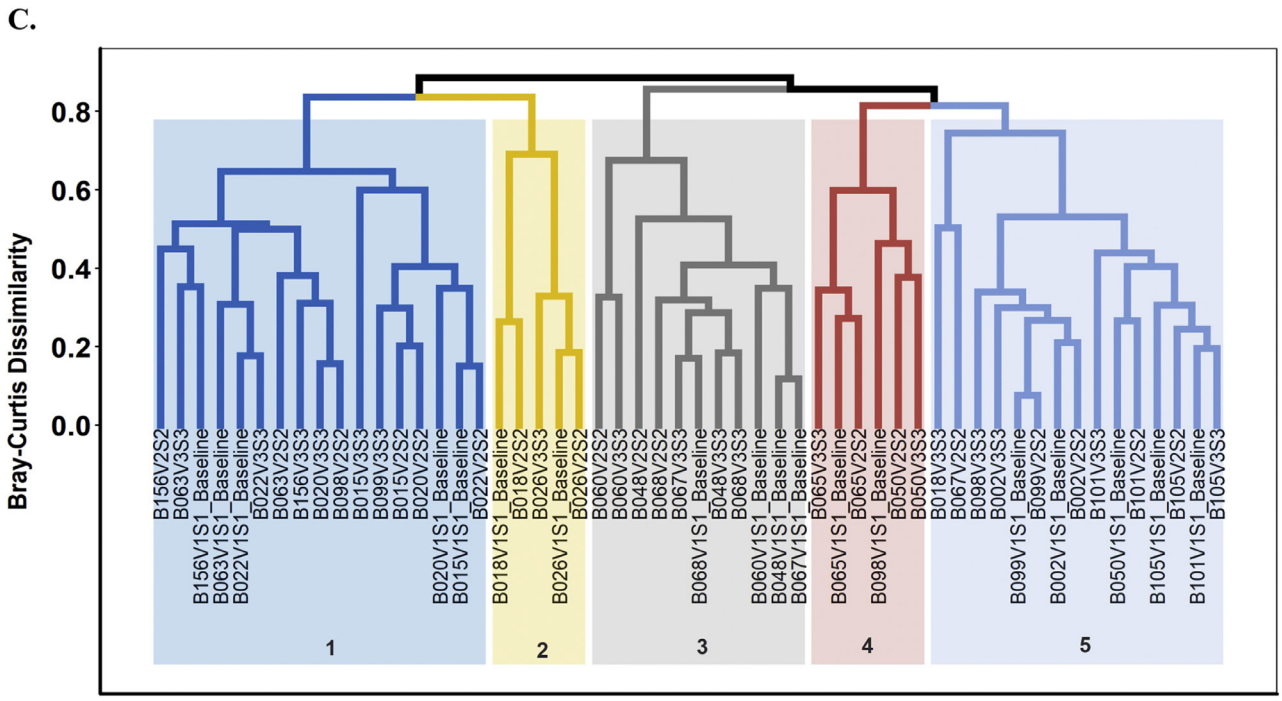

Fig. 2. Continued

of these taxa not only during stable and exacerbating periods but also following antibiotic treatment, as has been shown by culture-independent studies [7]. Furthermore, by completion of treatment, lung function had increased compared to the previous study visit. However, despite these changes, it was difficult to relate changes in clinical state to shifts in the community structure due to inter-patient variability (Fig. 3B), corroborating with previous findings [8].

Given that differences in bacterial community stability were observed between the longitudinal cohorts i.e. the within-patient samples were less stable in the pulmonary exacerbation cohort compared to the clinically stable cohort, we investigated, in an additional analysis, if extended-quantitative culture could provide prognostic information on the future risk of an exacerbation. Neither ecological indexes nor bacterial density predicted an exacerbation. However, the extended-quantitative culture measures included may not provide enough taxonomic resolution to be clinically informative e.g. the density of individual bacterial species might be important. Further, exacerbations could be triggered by viral infections, which were not tested for in this study. In contrast, clinical parameters (lung function, BMI and respiratory symptom score) were good predictors of a future exacerbation [22,25,26].

The bacterial genera reported here are typical of those detected using culture-independent methods [5–7]. In keeping with earlier findings, *Prevotella* and *Veillonella* were the most prevalent obligate anaerobes cultured from sputum [9,10]. The role of anaerobes in the CF airway remains contentious and might be affected by the lack of resolution in classification of taxa. Anaerobes potentially form part of a normal airway microbiota especially as they have been identified in respiratory samples from healthy participants, albeit at much lower TVC than in CF, and have been associated with milder disease when dominant in the community [12,14]. In contrast, there is also evidence that anaerobes contribute to a dysregulated inflammatory response or promote survival of recognised pathogens and therefore, influence disease pathogenesis [27,28].

A limitation of this study is that a number of patients were excluded from the longitudinal analyses (Fig. 1) due to a lack of extended-quantitative culture data at all study visits and this contributed to the small number of exacerbations studied; this reflects that some individuals were unable to expectorate any or only a small volume of sputum on some occasions. The majority of patients, who were included in the longitudinal analyses, were chronically infected with CF pathogens such as *P. aeruginosa* and *B. cepacia* complex and therefore, the findings may differ if the cohort included a larger number of patients without chronic airway infection. The duration of antibiotic treatment of an exacerbation and subsequent timing of the follow-up outpatient appointment was variable in the pulmonary exacerbation cohort analysed and how this may impact the findings is unknown. Moreover, analysis of sputum samples cannot reveal whether airway communities are spatially heterogeneous [30] or reflect whether an exacerbation occurs due to extension of infection into less affected areas. This inability to infer individual airway communities is a general limitation of sputum in both clinical care and research. Although there was no evidence of a universal signature of the lower airways bacterial community that predicted a future exacerbation, most of the participants included were adults with infection with recognised CF pathogens and all expectorated spontaneously indicating more advanced disease; therefore, findings may not extrapolate to younger populations [29]. It is also important to acknowledge that a general constraint of a quantitative microbiology approach is failure to detect viable but non-culturable bacteria.

5. Conclusions

In conclusion, extended-quantitative culture provides a detailed assessment of the viable lower airway bacterial community and shows that community composition varied between patients. Conserved bacterial communities were more characteristic of those with stable disease over many months whilst exacerbations were associated with a temporal fluctuation in bacterial density with antibiotics and a greater change in community structure. Although extended-quantitative culture parameters were not prognostic of exacerbations, it is unclear whether they could be used to track

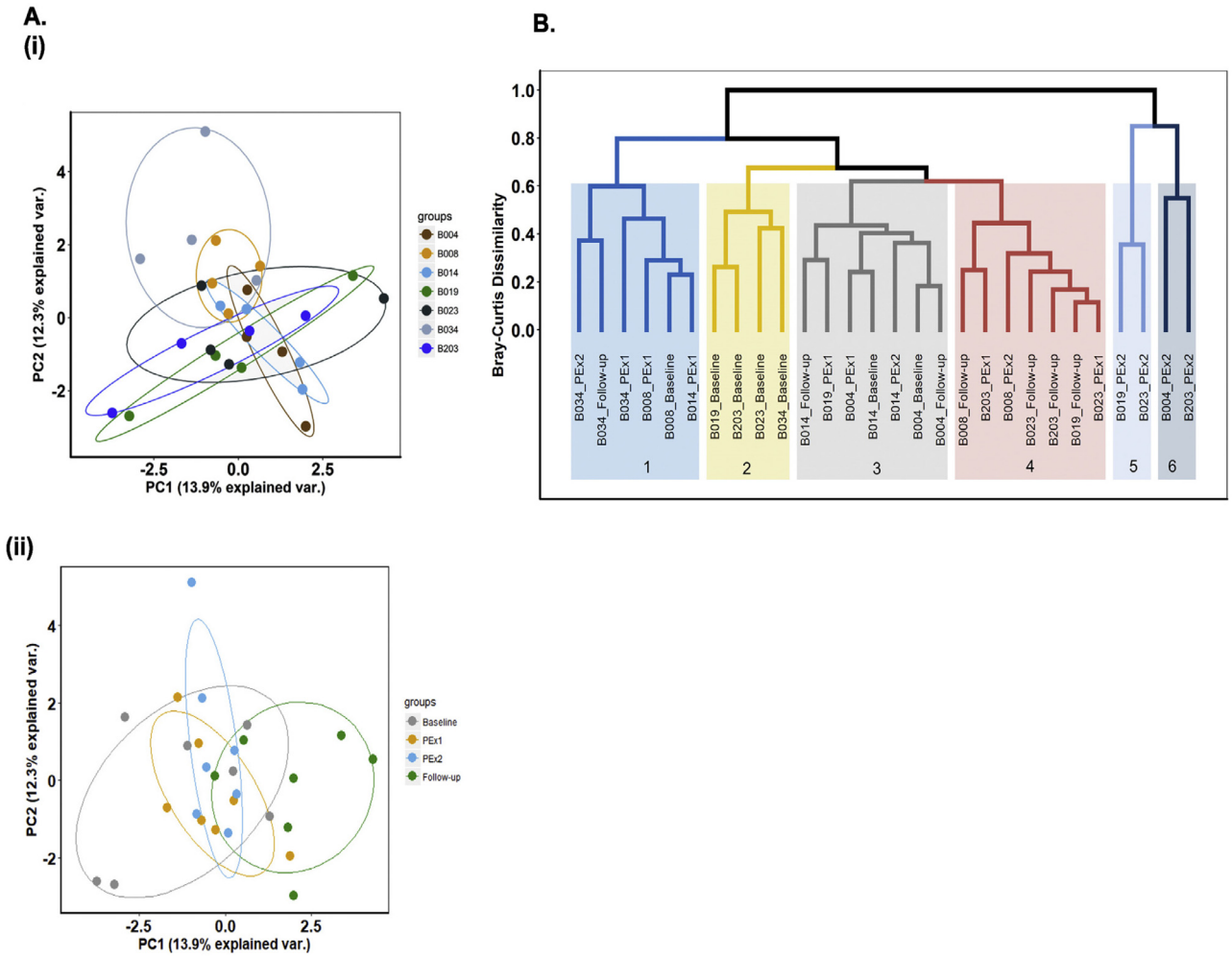

Fig. 3. Pulmonary exacerbation cohort ($n = 7$; hypothesis 2). A. Principal components analysis (i) individual patients, with different coloured points (samples) indicating the study visit or patient, respectively and (ii) Baseline, PEx1, PEx2 and Follow-up samples. The first principle component (PC1) accounts for most of the data variability. The proximity of the points on the plot indicates the similarity between bacterial communities with more similar communities being closer together. Elipses indicate 95% confidence intervals. PC2, second principle component. B. Dendogram displaying the realtionship between the community structure (bacterial membership [CFU/g]) of sputum samples according to the Bray-Curtis quantitative index of dissimilarity. Major clusters of individual community structures are shown by different coloured boxes (labelled 1–6). Bifurcation of clusters indicates the Bray-curtis dissimilarity score with scores closer to 1 indicating increasing dissimilarity. *Description of samples:* Baseline, sputum collected when clinically stable; PEx1, sputum collected at the initiation of treatment of a pulmonary exacerbation; PEx2, sputum collected at completion of treatment of a pulmonary exacerbation; Follow-up, sputum collected when clinically stable post-exacerbation.

and predict an individual's disease progression or guide antimicrobial therapy.

Contributions

Conceived and designed study, JSE, MMT, DFG, NGMcE, RCB, MSM; Collected samples and clinical data, EJ, KO'N, DGD, AR; Performed research, GGE, LM, SJM; Analysed data, LJS, GGE; Intellectual contributions, DGD, AR, DFG, NGMcE, RCB, MSM; Wrote the paper, LJS, GGE, JSE, MMT; All authors read and approved the final version of the manuscript.

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Conflict of interest statement

MMT and JSE report grants from Northern Ireland Health and Social Care Research and Development Office and MSM reports grants from National Institutes of Health HL084934, during the conduct of the study. NGMcE reports grants from US-Ireland partnership/Science Foundation Ireland/Health Research Board, during the conduct of the study. JSE and MMT also reports grants from the EU Innovative Medicines Initiative, outside the submitted work. MMT reports grants from Novartis, Basilea Pharmaceutica, Alaxia SAS, and Polyphor outside the submitted work. JSE reports grants, personal fees and clinical trial involvement with Vertex and clinical trial involvement with Celtaxsys and Corbus Pharmaceuticals, outside the submitted work. RCB reports personal fees from and has a patent pending with Parion Sciences, outside the submitted work. All other authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcf.2019.02.012.

References

[1] Elborn JS. Cystic fibrosis. Lancet 2016;388(10059):2519–31.
[2] Cystic Fibrosis Trust. Laboratory standards for processing microbiological samples from people with cystic fibrosis. 1st ed; September 2010.
[3] Cox MJ, Allgaier M, Taylor B, Baek MS, Huang YJ, Daly RA, et al. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. PLoS One 2010;5(6):e11044.
[4] Klepac-Ceraj V, Lemon KP, Martin TR, Allgaier M, Kembel SW, Knapp AA, et al. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and *Pseudomonas aeruginosa*. Environ Microbiol 2010;12(5):1293–303.
[5] Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, et al. Decade-long bacterial community dynamics in cystic fibrosis airways. Proc Natl Acad Sci U S A 2012;109(15):5809–14.
[6] Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, et al. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. PLoS One 2012;7(9):e45001.
[7] Cuthbertson L, Rogers GB, Walker AW, Oliver A, Green LE, Daniels TW, et al. Respiratory microbiota resistance and resilience to pulmonary exacerbation and subsequent antimicrobial intervention. ISME J 2016;10(5):1081–91.
[8] Carmody LA, Zhao J, Kalikin LM, LeBar W, Simon RH, Venkataraman A, et al. The daily dynamics of cystic fibrosis airway microbiota during clinical stability and at exacerbation. Microbiome 2015;3:12.
[9] Tunney MM, Klem ER, Fodor AA, Gilpin DF, Moriarty TF, McGrath SJ, et al. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. Thorax 2011;66(7):579–84.
[10] Tunney MM, Field TR, Moriarty TF, Patrick S, Doering G, Muhlebach MS, et al. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med 2008;177(9):995–1001.
[11] Sibley CD, Grinwis ME, Field TR, Eshaghurshan CS, Faria MM, Dowd SE, et al. Culture enriched molecular profiling of the cystic fibrosis airway microbiome. PLoS One 2011;6(7):e22702.
[12] Muhlebach MS, Hatch JE, Einarsson GG, McGrath SJ, Gilipin DF, Lavelle G, et al. Anaerobic bacteria cultured from cystic fibrosis airways correlate to milder disease: a multisite study. Eur Respir J 2018;52(1). doi:10.1183/13993003.00242-2018.
[13] Farrell PM, Rosenstein BJ, White TB, Accurso FJ, Castellani C, Cutting GR, et al. Guidelines for diagnosis of cystic fibrosis in newborns through older adults: Cystic Fibrosis Foundation consensus report. J Pediatr 2008;153(2):S4–S14.
[14] O'Neill K, Bradley JM, Johnston E, McGrath S, McIlreavey L, Rowan S, et al. Reduced bacterial colony count of anaerobic bacteria is associated with a worsening in lung clearance index and inflammation in cystic fibrosis. PLoS One 2015;10(5):e0126980.
[15] O'Neill K, Tunney MM, Johnston E, Rowan S, Downey DG, Rendall J, et al. Lung clearance index in adults and children with cystic fibrosis. Chest 2016;150(6):1323–32.
[16] O'Neill K, Bradley JM, Reid A, Downey DG, Rendall J, McCaughan J, et al. Airway infection, systemic inflammation and lung clearance index in children and adults with cystic fibrosis. Eur Respir J 2018;51(2). doi:10.1183/13993003.01704-2017.
[17] Fuchs HJ, Borowitz DS, Christiansen DH, Morris EM, Nash ML, Ramsey BW, et al. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. N Engl J Med 1994;331(10):637–42.
[18] Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J 2005;26(2):319–38.
[19] Stanojevic S, Wade A, Stocks J, Hankinson J, Coates AL, Pan H, et al. Reference ranges for spirometry across all ages: a new approach. Am J Respir Crit Care Med 2008;177(3):253–60.
[20] Quittner AL, Buu A, Messer MA, Modi AC, Watrous M. Development and validation of the cystic fibrosis questionnaire in the United States: a health-related quality-of-life measure for cystic fibrosis. Chest 2005;128(4):2347–54.
[21] Green DM, McDougal KE, Blackman SM, Sosnay PR, Henderson LB, Naughton KM, et al. Mutations that permit residual CFTR function delay acquisition of multiple respiratory pathogens in CF patients. Respir Res 2010;11:140.
[22] Quon BS, Dai DL, Hollander Z, Ng RT, Tebbutt SJ, Man SF, et al. Discovery of novel plasma protein biomarkers to predict imminent cystic fibrosis pulmonary exacerbations using multiple reaction monitoring mass spectrometry. Thorax 2016;71(3):216–22.
[23] Lee TW, Brownlee KG, Conway SP, Denton M, Littlewood JM. Evaluation of a new definition for chronic *Pseudomonas aeruginosa* infection in cystic fibrosis patients. J Cyst Fibros 2003;2(1):29–34.
[24] Cystic Fibrosis Trust. Antibiotic treatment for CF. 3rd ed; May 2009.
[25] Block JK, Vandemheen KL, Tullis E, Fergusson D, Doucette S, Haase D, et al. Predictors of pulmonary exacerbations in patients with cystic fibrosis infected with multi-resistant bacteria. Thorax 2006;61(11):969–74.
[26] van Horck M, Winkens B, Wesseling G, van Vliet D, van de Kant K, Vaassen S, et al. Early detection of pulmonary exacerbations in children with Cystic Fibrosis by electronic home monitoring of symptoms and lung function. Sci Rep 2017;7(1):12350.
[27] Mirkovic B, Murray MA, Lavelle GM, Molloy K, Azim AA, Gunaratnam C, et al. The role of short-chain fatty acids, produced by anaerobic bacteria, in the cystic fibrosis airway. Am J Respir Crit Care Med 2015;192(11):1314–24.
[28] Sherrard LJ, McGrath SJ, McIlreavey L, Hatch J, Wolfgang MC, Muhlebach MS, et al. Production of extended-spectrum beta-lactamases and the potential indirect pathogenic role of *Prevotella* isolates from the cystic fibrosis respiratory microbiota. Int J Antimicrob Agents 2016;47(2):140–5.
[29] Muhlebach MS, Zorn BT, Esther CR, et al. Initial acquisition and succession of the cystic fibrosis lung microbiome is associated with disease progression in infants and preschool children. PLoS Pathog 2018;14(1):e1006798.
[30] Brown PS, Pope CE, Marsh RL, et al. Directly sampling the lung of a young child with cystic fibrosis reveals diverse microbiota. Ann Am Thorac Soc 2014;11(7):1049–55.