THE LOWER AIRWAY MICROBIOTA IN EARLY CYSTIC FIBROSIS LUNG DISEASE: A LONGITUDINAL ANALYSIS – ONLINE SUPPLEMENT

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RESULTS

Participants: One-hundred and forty-two children were recruited into the initial study. Bronchoscopy and bronchoalveolar lavage (BAL) procedures were performed on a median of two (range 1-4) occasions each. 16S ribosomal RNA gene (16S rRNA gene) amplification and sequencing was performed on 148 available samples from 54 subjects. Fifty-three whole BAL samples from 34 subjects yielded <1000 16S rRNA gene sequences and were excluded from further analysis (Table S2). These samples were collected at median age 25.6-months (IQR 14.1-35.2-months). Twenty-nine (85%) of these subjects had at least one additional sample included in the study. Of the remaining five subjects, three produced three samples and two produced one sample, each of which yielded < 1000 reads.

The BAL samples that did not amplify yielded fewer bacteria in quantitative microbiological culture than those that returned >1000 reads. Only two (4%) had significant ($\geq 10^5$ cfu/mL) growth of pathogenic bacteria, whilst nine (17%) had no cultivatable organisms. Quantitative culture results of these BAL samples are summarised in Table S3. Interleukin-8 (IL-8), but not neutrophil elastase (NE), was lower in BAL samples that failed to amplify than in those with no dominant proinflammatory pathogen detected by 16S rRNA gene sequencing (IL-8, median (IQR): 84 pg/mL (47.5-315.5 pg/mL) and 323 pg/mL (84-875 pg/mL, t-test($\log_{10}$), p = 0.005; NE, 5.9 mcg/mL (2.5-12.4 mcg/mL) and 5.7 mcg/mL (2.5-14.4 mcg/mL), t-test($\log_{10}$), p = 0.39).

FIGURE LEGEND

FIGURE S1: Subject age at time of longitudinal bronchoalveolar lavage (BAL) samples. Individual subjects are displayed across the horizontal axis; age at serial sampling time is displayed on the vertical axis.

FIGURE S2: Evolution of individual subjects' lower airway microbiota over time. The patterns of change in the 16S rRNA gene sequencing of all subjects with data from serial bronchoalveolar lavage (BAL) samples are displayed. Relative abundance of individual genera are displayed in the stacked column are graphs (range 0-100%). Shannon diversity index (●) and neutrophil elastase (mcg/mL) (◇)included on the left and right of each column respectively. Clinical histories of the first three subjects are included below:

1. Serial BAL samples from a male subject (p.Phe508del/p.Phe508del), who was diagnosed with CF at 1.3-months of age by newborn screening. BALs were performed at 2.6, 13.5 and 24.7-months of age and demonstrated initial diverse, although different lower airway microbiota with later dominance of a typical CF pathogen (*Staphylococcus*). The subject was fed a combination of breast milk and formula and was not exposed to household cigarette smoke. He was first admitted to hospital at 1.5-months of age for a CF education programme. Best recorded FEV_1 and FVC values at 6-years of age were 97% and 110% predicted respectively. His first BAL was performed at 2.6-months, and the subject had a 2 week history of intermittent cough, coryza and wheeze that was treated with oral trimethoprim-sulphamethoxazole. Quantitative culture yielded alpha-haemolytic *Streptococcus* and non-haemolytic *Streptococcus* in concentrations of $5x10^3$ colony forming units (cfu)/mL and $2.5x10^3$ cfu/mL respectively. IL-8 was 79 pg/mL and NE was not

detected. SDI was 2.01. The relative abundance of individual genera, detected on 16S rRNA gene sequencing, is displayed.

The second BAL was performed at 13.5-months, during a 3-day hospitalisation for treatment of a respiratory illness. He had a 1-week history of cough and wheeze and had been treated with 1-week of oral cefaclor, followed by 3-days of intravenous flucloxacillin and gentamicin. Cultures grew $6x10^4$ cfu/mL alpha-haemolytic *Streptococcus*. IL-8 and NE concentrations in BAL fluid were 875 pg/mL and 21.1 mcg/mL, respectively. Nasopharyngeal secretions were positive for respiratory syncytial virus. SDI was 2.16. The lower airway microbiota remained diverse, although different from the previous sample.

His third BAL was performed at 24.7-months of age. He had a 1-week history of intermittent cough and was not receiving antibiotic therapy. Cultures yielded *S. aureus* $1.2x10^5$ cfu/mL. IL-8 and NE concentrations in BAL fluid were 491 pg/mL and 17.8 mcg/mL, respectively. SDI was markedly reduced (0.002) and the relative abundance of *Staphylococcus* detected by 16S rRNA gene sequencing was 99.97%.

2. Serial BAL samples from a male subject (p.Phe508del/p.Gly551Asp) diagnosed with CF by newborn screening at 1.8-months of age. Serial BALs were performed at 2.5, 12.6, 25.5 and 60-months of age. 16S rRNA gene sequencing was performed on the latter three samples, and demonstrated a stable, diverse lower airway microbiota over a period of at least 13-months, followed by dominance of a typical CF pathogen (*Pseudomonas*).The subject was breastfed. Cigarette smoke exposure was unknown. Best FEV_1 and FVC recordings at 6-years of age were 106% and 96% predicted respectively.

His first BAL was performed at 2.5-months of age. He was asymptomatic and not prescribed antibiotics at the time. Cultures grew alpha-haemolytic *Streptococcus* $2.8x10^4$ cfu/mL,

Neisseria sp. $1x10^4$ cfu/mL and *Haemophilus* sp. $2.1x10^3$ cfu/mL. IL-8 was 30 pg/mL and NE was 12 mcg/mL. 16S rRNA gene sequencing recovered <1000 reads.

The second BAL was performed at 12.6-months of age. The patient was asymptomatic and not receiving antibiotic therapy. Cultures grew upper respiratory tract commensals $1.1x10^6$ cfu/mL and *S. aureus* $8x10^1$ cfu/mL. IL-8 and NE concentrations in BAL fluid were 58 pg/mL and 12 mcg/mL, respectively. SDI was 2.05. The relative abundance of individual genera is displayed above.

His third BAL was performed at 25.5-months of age. He had had an intermittent cough for 8-weeks and had been treated with amoxicillin-clavulanic acid for 4-weeks. Cultures grew *M. cattarhalis* $3.5x10^5$ cfu/mL, IL-8 concentration was 3 pg/mL and NE was undetected in the BAL fluid. SDI was 1.88, and the baseline lower airway microbiota was similar to that of the previous sample, obtained 13-months before the procedure.

A fourth BAL was performed at 60-months of age. Clinical and inflammatory data were not available, but cultures grew *P. aeruginosa* $1.67x10^7$ cfu/mL. SDI was markedly reduced (0.05) and 16S rRNA gene sequencing demonstrated dominance of *Pseudomonas* (relative abundance 99.3%).

3. Serial BAL samples from a male subject (p.Phe508del homozygous), diagnosed with CF at 1.4-months of age by newborn screening. BALs were performed at 1.4, 15 and 34-months of age, and demonstrate initial dominance of *Staphylococcus*, with subsequent recovery of microbial diversity, followed by the reestablishment of *Staphylococcus* as the dominant genus. The subject received a combination of breast milk and formula and was exposed to household cigarette smoke. Best FEV_1 and FVC recordings at 6-years of age were 97% and 98% predicted respectively.

His first BAL was performed at 1.4-months of age, during a hospital admission for CF education. Whilst he was asymptomatic at the time of the procedure, he developed a cough soon afterwards, and was treated with oral flucloxacillin. Cultures yielded *S. aureus* and *Corynebacterium* in concentrations of $1x10^6$ cfu/mL and $1.9x10^5$ cfu/mL respectively. BAL IL-8 concentration was 1475 pg/mL and NE level was 9.9 mcg/mL. SDI was 0.87, and the relative abundance of *Staphylococcus* and *Corynebacterium* were 73.6% and 16.4% respectively. During the first-year of life, he had multiple episodes of moist cough, treated with oral antibiotics (flucloxacillin, cefaclor and amoxicillin-clavulanic acid).

His second BAL was performed at 15-months. He had had an intermittent cough for 1-week and was not taking antibiotics. Cultures grew alpha-haemolytic *Streptococcus* $2.7x10^3$ cfu/mL, *Neisseria* sp. $1.5x10^3$ cfu/mL, *Corynebacterium sp.* $2x10^3$ cfu/mL, and *Haemophilus* sp. $4x10^2$ cfu/mL. IL-8 and NE concentrations in BAL fluid were 266 pg/mL and 7.0 mcg/mL, respectively. SDI had increased to 2.43 and 16S rRNA gene sequencing demonstrated a diverse lower airway microbiota. He continued to experience intermittent respiratory symptoms, treated with oral amoxicillin-clavulanic acid.

His third BAL was performed at 34-months, at which time he was asymptomatic and not receiving antibiotic therapy. Cultures again grew low concentrations of alpha-haemolytic streptococci $1x10^1$ cfu/mL, *Neisseria* sp. $1.1x10^2$ cfu/mL and *Haemophilus* sp. $1.4x10^2$ cfu/mL. BAL IL-8 and NE concentrations were 210 pg/mL and 6.3 mcg/mL, respectively. Interestingly, SDI was markedly reduced (0.03) and 16S rRNA gene sequencing demonstrated near complete dominance of *Staphylococccus* (relative abundance 99.7%).

TABLES

TABLE S1: Characteristics of study samples compared with bronchoalveolar lavage samples where 16S rRNA V1-3 region amplification and sequencing yielded <1000 reads.

	Study samples (n=95)	**Samples yielding <1000 reads (n=53)**	***p***
Reads per sample, mean ± SD (range)	12422.3 ± 11371.8 (1000-18297)	409.7 ± 228.9 (21-934)	<0.0001[a]
Age at BAL in months, mean ± SD (range)	24.6 ± 17.9 (1.2-78.3)	24.5 ± 15.3 (1.2-63.0)	0.97[a]
Respiratory symptoms at BAL, n (%)[b]	54 (57%)	21 (40%)	0.025[c]
Antibiotics at BAL, n (%)[d]	45 (47%)	23 (43%)	0.56[c]

[a] Unpaired t-test
[b] Data unavailable for 9 study samples and 4 excluded samples
[c] Chi-square test
[d] Data unavailable for 7 study samples and 3 excluded samples

TABLE S2: Characteristics of bronchoalveolar lavage samples obtained in children with or without respiratory symptoms

	Asymptomatic (n=31)	**All symptomatic patients (n=54)**	***p***	**Cough, not hospitalised (n=43)**	**Respiratory hospitalisation (n=11)[b]**	***p***
Age at BAL in months, mean ± SD (range)	17.2 ± 12.3 (1.4-42.0)	27.8 ± 20.0 (1.2-78.3)	0.003[b]	28.0 ± 20.0 (1.4-78.3)	27.1 ± 20.9 (1.2-69.9)	0.91[b]
Age at BAL in months, median (IQR)	14.1 (7.6-27.1)	22.6 (13.7-39.0)		24.7 (14.2-42.9)	22.6 (13.3-32.9)	
Antibiotics, n (%)	2 (6.5%)	40 (74.1%)[c]	<0.0001[d]	30 (69.8%)[d]	10 (90.9%)[d]	0.05[d]

[a] Includes two BAL samples from one subject, obtained at 13.1 and 59.4 months respectively
[b] Unpaired t-test
[c] Data unrecorded for 2 subjects (1 "cough not hospitalised", 1 "respiratory hospitalisation")
[d] Chi-square test

TABLE S3: Microbiological culture results of the bronchoalveolar lavage samples (n=53) that yielded fewer than 1000 16S rRNA gene sequences

Quantitative microbiological culture	Number of BAL samples
S. aureus CFU 10^1-10^4/mL	 6
P. aeruginosa CFU 10^1-10^4/mL CFU 10^5-10^7/mL	 3 1
H. influenzae CFU 10^5-10^7/mL	 1
Haemophilus species CFU 10^1-10^4/mL	 10
Alpha haemolytic streptococci CFU 10^1-10^4/mL	 25
Beta haemolytic streptococci CFU 10^1-10^4/mL	 1
Neisseria species CFU 10^1-10^4/mL	 16
Upper respiratory tract flora CFU 10^1-10^4/mL	 9
Moraxella cattarrhalis CFU 10^1-10^4/mL	 1
E. coli CFU 10^1-10^4/mL	 5
Gram negative bacilli – not specified CFU 10^1-10^4/mL	 2
Coagulase negative staphylococci CFU 10^1-10^4/mL	 15
Diptherioids CFU 10^1-10^4/mL	 5

FIGURE S1:

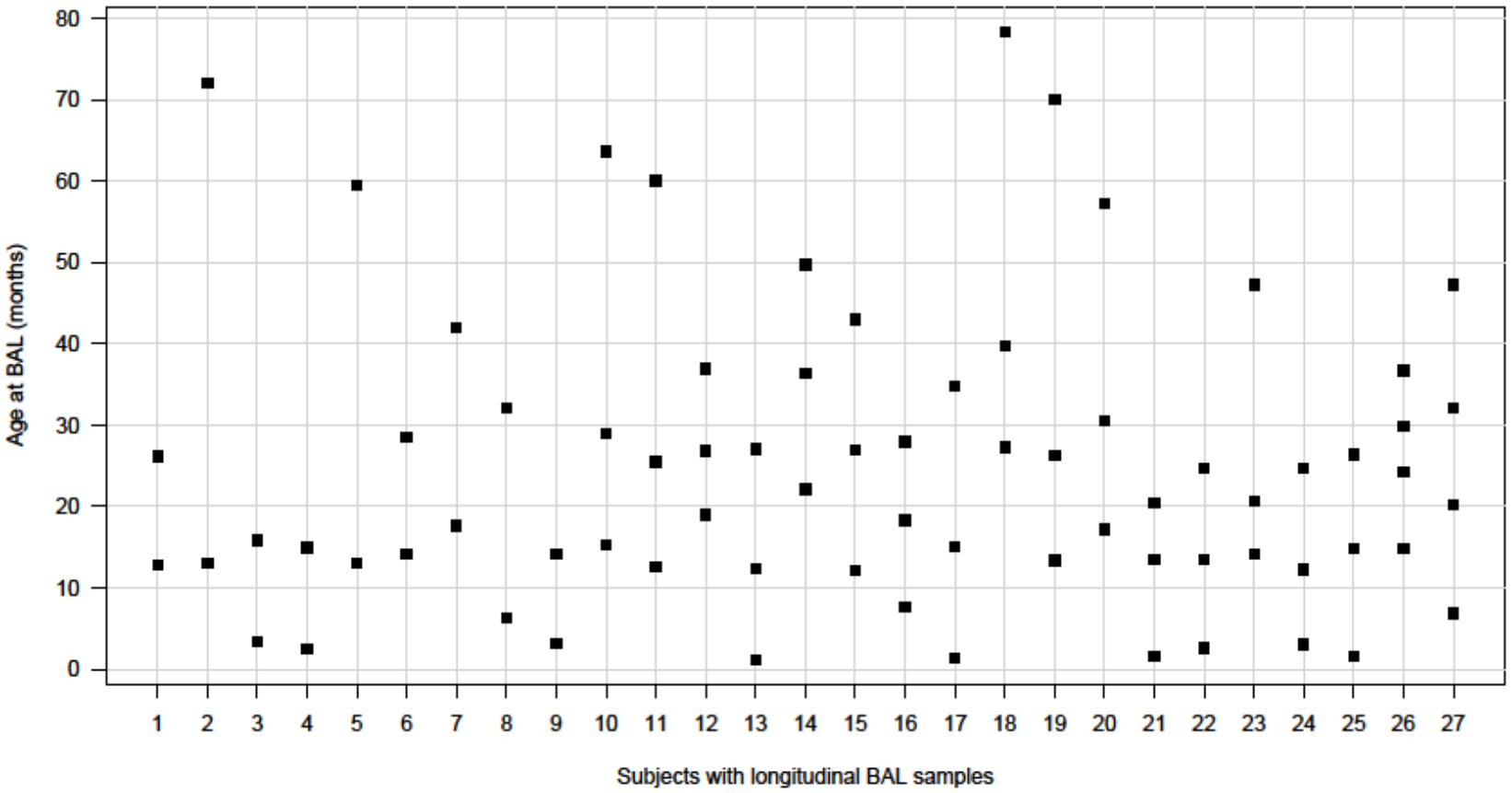
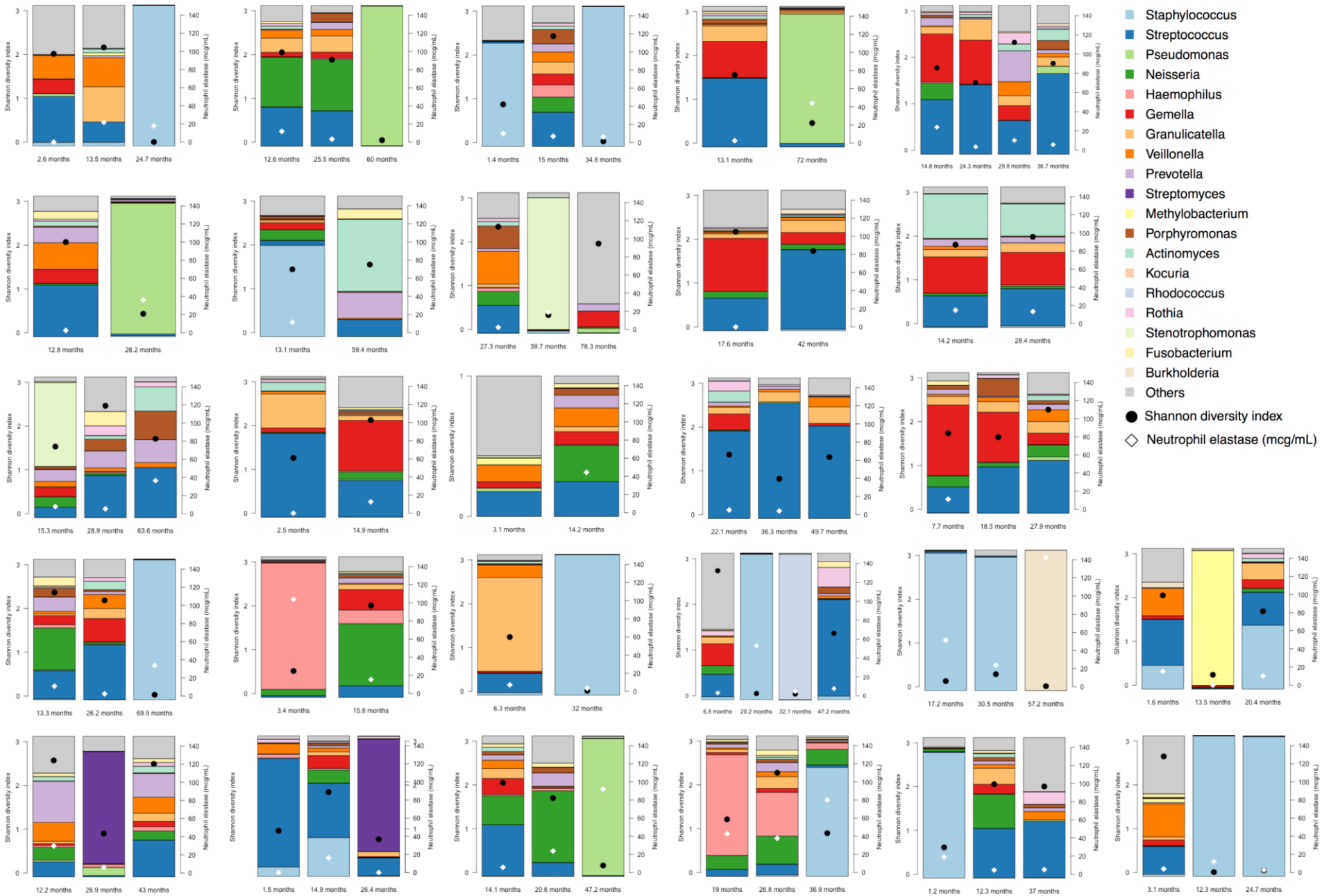

Age at BAL (months)
0
10
20
30
40
50
60
70
80
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Subjects with longitudinal BAL samples

FIGURE S2:


Staphylococcus
Streptococcus
Pseudomonas
Neisseria
Haemophilus
Gemella
Granulicatella
Veillonella
Prevotella
Streptomyces
Methylobacterium
Porphyromonas
Actinomyces
Kocuria
Rhodococcus
Rothia
Stenotrophomonas
Fusobacterium
Burkholderia
Others
Shannon diversity index
Neutrophil elastase (mcg/mL)
Shannon diversity index
Neutrophil elastase (mcg/mL)