ORIGINAL ARTICLE

Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience

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ABSTRACT

Background Culture-independent analysis of the respiratory secretions of people with cystic fibrosis (CF) has identified many bacterial species not previously detected using culture in this context. However, little is known about their clinical significance or persistence in CF airways.

Methods The authors characterised the viable bacterial communities in the sputum collected from 14 patients at monthly intervals over 1 year using a molecular community profiling technique—terminal restriction fragment length polymorphism. Clinical characteristics were also collected, including lung function and medications. Ecological community measures were determined for each sample. Microbial community change over time within subjects was defined using ecological analytical tools, and these measures were compared between subjects and to clinical features.

Results Bacterial communities were stable within subjects over time but varied between subjects, despite similarities in clinical course. Antibiotic therapy temporarily perturbed these communities which generally returned to pretreatment configurations within 1 month. Species usually considered CF pathogens and those not previously regarded as such exhibited similar patterns of persistence. Less diverse sputum bacterial communities were correlated to lung disease severity and relative abundance of *Pseudomonas aeruginosa*.

Conclusion Whilst not true in all cases, the microbial communities that chronically infect the airways of patients with CF can vary little over a year despite antibiotic perturbation. The species present tended to vary more between than within subjects, suggesting that each CF airway infection is unique, with relatively stable and resilient bacterial communities. The inverse relationship between community richness and disease severity is similar to findings reported in other mucosal infections.

Key messages

What is the key question?

- To what degree do the bacterial species present in cystic fibrosis (CF) sputum represent chronic colonisation of the lower airways?

What is the bottom line?

- The CF bacterial community, including species only recently reported, as well as known pathogens such as *Pseudomonas aeruginosa*, is in many instances stable and resilient over extended periods.

Why read on?

- These findings provide a basis for understanding the clinical importance of the bacterial community in disease progression, and a context for studies that assess the impact of antimicrobial therapy.

INTRODUCTION

Whilst advances in longevity continue, respiratory failure remains the leading cause of death in cystic fibrosis (CF). The lung damage that leads to respiratory failure results from the chronic bacterial infection and host immune response that is typical by adulthood, if not before. Infection by certain bacterial species has been associated with poorer clinical outcomes.[1 2] These species have been detected previously using selective culture-based approaches that are inherently biased. More recent studies are increasingly characterising the bacteria present in the CF airways using culture-independent approaches that avoid these biases.[3–6] In this way, much greater bacterial diversity has been found typically in these airway secretions than previously appreciated. It is also important to note that bacterial species, many of them anaerobes, identified using culture-independent methods are frequently of abundances comparable to species routinely cultivated and treated as pathogens in the CF airway.[6–8]

From the earliest of these culture-independent studies, it has been postulated that species present in the lower airways represent an interacting community,[9] implying roles for 'non-traditional' species in CF lung disease pathogenesis and treatment response. However, support for such a community model requires determination of the

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persistence of the various community members, the community response to perturbations, for example antibiotics, and its relationship with disease severity. Some species may remain in the lower airways for relatively short periods, while others may establish chronic infections. Infection over longer periods may also be more likely to adversely impact upon respiratory health. In support of these concepts, CF airways communities have been suggested to contain both core and transient species,[10] with community structure and membership related to age and lung disease severity.[11] However, such studies are generally cross sectional or short term,[12] and a longitudinal analysis of multiple subjects has not yet been performed.

Longitudinal analysis has followed how specific CF pathogens change with time during chronic airway infection.[13 14] This approach must now be applied to the entire community. The first step is to define the variability of CF airway microbiota over time, during clinical changes, and with antibiotic treatment. Community constituency could change in a number of ways. At one extreme, the composition of the community may change frequently, with the community found in one specimen not resembling those in subsequent samples from the same subject. At the other extreme, the community may be so stable that the same species are recovered at any sampling point. CF airway disease is typified by periods of relative respiratory health punctuated by exacerbation; with augmented treatment, usually requirement for antibiotics. As such, we hypothesised that the varying treatment that a patient with CF receives over the course of a year, and the dynamic nature of respiratory and general health, would be associated with detectable changes in the bacterial community and a 'highly variable' composition. To test this hypothesis required the collection of samples from multiple subjects spanning an extended period.

Here, we report the culture-independent analysis of viable bacteria present in monthly samples from airways of 14 adults with CF over a year. These data were collected alongside detailed clinical metadata. A series of analytical tools tested the compositional stability of these communities over time.[10] We also analysed the relationship between community structure and lung disease severity.

MATERIALS AND METHODS

Clinical samples

This observational study of 14 adult patients with CF over 12 months was undertaken with ethical approval from Southampton and South West Hampshire Research Ethics Committee (06/Q1704/26). Eligible subjects were aged 18 or over and had experienced at least three pulmonary exacerbations (CFPEs) requiring antibiotic treatment in the prior 12 months. Samples from these patients were previously used in an analysis of core and satellite bacterial taxa.[10]

Patient clinical details are summarised in supplementary table 1. The start of a CFPE was defined by the clinician's decision to initiate antibiotic therapy for deteriorating clinical status, broadly based on factors described previously.[15] The end of CFPE was defined by the decision to cease antibiotic therapy due to stabilisation or improvement in signs and symptoms. A total of 39 episodes of CFPE were experienced by these patients over the year, ranging from 0 (patients 3 and 8) to 6 (patient 9) with a mean of 2.8, SD 1.4. Overall, 17 different antibiotic combinations were used with five 'elective' antibiotic courses given for reasons other than worsening respiratory symptoms.

One sputum sample was obtained from each patient at approximately monthly intervals. The majority of samples (65%) were collected at least 21 days prior to, or 21 days after cessation of, antibiotics for CFPE (supplementary table 2). All sputa were stored at 4°C immediately after expectoration, shipped at 4°C (in accordance with handling guidelines[16]) and stored at −80°C prior to processing, or cultured by the Health Protection Agency South East, UK following standard operating procedures.

Clinical data

Clinical measures (including forced expiratory volume in 1 s (FEV_1) and forced expiratory volume in 6 s (FEV_6) in litres, temperature, and patient reported outcome (PRO) scores) were recorded at sample collection. PRO scores were recorded using visual analogue scores for individual symptoms ('breathlessness', 'cough severity', 'sputum production' and 'general well-being') from 0 (no symptoms) to 100 (worst symptoms).

Culture-independent analysis

Exclusion of DNA from non-viable cells in sputa via cross-linking using propidium monoazide[17 18] and subsequent nucleic acid extraction were performed as described previously.[19] PCR and terminal restriction fragment length polymorphism (T-RFLP) profiling were carried out as previously described[3] (see supplementary materials and methods).

Statistical analysis

Bacterial species richness (the number of bacterial species in a sample) was inferred from T-RF band number as previously described.[20] Cumulative bacterial taxon richness (the total number of different bacterial taxa identified after each successive time point) was assessed using taxa–time relationships (TTRs); these describe how richness increases with the time over which the community is monitored[20 21] (see supplementary materials and methods).

Distance–decay relationships (DDRs) of bacterial community similarity with time (temporal differences in days) were determined. DDRs describe how similarity in taxa composition between two communities varies with a measure of distance (here, the time between pairs of sample collections)[22] (see supplementary materials and methods).

Principal component analysis (PCA), used to identify differences in community structures, was carried out with software developed in house, using the Python programming language with the Numpy package. The principal components were calculated using eigenvalue decomposition of mean-centred and auto-scaled data.

RESULTS

Measures of community species constituency, and clinical data, were compared for sputa collected monthly over a year from 14 patients. Bacterial community measures assessed the changes in the relative abundance of species detected both within individuals over time, and between subjects.

Bacterial diversity

Bacterial species dynamics were determined using selective culture-based analysis and DNA-based profiling. Culture-based microbiological analysis (supplementary table 3) showed that *Pseudomonas aeruginosa* and *Pseudomonas* spp. were the most frequently reported (70.8% of samples). Routine culture also commonly identified 'oral flora' (56.0%). 'Unidentified isolates' (1.2%), 'coliform' (2.4%), '*Stenotrophomonas maltophilia*' (0.6%), 'methicillin-sensitive *Staphylococcus aureus* (MSSA)' (10.7%), '*Staphylococcus* sp.' (1.8%) and '*Streptococcus* Group F' (0.6%) were also reported. No significant correlations were found between

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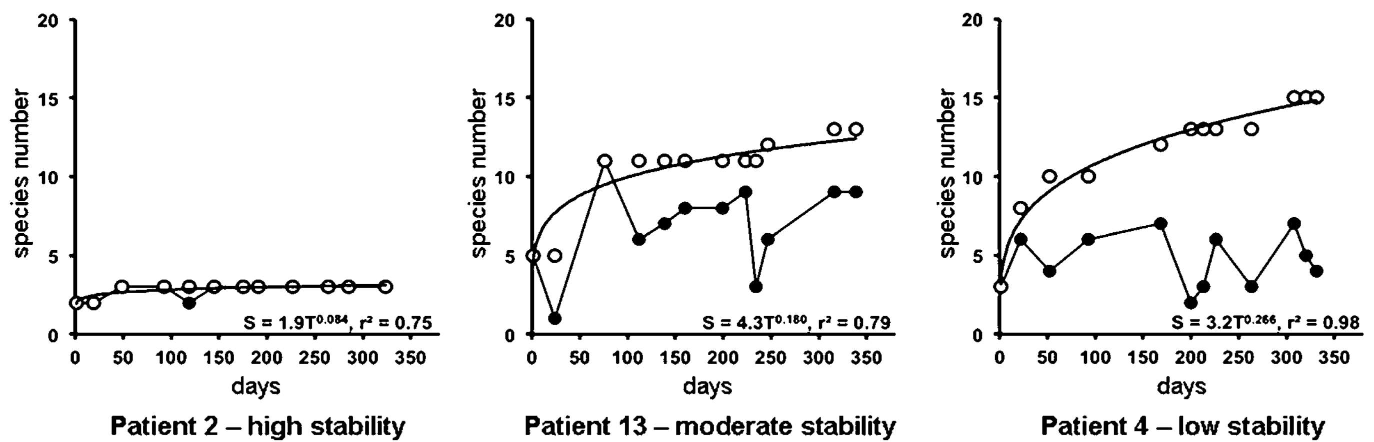

Figure 1 Species richness dynamics and taxa–time relationship. Species richness (black circles, black line) and the cumulative species richness (white circles, no line) are shown. Power regression lines were fitted to the cumulative species richness. Also given are the taxa–time relationship power law equation ($S = cT^w$), and coefficients of determination (r^2). All regression coefficients were significant ($p<0.001$).

species detected by this method and clinical status, or in the coculture of separate species (Pearson correlations with Bonferroni corrections).

The second tool used to analyse bacterial species dynamics was the culture-independent technique, T-RFLP. T-RFLP provides measures of species constituency when based on 16S rRNA gene analysis. In the 168 samples, a total of 1158 T-RF bands were detected, representing 37 distinct T-RF lengths (each representing one or more species). The mean number of T-RF bands in profiles from individual patients over the study ranged from 2.6 (SD 2.4, n=12) (patient 12) to 12.7 (SD 1.6, n=12) (patient 10), with an overall mean of 6.9 (SD 3.9, n=168).

T-RFLP data were used to characterise which species were present over the study period in each subject. In the following sections, data for three patients are selected to represent the types of community dynamics observed; all other data are presented as supplementary information. These patients represented the highest (patient 2) and lowest (patient 4) relative bacterial community stability (Bray-Curtis similarity index), and the relative stability closest to the group mean (patient 13).

Community change over time

TTR analysis identified the cumulative number of species detected in each sample (figure 1 for patients 2, 4 and 13; remainder in supplementary figure 1). For the overall dataset, scaling component values (*w*) were observed to range from 0.05 (patient 6) to 0.38 (patient 12) (supplementary figure 1). This indicated that some patients acquired new species more frequently than others. No significant relationship was identified however between the number of species at the beginning of the study and the subsequent rate of new species acquisition (pairwise Pearson correlation across all patients, p=0.699, r^2=0.5).

The overall change in community composition over the 12-month sampling period was assessed using the inverse of the Bray-Curtis similarity index. This was applied to all possible pairs of samples from individual subjects and the mean of each subject's resulting values calculated for the year (n=11) (results expressed as % change; supplementary table 4). While the mean change in bacterial composition for the group overall was 35.5% (SD 18.2%, n=154 samples), low levels of change were seen in certain patients (eg, patients 2, 3 and 5 had 4.3%, 18.1% and 18.8% change respectively; supplementary table 4), with higher relative levels of change seen in other patients (eg, patients 4 and 14 had 70.1% and 64.3% change, respectively; supplementary table 4). Together, these results indicated that bacterial community variation is inherently different among patients with CF. No relationship was identified between mean richness of a patient's community and its variability over time (Pearson correlation, p=0.252, R=0.389).

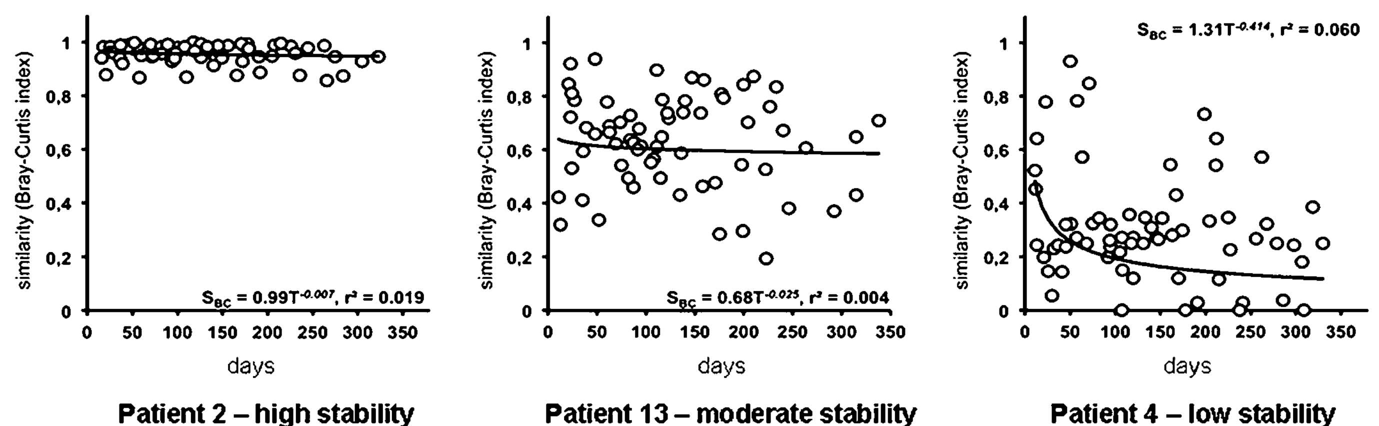

Figure 2 Distance–decay relationships. The distance–decay of bacterial community similarity with time. Power regression lines were fitted, and power law regression equations ($S_{BC} = cT^d$) and coefficients of determination (r^2) are given in each instance. Only two relationships were significant (p=0.040 for patients 4 and 7).

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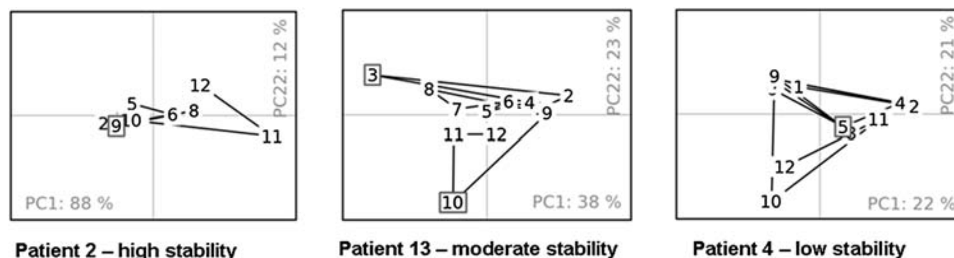

Figure 3 Principal components analysis of individual patients. Plots for community profiles by component 1 (PC1) and component 2 (PC2) are shown. The percentage of variance in the data explained by each component is indicated on the relevant axis. Sampling points are indicated with numbers 1–12, with samples collected during pulmonary exacerbations indicated by boxed numbers.

To test whether similarity between samples was due solely to the time interval between sample collection, DDR were used (figure 2 and supplementary figure 2). A statistically significant distance-decay relationship was identified for two subjects (4 and 7, p=0.040 for both). For all other subjects, no relationship between community composition at two given sample points and time interval between them was found (supplementary table 5). This suggests that the community stability observed was not simply a function of sampling frequency.

PCA was performed on community data from each subject (figure 3 and supplementary figure 3). While the communities in each patient varied over time, no trends within patients were observed consistently across the patient group as a whole. Further, samples collected during periods of exacerbation were not found consistently to be significantly different from non-exacerbation communities (Pearson correlations for community richness, p=0.216, R=0.501, structure (slope), p=0.056, R=0.765, and *P aeruginosa* relative abundance, p=0.023, R=0.648). PCA analysis on data from all subjects are shown in figure 4. When the two components that explain the highest levels of variance are examined, the sample points from individual patients cluster more tightly with each other than with communities from other subjects in a number of cases. The relatively small proportion of total variation represented by any individual component (14% for component 1) indicates that bacterial community differences between patients cannot be ascribed to differences in a small number of correlated factors, but rather are likely to represent many unrelated factors, each contributing a small amount of variance.

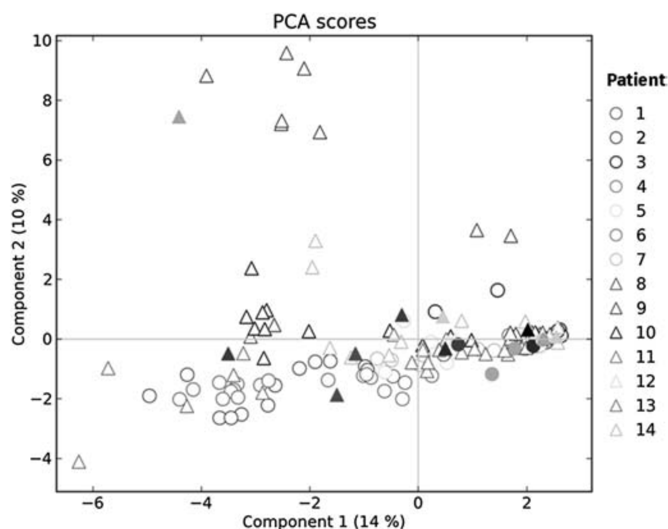

Figure 4 Principal components analysis (PCA) of all subjects. The variance explained by each component is shown in brackets. Solid shapes indicate samples collected during pulmonary exacerbations.

Clinical data

Figure 5 shows the variation in FEV_1, PRO measures and antibiotics for patients 2, 4 and 13 respectively; data for all patients are in supplementary figure 4, with FEV_6 data shown in supplementary figure 5. The FEV_1 (% predicted) values ranged from 5.6% (patient 9) to 94.2% (patient 10), mean 49.1% (SD 17%, n=163). FEV_6 (litres) values ranged from 0.56 (patient 2) to 3.85 (patient 10), mean 2.27 (SD 0.79, n=162). To reduce the effect of short-term lung function fluctuations, a weekly mean for FEV_1 and FEV_6 was also calculated (here, the mean was calculated for all measurements 3 days preceding and following each sampling point). PRO scores are also displayed as a sum of the respective values for the component symptom scores (breathlessness, sputum production, cough and general wellbeing).

Apart from patients 1, 2 and 11, for whom a substantial decline was observed, lung function for the other patients was relatively stable over the year. PRO scores varied greatly between patients, however no clear relationship was observed in relation to lung function changes.

Correlating clinical and microbial datasets

Airway community characteristics were correlated with clinical characteristics. Subjects were separated into higher and lower halves based on FEV_1, with these resulting groups examined for community characteristic differences. Five of the six patients with FEV_1 above the median (patients 1, 6, 10, 11 and 14) shared three or more of the following characteristics: species number >7, a mean relative *P aeruginosa* abundance of <35%, a TTR value of <0.1, and a DDR value of <0.1 with no statistical significance. All of these patients had experienced at least three CFPEs, except patient 6 (two CFPEs), and showed a change in community composition over the year above the median (31.7%), except patient 6 (24.8%). In contrast, only one subject with a low mean FEV_1 (patient 9) had four of these criteria.

The statistical significance of these relationships was tested through Pearson's coefficient correlation analyses (table 1). Three significant relationships were identified after the application of Bonferroni correction; higher PRO scores reported by women than men (p=0.002, R=0.758); a steeper rank abundance slope (the slope of the regression line fitted to species abundance ordered from highest to lowest abundance) with detection of low species number (p=0.002, R=−0.763) and a steeper rank abundance slope correlated with high *P aeruginosa* relative abundance (p=0.002, R=−0.763).

In addition, pairwise comparisons between clinical and microbiological data were performed across all 168 samples independent of patient identity. Significant relationships after Bonferroni correction between FEV_1 and FEV_6 and higher species number per sample, steeper rank abundance slope, and lower *P aeruginosa* relative abundance were identified (table 1).

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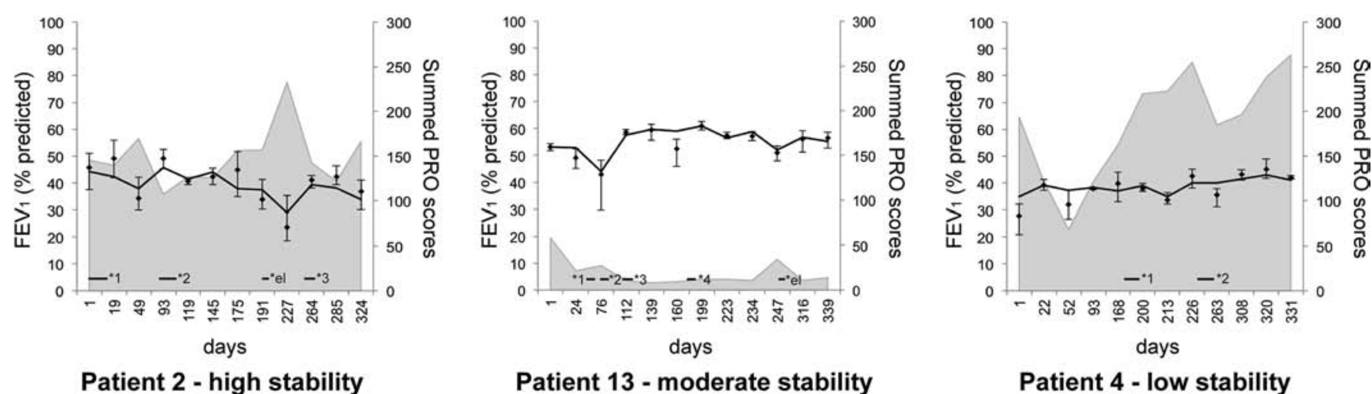

Figure 5 Forced expiratory volume in 1 s (FEV_1), patient reported outcomes (PROs) and antibiotic treatment at sampling. Lung function FEV_1 (litres) (black diamonds) is shown at each sampling point, and a calculated weekly mean FEV_1 (black solid line). The individual patient reported outcome scores (breathlessness, sputum production, cough, general wellbeing) were summed for each sampling point and are shown as grey areas (the higher the score, the worse the symptoms). Pulmonary exacerbations with antibiotic treatments are indicated as black dashes at the bottom of the graph with the details for each pulmonary exacerbation (marked with an asterisk and the respective number) shown in supplementary table 1.

DISCUSSION

This study defined the stability of communities over time in relation to host and clinical characteristics. In general, while some degree of overlap was observed, the airways of individuals with CF contained communities that differed from those in other subjects, often despite similar clinical courses. In contrast, subjects' bacterial communities changed little over the year, despite intervening respiratory exacerbation periods; the changes observed during antibiotic treatment did not persist, returning to approximate pretreatment structures within a month.

Of the previous studies that applied culture-independent methods to examine the microbiota of CF airways,[3 5 6 23 24] two focused on the relationship with lung disease severity. In agreement with these studies,[10 11] subjects with higher lung function in our study tended to have more diverse airway communities and vice versa. Furthermore, as also shown previously,[10 11 23] *P aeruginosa* was associated with lower community richness and lower lung function.

The feature that distinguished this study from previous work was its longitudinal analysis of viable cells. Most earlier studies identified the bacteria in individual CF samples in cross-sectional analyses or, less often, in two or more samples taken over a short time period (eg, in treatment of exacerbation).[8 12] Tunney *et al*[12] applied a combination of T-RFLP and culture to demonstrate that sputum communities changed little during and soon after antibiotic treatment. While this and other short-term studies were revealing, a longer-term study of multiple subjects was required to define long-term airway bacterial dynamics. We

Table 1 Summary of significant correlations*

Correlate 1	Correlate 2	Pearson correlation coefficient (r)	Significance (p)	n
Significant patient-specific pairwise correlations				
Clinical: clinical correlations				
FEV_1	Temperature	0.539	0.047	14
FEV_6	No. CFPEs	−0.573	0.032	14
No. CFPEs	Age	−0.533	0.05	14
Genotype	Age	0.652	0.012	14
PRO sum	Gender	0.758	0.002*	14
BMI	Nebulised colomycin	0.621	0.018	14
Clinical: microbiological correlations				
FEV_6	Rank abundance slope	−0.602	0.023	14
FEV_6	Species number	0.549	0.042	14
Microbiological: microbiological correlations				
Pseudomonas aeruginosa T-RF %	Rank abundance slope	−0.763	0.002*	14
P aeruginosa T-RF %	Species number	−0.763	0.002*	14
P aeruginosa T-RF %	Bray-curtis similarity	−0.715	0.004	14
Species number	Rank abundance slope	−0.711	0.004	14
Significant patient independent pairwise correlations across all samples				
FEV_1	Species number	0.771	0.0001	168
FEV_6	Species number	0.748	0.0001	168
FEV_1	Rank abundance slope	−0.672	0.0001	168
FEV_6	Rank abundance slope	−0.712	0.0001	168
FEV_1	*P aeruginosa* T-RF %	−0.706	0.0001	168
FEV_6	*P aeruginosa* T-RF %	−0.691	0.0001	168

*Correlations for which $p<0.05$ after Bonferroni correction.
BMI, body mass index; CFPE, cystic fibrosis pulmonary exacerbation; FEV_1, forced expiratory volume in 1 s; FEV_6, forced expiratory volume in 6 s; PRO, patient-reported outcome; T-RF, terminal restriction fragment.

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sought to distinguish transient from chronic infectious characteristics, as the latter are more likely to be associated with lung disease progression. While Sibley *et al*[6] previously analysed bacterial dynamics over a period comparable to that examined here, their study focused on a single individual, precluding generalisation and inter-subject comparison.

Further, we focused on only viable cells[18 19] to ensure an accurate characterisation of community stability. This minimised the contribution of dead bacteria, for example, from antibiotic treatment or immune response. Thus, bacterial species consistently observed can be described with some confidence as being chronically present in the CF lower airways.

Given the many perturbations experienced by the airway microbiota in an adult patient (including antibiotic therapy, immune response, and cough clearance), we hypothesised that each subject's bacterial community would vary greatly in membership over time. Cross-sectional analyses of bacterial communities in CF airways have reported a wide range of bacteria.[10 11] Such variation could be explained by high species turnover. Surprisingly though, we found CF lung bacterial communities to be, to a large extent, stable over a year.

In contrast, the composition of airway communities differed among patients. Interestingly, this was reminiscent of findings from other mucosal microbial communities, for example, the gastrointestinal tract.[25] The reasons for this stability within patients, and divergence between patients, are unknown; however, disease severity and its correlate, treatment history, may play selective roles. We also observed that CF airway bacterial communities were resilient. While antibiotic therapy can have a significant short-term impact on CF lung communities,[8 12] our data indicate that these communities approach pretreatment configurations rapidly.

Our results also extend the previous finding that CF lung community richness is inversely correlated with lung disease severity.[10] Neither community richness nor lung function changed significantly within subjects during the study. As such, we could not define the contribution of individual species, or specific communities, to lung disease pathogenesis. However, we did find an inverse relationship between community richness and lung function, similar to previous cross-sectional studies.[11] This is reminiscent of relationships found for other mucosal microbial communities.[26 27] In this regard, while no causality can or should be inferred, the current results may indicate that CF lung health is highest in the presence of a diverse microbiota. Alternatively, repeated antibiotic exposure, or the toxic products of pathogens such as *P aeruginosa*, could be responsible for community simplification. Antibiotic treatment could not be controlled for in this study. As such, further investigation of the relationships observed between community richness and disease severity is now warranted.

In summary, we found that the species that constitute CF airway communities are present for prolonged periods, and that communities can change little over a year, despite antibiotic treatment. This stability indicates that much of the wider bacterial diversity seen in airway samples does not represent short-term colonisation.

Contributors FS, GR, CvdG, PM, LV, MC, LH, TD, NP, BF and KB were all involved in the conception, design, interpretation of data, and revising and final approval of the article. In addition, CvdG and LV were responsible for statistical analysis, and TD for sample and metadata collection. GR is the guarantor. In addition, collaborator KD was involved in the processing of samples, PC in data processing.

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Competing interests None.

Patient consent Detail has been removed from this case description/these case descriptions to ensure anonymity. The editors and reviewers have seen the detailed information available and are satisfied that the information backs up the case the authors are making.

Ethics approval Ethics approval was provided by Southampton and South West Hampshire Research Ethics Committee.

Provenance and peer review Not commissioned; externally peer reviewed.

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Expanded Materials and Methods

Clinical Samples

This observational study of 14 adult CF patients over 12 months was undertaken with ethical approval from Southampton and South West Hampshire Research Ethics Committee (06/Q1704/26). Eligible subjects were aged 18 or over and had experienced a minimum of 3 pulmonary exacerbations (CFPE) requiring antibiotic treatment in the prior 12 months. Samples from these patients were previously used in an analysis of core and satellite bacterial taxa [10].

Patient clinical details are summarised in Supplementary Table 1. The start of a CFPE was defined by the clinician's decision to initiate antibiotic therapy for deteriorating clinical status, broadly based on a range of factors described previously [15]. In turn, the end of CFPE was defined by the decision to cease antibiotic therapy due to stabilisation or improvement in signs and symptoms. A total of 39 episodes of CFPE were experienced by these patients over the year. The number of CFPE ranged from zero (Patients 3 and 8) to 6 (Patient 9) with a mean of 2.8 ± 1.4. In total, 17 different antibiotics combinations were used with five "elective" antibiotic courses given for reasons other than worsening of respiratory symptoms (Supplementary Table 1).

One sputum sample was obtained from each patient at approximately monthly intervals. The majority of samples (65%) were collected at least 21 days prior to or 21 days after stop of antibiotics for CFPE (timings shown in Supplementary Table 2). However, it was not always possible to obtain samples within these limits and 10% of samples were collected that lay 14-20 days prior or post CFPE, 12% of samples were collected 7-13 days prior or post CFPE, 5% of samples were collected 0-6 days prior or post CFPE and 8% of samples were taken during antibiotic courses for CFPE. All sputum samples were stored at 4°C (immediately after expectoration), shipped at 4°C (in accordance with the sputum handling guidelines [16]) and stored at -80°C prior to processing or cultured by the UK Health Protection Agency (HPA) South East, in accordance with HPA standard operating procedures.

Clinical data

Clinical measures (FEV_1 and FEV_6, temperature, and patient reported outcome (PRO) scores (“breathlessness”, “cough severity”, “sputum production”, and “general well-being”) were recorded using visual analogue scores (VAS) by means of a symptom sheet. VAS were recorded in millimetres from 0 (no symptoms) to 100 (worst symptoms).

Culture-independent analysis

Exclusion of DNA from non-viable cells in sputa via cross-linking using propidium monoazide [17,18] and subsequent nucleic acid extraction were performed as described previously [19]. Specifically, PMA was dissolved in 20% dimethyl sulfoxide to create a stock concentration of 20 mmol/L with this added to 500 µL of heat-killed cells to give a final concentration of 50 µmol/L. Sputum samples and bacterial suspensions were transferred to 24-well, flat-bottomed, cell culture cluster plates (Corning, Corning, NY) for exposure to light. Following an incubation period of 30 min in the dark with occasional mixing, samples were light exposed using LED Active Blue equipment (IB - Applied Science, Barcelona, Spain). After photo-induced cross-linking, cells were transferred to 1.5-mL microfuge tubes and pelleted at 5000 × g for 5 min prior to DNA isolation.

Nucleic acid extractions were performed on 100 µL portions of sputum. Guanidinium thiocyanate–EDTA–sarkosyl (500 µL) and PBS (500 µL), pH 8.0, were added to samples. Cell disruption was achieved using a Fastprep-24 Instrument (MP Biomedicals Europe, Illkirch, France) 6.5 m/s, 60 s, followed by incubation at 90 °C for 1 min and −20 °C for 5 min. Cell debris was pelleted by centrifugation at 12 000 × g for 2 min at 4 °C. Supernatant was transferred to a fresh microfuge tube. NaCl (to a final concentration of 0.5 mol/L and polyethylene glycol (to a final concentration of 15%)were added and DNA precipitated at 4 °C for 30 min. DNA was pelleted by centrifugation at 12 000 × g for 2min at 4 °C and resuspended in 300 µL of sterile distilled water. Samples were heated at 90 °C for 30 s and vortexed. Phenol/chloroform (1:1) (300 µL) was added, and samples were vortexed for 20 s before centrifugation at 12 000 × g at 4 °C for 3min. The upper

phase was then transferred to a fresh microfuge tube. Total DNA was then precipitated by the addition of an equal volume of isopropanol, a 0.1-volume 10 mol/L ammonium acetate, and 1 µL of GenElute linear polyacrylamide (Sigma-Aldrich, Gillingham, UK) and incubated at −20 °C for 25 min. DNA was pelleted by centrifugation at 12 000 × g at 4 °C for 5 min. Pelleted DNA was then washed 3 times in 70% ethanol, dried, and resuspended in 50 µL of sterile distilled water. DNA extracts were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK).

PCR amplification and T-RFLP profiling were carried out as previously described [1]. Specifically, the oligonucleotide primers used to amplify a region of the 16S rRNA gene for members of the domain Bacteria, 8f700 and 926r (5'-CCG TCA ATT CAT TTG AGT TT-3'), were described previously (24). Primer 8f700 was labelled at the 5' end with IRD700; primer 926r was unlabeled. PCR mixtures comprised 1x PCR buffer, 1.5 mM MgCl2, each deoxynucleoside triphosphate at a concentration of 0.2 mM, each primer at a concentration of 0.2 mM, and 1 U of RED*Taq* DNA polymerase (Sigma-Aldrich) in a final volume of 50 µl. An initial denaturation step of 94°C for 2 min was followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min, with a final extension step at 72°C for 10 min. Amplification was carried out by using a GeneAmp PCR System 2400 (Perkin-Elmer), with PCR products for T-RFLP analysis stored at -20°C after verification on Tris-acetate-EDTA–agarose gels as described above. PCR products (ca. 20 ng) were digested by using the restriction endonuclease CfoI (Roche, Lewes, United Kingdom) for 3 h at 37°C with the reaction buffer supplied by the manufacturer. All restriction endonuclease digestions were carried out to complete digestion as shown by comparing PCR products after various digestion incubation times (data not shown). The restriction endonuclease was inactivated by heating at 90°C for 20 min. An approximately 0.7-µg portion of T-RFLP PCR products was separated by length by using a 25-cm SequagelXR denaturing acrylamide gel (National Diagnostics) prepared in accordance with the manufacturer’s instructions with the addition of 8.3 M urea and 10% (final concentration) formamide and by using an IR2 automated DNA sequencer (LI-COR Biosciences) at 55°C and 1,200 V.

T-RFLP gels were analyzed by using Phoretix one-dimensional advanced software, version 5.10 (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). The sizes of the bands resolved by T-RFLP were determined by comparing their relative positions with two sets of size markers, one set that formed bands at 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, and 1,000 bases (microSTEP 15a [700 nm]) and one set that formed bands at 155, 209, 214, 238, and 364 bases (microSTEP custom GR [700 nm]). Both sets of size markers were obtained from Microzone (Lewes, United Kingdom). In addition, this software was also used to determine the volume of each band (with band volume being the product of the area over which a band was detected and the intensity of signal recorded over that area). Band volume was expressed as a percentage of the total volume of bands detected in a given electrophoretic profile. T-RFLP bands were resolved over the region between 50 and 958 bases. No bands shorter than 50 bases in length were recorded, as they were in the region susceptible to high levels of signal stemming from the IR tag on either unattached or nonused primer 8f700IR. In this study, the threshold used to detect bands was 0.01% of the total signal between the 50- and 958-base region.

MapSort (Wisconsin Package, version 10.3; Accelrys), was used to identify *Hha*I restriction sites *in silico* in all of the 2,137 16S rRNA gene sequences from clone library construction, and to predict T-RFLP band lengths for each clone. Species or genus level identities were assigned to T-RF bands from sputum sample profiles on the basis of these predicted band lengths. 16S rRNA gene clone data generated previously from a subset of the samples analysed here was published previously [6].

Statistical analysis

Bacterial species richness (the number of bacterial species in a sample) was inferred from the number of T-RF bands present in a sample as previously described [20]. The cumulative bacterial taxon richness (the total number of different bacterial taxa identified after each successive time point) was assessed using taxa-time relationships (TTR), which describe how the taxon

richness of a community increases with the length of time over which the community is monitored [20,21]. TTR are modelled with the power law equation, $S = cT^w$, where S is the cumulative number of observed taxa over time T, c is an empirically derived taxon- and time-specific constant, and w is the slope of the fitted curve or temporal scaling exponent (the rate of observation of new bacterial taxa over the course of sampling); therefore, increasing values of w reflect greater turnover rates. Power law regression coefficients of determination (r^2) and significance (P) values were calculated using Minitab software (version 14.20; University Park, PA).

To examine similarities and differences in bacterial community composition over time, we employed distance-decay relationships (DDR). DDR describes how similarity in taxa composition between two communities varies with a measure of distance (here, the time interval between pairs of sample collections) [22]. Similarities and differences in community composition were determined using the Bray-Curtis quantitative index of similarity using the PAST program available from the University of Oslo website link (http://folk.uio.no/ohammer/past) run by Øyvind Hammer. Power law coefficients of determination (r^2) and significance (P) were calculated using Minitab software (version 14.20, Minitab, University Park, PA, USA).

Pairwise Pearson's correlation analyses were performed to assess whether clinical or microbiological factors showed significant relationships i) across patients, and ii) across samples independent of the patient from which they came. All Pearson's correlation analysis and Bonferroni corrections where applied were carried out in SPSS version 15.0.1 (SPSS Inc., Chicago, IL). Correlations across the patients (n = 14) assessed the following parameters – clinical: age, genotype, gender, BMI, number of CFPE, mean FEV_1, mean FEV_6 and mean temperature over the 12 month study period, mean individual and summed PRO scores over the year, sample status, antibiotics and long-term medications administered over the year and time, - microbiological: averaged diagnostic microbiology results, mean species number, mean Bray-Curtis similarity index, TTR and SDR slope, mean rank abundance slope and mean relative abundance of *P. aeruginosa* in the T-RFLP profile. Correlations across all samples (n = 168) assessed relationships between data

corresponding to each of the 12 sampling points for clinical parameters - FEV_1, FEV_6, temperature, individual and sum of the PRO scores, sample status, and antibiotics and long-term medications administered; and microbiological variables - diagnostic microbiology results, species number, Bray-Curtis similarity index, rank abundance slope of the community and relative abundance of *P. aeruginosa* in the T-RFLP profile.

To test the robustness of Pearson's correlations, Mantel tests were performed. Specifically, Mantel proximity matrices were constructed for community similarity (Bray-Curtis index of similarity) and then FEV_1, FEV_6, temperature, summed PRO scores, sample status (whether samples were taken during clinical stability or how long (days) before/after CFPE), antibiotics (or combinations thereof) administered, temporal distance between samples, species number, rank abundance slope (see below), and relative abundance of *P. aeruginosa* within in the T-RFLP profile, performed as previously described [10] using XLSTAT 2010 (Addinsoft, France). The results of these Mantel tests corroborated the findings from the Pearson's correlations, with no evidence of auto-correlation was observed ($P > 0.05$).

Principal component analysis (PCA), used to identify and quantify differences in microbial community structures, was carried out with software developed in-house, using the Python programming language (version 2.7.2) with the Numpy package (version 1.5.1) as described previously (Louic S Vermeer, Gilbert O. Fruhwirth, Pahini Pandya, Tony Ng, and Andrew James Mason. NMR metabolomics of MTLn3E breast cancer cells identifies a role for CXCR4 in lipid and choline regulation *J. Proteome Res* In press). Information of the development of these packages is available at www.python.org and www.numpy.scipy.org, respectively. The principal components were calculated using eigenvalue decomposition of mean-centred and auto-scaled T-RFLP profile band percentage values. Source code for in-house software can be provided on request.

Supplementary Table 1. Patient information[1].

subject	age	gender	genotype I	genotype II	mean FEV_1 (%predicted) for the year	BMI	diabetes	number of CFPE over the period months studied	CFPE	CFPE occurrence (days) from study start, across full collection period	short term medications		long term medications								
											PO antibiotics	IV antibiotics	macrolide	colomycin neb	tobramycin neb	col/tob neb (alt months)	itraconazole	oral corticosteroids	inhaled corticosteroids	DNAse	hypertonic saline
1	30	male	phe508del	unknown	54.9	29	no	3	1	95-130	doxy, azit, cipr	fluc, gent, tobr, mero	no	no	no	no	no	no	yes	yes	no
									2	151-161	cipr, fluc										
									3	237-256	cipr, doxy										
2	45	female	phe508del	unknown	40.2	18.5	yes	3 (+1[a])	1	1- 14		ceft, tobr	yes	yes	no	no	no	no	yes	no	no
									2	88-105		colo, tobr									
									El	191-198	doxy										
									3	254-268		colo, tobr									
3	47	male	phe508del	unknown	33.9	20.7	yes	0	0				yes	yes	no	no	no	no	yes	yes	no
4	30	female	phe508del	711+3A7G	38.2	25	no	3	1	175-189	co-am, fluc		yes	no	no	no	no	no	yes	yes	no
									2	235-249	doxy										
									3	349-362	doxy										
5	22	female	phe508del	phe508del	36.2	19	no	5	1	62-105		colo, ceft, mero, tobr, amik	yes	yes	no	no	yes	no	yes	yes	no
									2	126-135	doxy										
									3	153-167	doxy										
									4	215-239	cipr	mero, amik									
									5	322-345	doxy	mero, amik									
6	55	male	phe508del	G85E	52.2	24.5	no	2	1	210-220	cipr		yes	yes	no	no	no	no	yes	yes	no
									2	251-261		ceft, gent									

[1] "neb" – nebulised, "El" – elective antibiotics, [a] - for sinus infection, [b] - for skin infection, [c] - as maintenance therapy, [d] - for haemoptysis. "amik" – amikacin, "amox" – amoxicillin, "azit" – azithromycin, "ceft" – ceftazidime, "cipr" – ciprofloxacin, "clar"– clarithromycin, "co-am" – co-amoxiclav, "colo" – colomycin, "doxy" – doxycycline, "eryt" – erythromycin, "fluc" – flucloxacillin, "mero" – meropenem, "metr" – metronidazole, "pipe" – piperacillin, "tazo" – tazobactam

7	21	female	phe508del	phe508del	56.6	19	no	4	1	68-78		mero	yes	yes	no	no	no	no	yes	yes	no
									2	151-160	colo, mero										
									3	237-250		doxy									
									4	320-334		doxy									
8	40	male	phe508del	phe508del	50.6	19.4	yes	0 (+1[b])	El	274-280	fluc		yes	yes	no	no	no	yes	yes	no	no
9	22	male	phe508del	phe508del	16.5	17.9	yes	6 (+1[c])	1	85-93	amox	ceft	yes	yes	no	no	no	no	yes	yes	yes
									2	139-151		colo, ceft									
									3	198-212		mero, colo									
									4	256-279	co-am	mero, colo									
									5	336-357		mero, colo									
									El	396-410		mero, colo									
									6	440-442		mero, colo									
10	18	female	phe508del	phe508del	84	22.5	no	2 (+1[c])	El	106-124		ceft, gent	yes	no	no	yes	no	no	yes	yes	no
									1	247-263	cipr										
									2	334-344		ceft, gent									
11	24	female	phe508del	G542X	72.5	23.4	no	3	1	79-93	eryt		yes	yes	no	no	no	no	yes	yes	no
									2	177-194	clar										
									3	205-222		colo, pipe/tazo									
12	20	male	phe508del	phe508del	26.8	30.4	no	4	1	13-25		ceft, gent	yes	no	yes	no	no	no	no	no	no
									2	118-133	cipr										
									3	159-174	cipr, metr										
									4	192-203		ceft, gent									
13	20	male	phe508del	phe508del	54.4	21	no	4 (+1[b])	1	39-49		ceft, tobr	no	no	no	yes	yes	yes	yes	yes	no
									2	70-83		ceft, gent									
									3	103-119	co-am										
									4	173-184		ceft, gent									
									El	245-261		ceft, gent									
14	23	male	phe508del	phe508del	53.6	20.7	yes	3 (+1[d])	1	1- 27		mero, tobr	no	yes	no	no	no	no	yes	no	no
									2	123-133		meropene, tobr									
									3	237-254		ceft									
									El	299-309		meropene, tobr									

Supplementary Table 2. Sample information.[2]

subject	sample	days since study start	clinical status	subject	sample	days since study start	clinical status
1	1	1*	stable	**08**	1	1*	stable
	2	24	stable		2	18	stable
	3	50	stable		3	46	stable
	4	80	3 days prior CFPE		4	76	stable
	5	120	8 days post CFPE		5	109	stable
	6	141	start of CFPE (prior to abx)		6	158	stable
	7	188	stable		7	176	stable
	8	220	5 days prior CFPE		8	211	stable
	9	260	15 days post CFPE		9	237	stable
	10	286	stable		10	270	exacerbating (day 6)
	11	323	stable		11	305	stable
	12	344	stable		12	349	stable
2	1	1	16 days post CFPE	**09**	1	1	stable
	2	19*	stable		2	10*	stable
	3	49	13 days prior CFPE		3	31	stable
	4	93	17 days post CFPE		4	57	stable
	5	119	stable		5	89	stable
	6	145	stable		6	176	12 days prior CFPE
	7	175	stable		7	218	15 days post CFPE
	8	191	stable		8	234	12 days prior CFPE
	9	227	exacerbating (day 3)		9	291	stable
	10	264	stable		10	305	stable
	11	285	stable		11	311	15 days prior CFPE
	12	324	stable		12	332	exacerbating (day 6)
3	1	1*	stable	**10**	1	1*	stable
	2	38	stable		2	29	stable
	3	70	stable		3	57	stable
	4	103	stable		4	83	13 days prior CFPE
	5	131	stable		5	134	19 days post CFPE
	6	161	stable		6	144	stable
	7	189	stable		7	181	stable
	8	220	stable		8	204	stable
	9	257	stable		9	221	16 days prior CFPE
	10	283	stable		10	270	16 days post CFPE
	11	306	stable		11	291	stable
	12	348	stable		12	321	3 days prior CFPE
4	1	1*	stable	**11**	1	1*	stable
	2	22	stable		2	31	stable
	3	52	stable		3	52	stable
	4	93	stable		4	83	exacerbating (day 10)
	5	168	exacerbating (day 2)		5	125	stable
	6	200	19 days post CFPE		6	150	stable
	7	213	13 days prior CFPE		7	162	9 days prior CFPE
	8	226	start of CFPE (prior to abx)		8	209	exacerbating (day 10)
	9	263	stable		9	239	stable
	10	308	20 days prior CFPE		10	269	stable
	11	320	9 days prior CFPE		11	304	stable
	12	331	stable		12	337	stable
5	1	1*	stable	**12**	1	1*	13 days prior CFPE
	2	22	stable		2	41	14 days post CFPE
	3	51	7 days prior CFPE		3	62	stable
	4	87	stable		4	98	stable

[2] * - sequenced for assignment of species identities to T-RF band lengths. Where samples were collected between two CFPE less than 42 days apart, the days to the nearest CFPE in time are shown. Samples taken at the start of CFPE were obtained before antibiotics (abx) were given.

	5	113	12 days post CFPE
	6	138	7 days post CFPE
	7	176	13 days post CFPE
	8	204	7 days prior CFPE
	9	228	exacerbating (day 17)
	10	255	19 days post CFPE
	11	285	stable
	12	311	stable
6	1	1*	stable
	2	19	stable
	3	52	stable
	4	84	stable
	5	113	stable
	6	138	stable
	7	171	stable
	8	190	9 days prior CFPE
	9	229	19 days post CFPE
	10	264	13 days post CFPE
	11	311	stable
	12	351	stable
7	1	1	stable
	2	29*	stable
	3	48	stable
	4	80	stable
	5	111	stable
	6	143	exacerbating (day 3)
	7	171	stable
	8	192	stable
	9	213	13 days prior CFPE
	10	272	stable
	11	310	exacerbating (day 1)
	12	351	stable
	5	132	exacerbating (day 13)
	6	148	exacerbating (day 12)
	7	170	2 days post CFPE
	8	193	2 days prior CFPE
	9	225	20 days post CFPE
	10	245	stable
	11	358	stable
	12	371	stable
13	1	1*	stable
	2	24	16 days prior CFPE
	3	76	exacerbating (day 5)
	4	112	stable
	5	139	stable
	6	160	14 days prior CFPE
	7	199	13 days post CFPE
	8	223	stable
	9	234	12 days prior CFPE
	10	247	exacerbating (day 1)
	11	316	stable
	12	339	stable
14	1	1	exacerbating (day 1)
	2	50	stable
	3	62*	stable
	4	90	stable
	5	120	exacerbating (day 3)
	6	155	stable
	7	181	stable
	8	207	stable
	9	231	start of CFPE (prior to abx)
	10	267	stable
	11	316	13 days post CFPE
	12	351	stable

Supplementary Table 3. Microbiological culture data.

patient	sample	gram negative bacilli					gram positive cocci			mixed organisms		fungi and yeasts			
		coliform	*P. aeruginosa* non mucoid	*P. aeruginosa* mucoid	*Pseudomonas* sp.	*S. maltophilia*	*S. aureus* (MSSA[3])	*Staphylococcus* sp.	*Streptococcus* Group F	oral flora	unidentified	*Aspergillus fumigatus*	*Aspergillus flavus*	*Aspergillus* sp.	yeasts
1	1														
	2				1		1					1			
	3									1					
	4														
	5		1		2							1			
	6				1	1				1					
	7						1			1					
	8	1					1			1					
	9	1								1					
	10				1			1			1			1	
	11				1			1			1				
	12						1			1					
2	1		1	1											
	2		1							1		1			
	3				2										
	4														
	5														
	6														
	7				2										
	8		1	1	1					1				1	1
	9														
	10				2		1			1		1			
	11		1	1			1					1			
	12		1		2		1			1		1			
3	1			1	1					1					
	2			1	1					1					
	3			1	1					1					
	4			1	2										
	5				2					1					
	6														
	7														
	8				2										
	9														
	10				2					2					
	11				2					1					1
	12				3					1					
4	1									1					
	2						1			1					
	3														
	4				1										
	5														
	6				1										
	7				1		1			1					
	8									1					

[3] Methicillin sensitive *Staphylococcus aureus*

	9											
	10	2				1		1				
	11				2			1	1			
	12					1	1	2				
5	1		1	1				1				
	2				1			1				
	3			1	1			1				
	4	1			1							
	5				1				1			1
	6				4			1	1			1
	7				1							
	8			1	1							
	9											
	10			1	1			1	1			1
	11			1	1			1	1			
	12				2			1		1		
6	1				1				1			1
	2		1		1				1			1
	3				1			1	1			1
	4											
	5											
	6											
	7				3			1			1	1
	8		1	1	1				1			1
	9											
	10				1						1	1
	11		1		1			1	1			1
	12											
7	1				1			1				
	2			1								
	3		1		1			1				
	4											
	5											
	6		1		1			1	1			
	7				2							
	8		2		2			1				
	9		2					1				
	10				2			1				
	11				2			1				
	12											
8	1				1			1				
	2				2	1		1				
	3			1	1	1			1			1
	4		1	1				1	1			1
	5			1				1	1			
	6			1		1						
	7			1	2	1		1				1
	8											
	9			1	1	1		1				
	10			1	1			1				
	11				2	1		1				
	12				3	1		1				1
9	1				1			1				
	2				1							
	3			1	1							
	4			1	1							

	5		1			1		
	6			2	1	1		
	7							
	8							
	9		1			1		
	10		1	1		1		
	11							
	12		1	1				
10	1		1	1		1		1
	2			1		1		1
	3			1		1		1
	4					1		1
	5			1				
	6		1			1		1
	7							
	8							
	9			3		1		1
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	11							
	12					1		1
11	1		1	1			1	1
	2							
	3			2		1		1
	4							
	5			2		1		1
	6			1		1		1
	7							
	8			2				1
	9			2		1		1
	10			1		1		
	11							
	12		1	1		1		1
12	1			2		1		
	2							
	3							
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	6							
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	4			2				
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	6							
	7	1		1				
	8		1	2		1	1	
	9	1	1	1		1	1	
	10	1	2			1		
	11		1	1		1	1	
	12		1	1		1	1	

14	1			1	1	
	2			1	1	
	3			2	1	
	4		1	2		
	5			2	1	
	6			2	1	
	7		1	2	1	
	8			2	1	
	9			2	1	
	10			2	1	
	11	2			1	1
	12			1	1	

Supplementary Table 4. Overall change in community composition.

subject	overall (mean %) change of the bacterial community over year[4]	standard deviation (%)
1	52.9	15.0
2	4.3	3.3
3	18.1	16.5
4	70.0	16.4
5	18.7	11.2
6	24.8	13.4
7	31.7	19.7
8	25.0	24.7
9	39.5	22.2
10	38.9	18.4
11	45.7	26.4
12	27.4	21.9
13	35.8	19.6
14	64.3	18.2

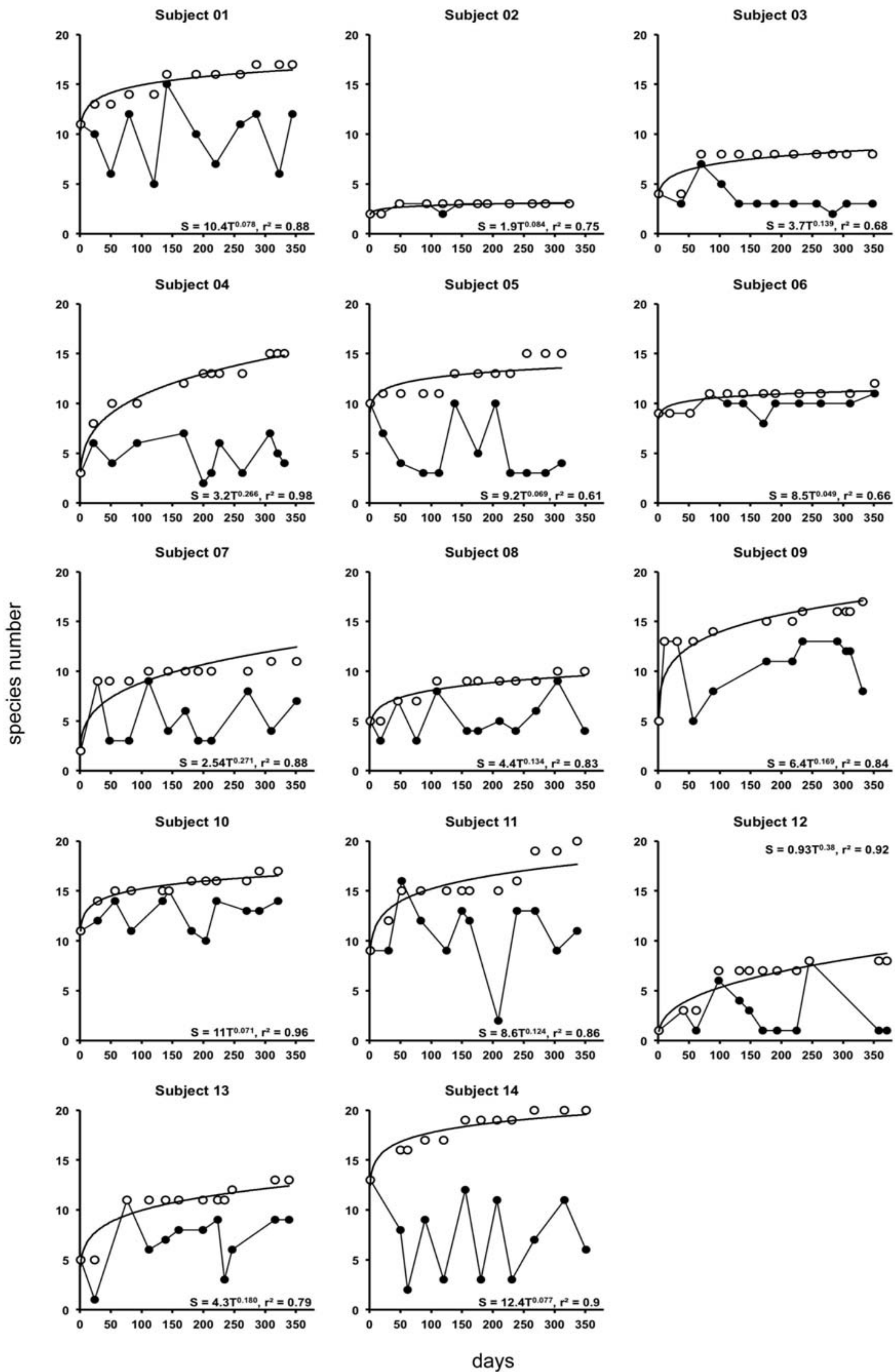
mean inverse of the Bray-Curtis similarity index

Supplementary Table 5. Summary of bacterial community characteristics.[5]

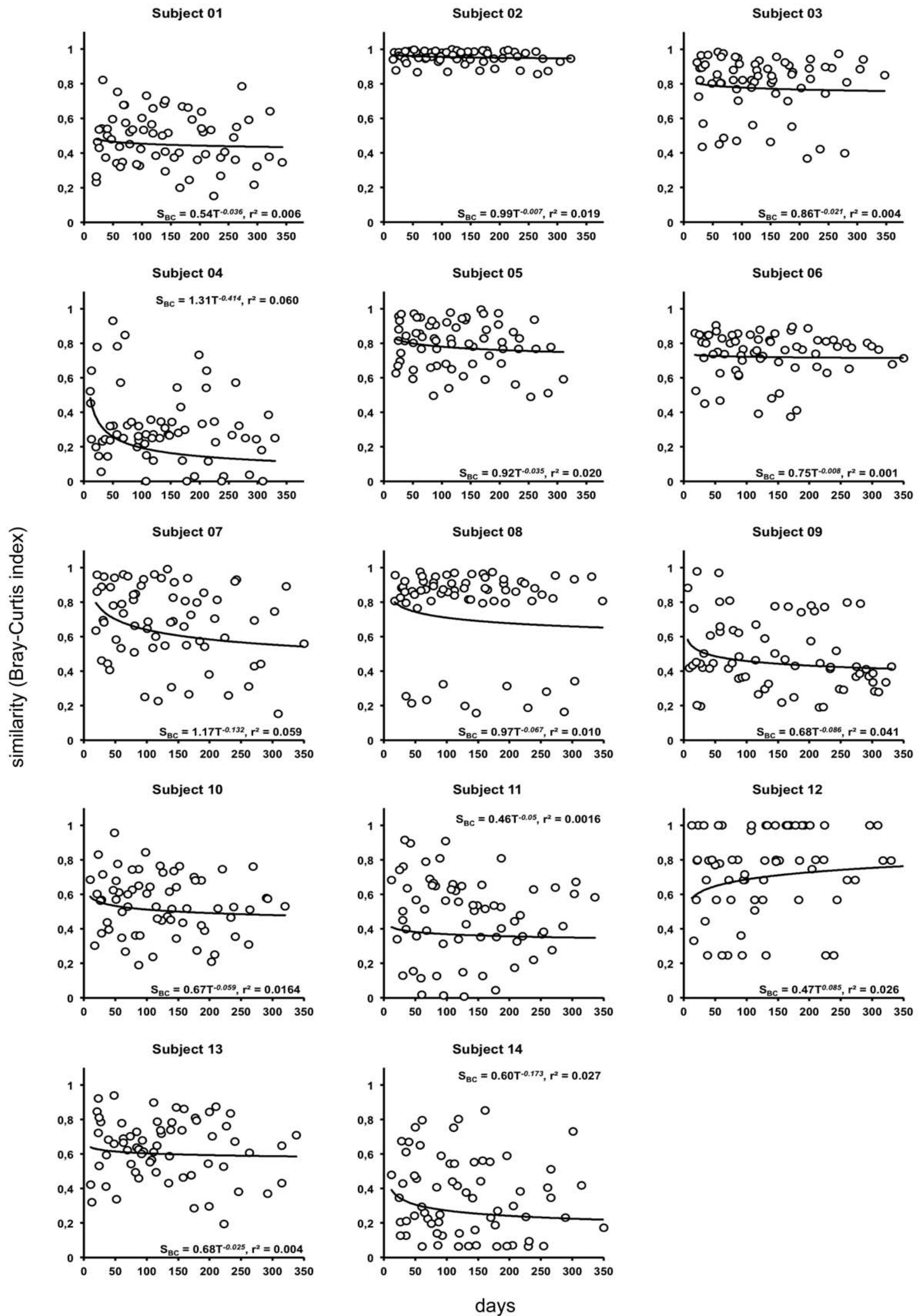
Subject	mean FEV_1 (L)	mean species number	mean *P. aeruginosa* T-RF band %	TTR scaling component (w)	significance of TTR scaling component (*P*)	DDR scaling component	significance of DDR scaling component (*P*)
1	2.13	9.8	4.65	0.078	0.0001	-0.036	0.533
2	0.95	2.8	93.59	0.084	0.0001	-0.007	0.275
3	1.08	3.5	77.22	0.139	0.001	-0.021	0.624
4	1.15	4.7	12.31	0.266	0.0001	-0.414	0.040
5	1.15	5.4	78.63	0.069	0.003	-0.035	0.254
6	1.71	9.8	17.49	0.049	0.001	-0.008	0.810
7	1.82	5.1	64.66	0.271	0.0001	-0.132	0.040
8	1.68	5.2	64.27	0.134	0.0001	-0.067	0.422
9	0.62	10.3	26.48	0.169	0.0001	-0.086	0.102
10	2.81	12.7	21.96	0.071	0.0001	-0.059	0.306
11	2.14	10.7	25.62	0.124	0.0001	-0.05	0.750
12	1.06	2.6	84.11	0.38	0.0001	-0.846	0.197
13	1.87	6.8	51.83	0.18	0.0001	-0.025	0.607
14	2.40	7.3	34.63	0.077	0.001	-0.173	0.186

[5] Shading denotes patients with a mean FEV_1 above the median (1.68 L) that shared at least 4 out of 5 of the following community characteristics: a mean species number > 7, a mean relative abundance of *P. aeruginosa* of < 35%, TTR and SDR values of < 0.1 with no statistical significance.

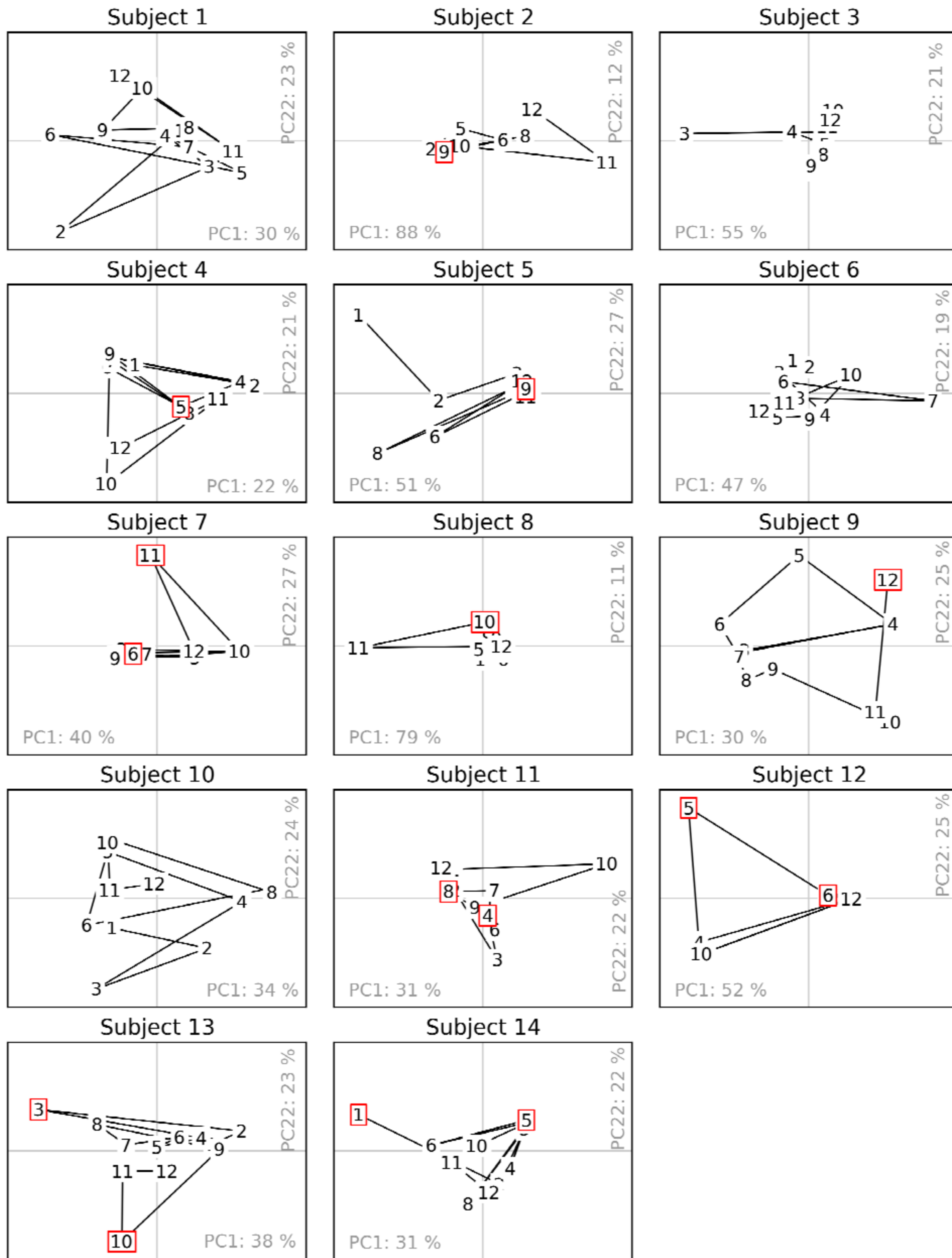
Supplementary Figure 1



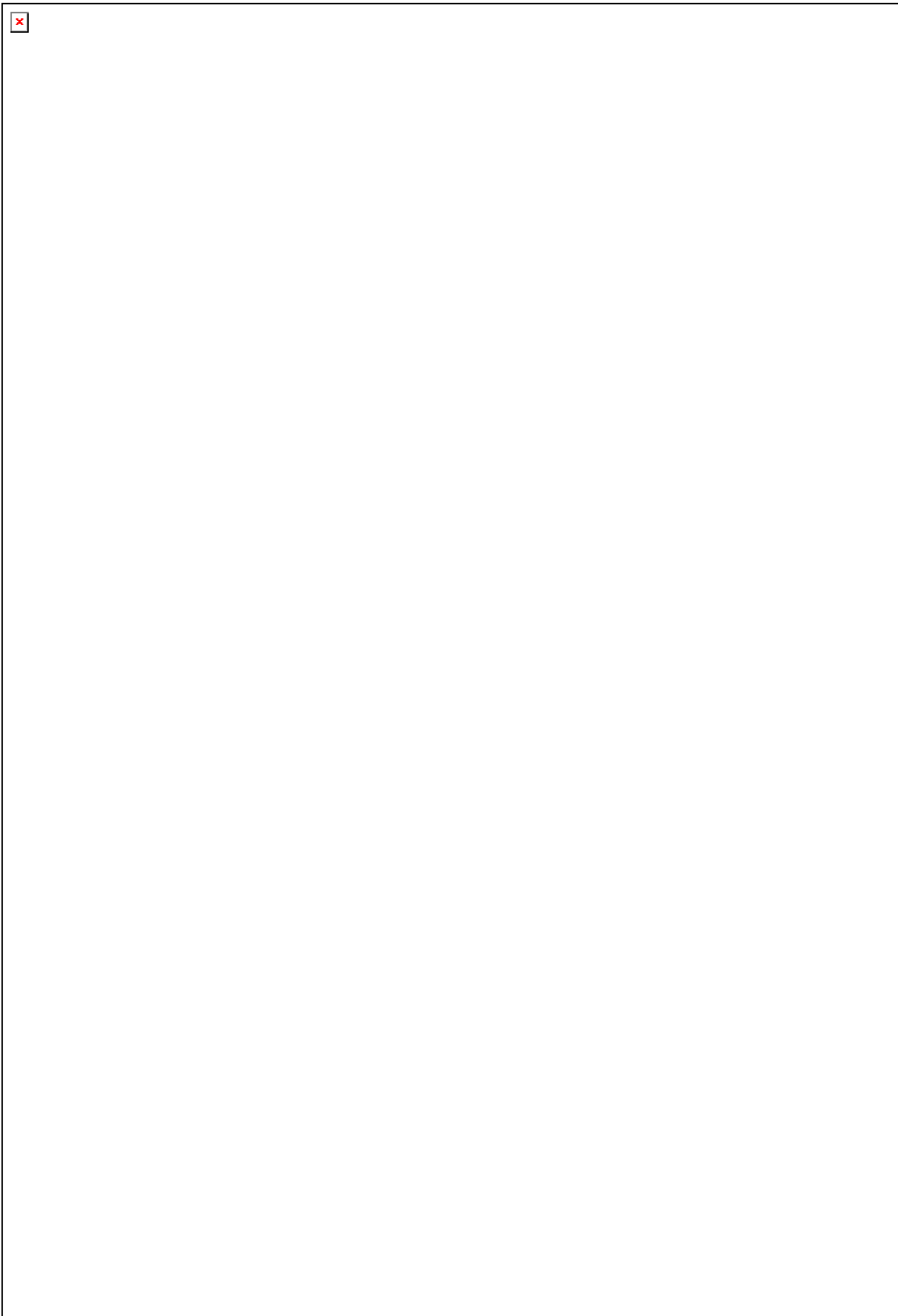
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5