ONLINE DATA SUPPLEMENT

Concordance between upper and lower airway microbiota in infants with Cystic Fibrosis.

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SUPPLEMENTAL METHODS

Study population

The study area of CF patients covered two CF tertiary care centers in the Netherlands (Center A: Erasmus Medical Center Sophia Children's Hospital; Center B: Wilhelmina Children's Hospital Utrecht). Parents were informed about the study by written information in their newborn's first weeks of life and asked to participate. CF infants younger than three months detected by newborn screening [1, 2] with confirmation of the diagnosis by sweat chloride test, and CF infants younger than seven months that were missed by newborn screening, were eligible for inclusion. Patients with prior history of preterm birth or coexisting heart, lung, metabolic, bone, or neuromuscular disease were excluded. Clinical CF-care took place according to a Dutch clinical surveillance protocol (included at the end of the Supplementary information) including imaging, bronchoalveolar lavage, clinical visits and obtaining upper respiratory tract cultures at standard time-points [2-5].

Bronchoscopy procedure

Flexible fiber-optic bronchoscopy for BAL was performed under general anesthesia in accordance with standard safety and monitoring procedures for bronchoscopy in children, according to the European Respiratory Society (ERS) rules [6-8].

Before the start of the bronchoscopy the tip of the scope was wiped with sterile gauze to serve as a negative control for the bacterial DNA background signal. At the laboratory, the gauze was washed with 5 ml of phosphate-buffered saline to extract any contaminating bacteria. We will refer to 'tip of the bronchoscope' DNA controls as 'gauze-controls'.

The bronchoscope (Pentax model Fl-9RBS, insertion tube diameter 3.1 mm, instrument channel diameter 1.15 mm, Pentax Europe GmbH, Hamburg, Germany) was introduced into the lower airway through a laryngeal mask, avoiding the use of the suction channel until the tip of the bronchoscope was below the carina. BAL was carried out in three (range 2-5) lung segments (the right middle lobe, the lingula, left lower lobe and another lung segment) by using normal sterile saline previously warmed up to body temperature (37°C) in amounts of 1 mL/kg with a maximum of 20 mL per portion. The saline was immediately aspirated into a sterile suction set over 10 – 20 s using negative pressures of 25 – 100 mmHg. In one of the two study centres, BAL fluids from different lobes per individual were pooled at the end of the procedure for logistic reasons.

Bacterial culture method

One aliquot of liquid Amies medium of the NP and OP samples and one aliquot of BAL fluid were used for bacteriological cultures. We performed parallel plating on the following media: Columbia with 5% sheep blood agar (CNA, detection of S. *pneumoniae*, *M. catarrhalis* and small colony variants of *S. aureus* (SCVSA) [9]), Haemophilus Chocolate agar 2 with Bacitracin (HAE2, *H. influenzae*), MacConkey agar (non-fastidious gram-negative rods, particularly members of the family Enterobacteriaceae and non-fermenting bacteria and the genus *Pseudomonas*), Bur-cep plates (*Burkholderia cepacia*) and Mannitol Salt Agar (*S. aureus*) [10]. All plates were incubated for up to 72 hours at 37°C. We identified by standard microbiological testing different colonies growing on the various media [11]. Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry was used for rapid and proper identification of atypical organisms e.g (micro-) colonies of SCVSA [1]. To determine which *Streptococcus* species were present in the samples, we re-cultured [3] all streptococci from a representative set of samples and determined the streptococcal species by optochin susceptibility testing, LytA quantitative PCR [6] and MALDI TOF Mass Spectroscopy [12].

Construction of Phylogenetic Library

Our samples were processed for 454 GS-FLX-Titanium sequencing of the V5-V7 region of the 16S rRNA gene.

Extraction of Bacterial DNA

DNA was extracted from 200 µl of raw nasopharynx and 200 µl oropharynx Amies medium [6], 300 µl BAL fluid and from our environmental control samples, including DNA isolation controls (200 µl) and gauze-controls) (300 µl) by using a modified mag forensics extraction kit protocol (LGC Genomics, Berlin, Germany). All samples were thawed on ice, vigorously vortexed for 20 seconds then transferred to 1.5 ml Eppendorf tubes containing 650 µl 0.1 mm zirconium beads (Biospec Products, Bartlesville, OK) in lysis buffer and 550 µl phenol (Sigma-Aldrich, St Louis, MO). Samples were mechanically disrupted through two cycles of 2 minutes bead beating (Mini-Beadbeater-24, Biospec Products) and 2 minutes on ice, before centrifugation at 5000g for 10 minutes. The aqueous DNA phase was then transferred to new Eppendorf tubes pre-filled with 10µl of magnetic beads suspension in 1.3 ml binding buffer. After 30 minutes of incubation in a shaker at room temperature, the beads were washed with 200 µl wash buffers 1 and 2, and then dried for 20 minutes at 55°C. DNA was eluted in 50 µl Elution buffer and stored at -80°C[13, 14].

Real-time PCR for Bacterial DNA

Bacterial DNA was quantified for each respiratory sample by qPCR to determine eligibility for subsequent analysis. The quantity was measured by using quantitative PCR and a universal primer-probe set specific for the 16S rRNA gene as described, [15] containing forward primer 16S-F1 (5′-CGA AAG CGT GGG GAG CAA A −3′), reverse primer 16S-R1 (5′-GTT CGT ACT CCC CAG GCG G-3′) and probe 16S-P1 (FAM-ATT AGA TAC CCT GGT AGT CCA-MGB). The PCR mixture consisted of 12.5 µL of 2× master mixture (Universal Mastermix, catalog no. GMO-UN-A100; Europe Diagenode SA, Liège, Belgium), 1 µL of each primer (10 µmol/L), 1 µL of probe (5 µmol/L), 6.5 µL DNAase-free water, and 3.0 µL of template DNA and a NTC (no template control) as procedural control. The machine used for the amplifications was a Step One Plus Real-Time PCR machine, catalog.no. 4376600, Applied Biosystems, Foster City, CA, USA with the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Obtained cycle

threshold values were related to a standard curve of bacterial DNA obtained from human saliva spiked with DNA of 6 bacterial species (*Streptococcus mutans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Porphyromonas catoniae*, *Propionibacterium propionicum*, and *Tannerella forsythia*). We used a standard DNA range of 0.1 pg/μL through 1 ng/μL.

Amplicon library preparation

An amplicon library was generated by amplification of the V5–V7 hypervariable region of this gene as described [13, 15]. We used forward primer 785F (5 ′ -GGA TTA GAT ACC CBR GTA GTC-3′) and reverse primer 1175R (5′-ACGTCRTCCCCDCCTTCCTC-3′). The primers were fitted with the 454 Life Sciences Adaptor A (forward primer) and B (reverse primer) and fused to the 5′ end of the 16S rDNA bacterial primer sequences. The reverse primer also included a unique decanucleotide sample identification key. The amplification mixture contained 2 units of Pfu Ultra II Fusion HS DNA polymerase (Stratagene, La Jolla, CA, USA) and 1× *PfuUltra* II Reaction Buffer (Stratagene), 200 μmol/L dNTP PurePeak DNA Polymerase Mix (catalog no. NU606001; Pierce Nucleic Acid Technologies Milwaukee, WI, USA), and 0.2 μmol/L of each primer. After denaturation (94°C for 2 min), 30 cycles were performed that consisted of denaturation (94°C for 30 s), annealing (50°C for 40 sec), and extension (72°C for 80 sec). Amplicons were size checked and quantified by gel electrophoresis and Quant-iT PicoGreen dsDNA Assay (Invitrogen, Carlsbad, CA, USA) by using a CLARIOstar® High Performance Microplate Reader (BMG LABTECH, 13000 Weston Parkway, Suite 109, Cary, NC 27513, United States). Amplicons of the individual samples were equimolar pooled, purified by agarose gel electrophoresis, and isolated from gels by using the QIAquick Gel Extraction Kit Protocol (QIAGEN, Hilden, Germany). The amplicon library was sequenced unidirectional by using 454 GS-FLX-Titanium Sequencer (Life Sciences, Roche, Hongkong, China).

Quality control

Only samples with a bacterial density of at least 0.3 pg/μl above environmental controls, including DNA isolation controls and gauze-controls as measured by Real-Time PCR were considered, in order to avoid interference of background DNA. To investigate the possibility of cross-contamination of low bacterial density samples by environmental bacteria, we ran an additional analysis, comparing the microbiota profiles of our lowest density samples and DNA isolation blanks and gauze-controls (see page 4 and 6 below).

Bioinformatic processing of sequences

The raw sequences obtained were processed using QIIME version 1.8 [16]. Sequences were checked for quality using split_library.py using default parameters; sequences with ≥1 error in the primer or >1 error in the barcode, a sequence length of <200 and >1000 base pairs (bp), >6 ambiguous bp or >6 homopolymers were removed from the database. Subsequently, barcodes and primers were trimmed off, chimeric sequences were identified and removed using chimeraslayer (identify_chimeric_seqs.py and filter_fasta.py). Next, a representative set of sequences was picked; reads were aligned and clustered into operational taxonomic units (OTUs) (pick_de_novo_otus.py, make_otu_table.py) using UCLUST at 97% similarity. Aligned sequences were taxonomically annotated using the Greengenes 16S-rRNA database (version 13.8).

***Post-hoc sequence analysis of our lowest density samples and controls to assess impact of environmental cross-contamination*.**

Sample selection. We selected 22 samples (9 NP and 13 BAL-samples), and 27 controls (17 gauze-controls, 10 DNA isolation controls) to assess the influence of environmental cross-contamination on the bacterial community composition of our lowest density NP-/BAL-samples. Methods of sampling and DNA-isolation are identical to those reported in the main text.

Post-hoc 16S rRNA MiSeq sequencing and bio-informatic processing. Generation of 16S ribosomal RNA amplicon libraries and MiSeq gene-sequencing was executed as previously described[17]. Amplicon pools were paired-end sequenced in two runs using an Illuminia MiSeq instrument (Illumina Inc., San Diego, CA, USA), as opposed to the 454 GS FLX Titanium sequencer, which was used to generate sequence data reported in the main text. An adaptive, window-based trimming algorithm (Sickle, version 1.33)[18] was applied to remove low-quality (regions of) reads. Subsequently, an error correction algorithm was applied (BayesHammer, SPAdes, version 3.5.0)[19]. Then, reads were assembled (PANDAseq, version 2.9)[20, 21] and demultiplexed (Qiime, version 1.9.1)[16]. Chimeric sequences were removed using UCHIME. VSEARCH abundance-based greedy clustering was used to bin sequences into OTUs at a 97% identity threshold [22]. OTU-annotations was performed using the RDP classifier (version 2.2)[23] and the SILVA reference database. OTUs present at ≥0.1% relative abundance (i.e. confident level of detection) in at least 2 samples were included, resulting in an OTU-table consisting of 168 OTUs (retaining 98.5% of reads). The table was rarefied at a sequence depth of 1,600 reads, which was then used as input for downstream analyses (i.e. visualization, non-metric multidimensional scaling and clustering).

Data-analyses. All analyses were performed in the R version 3.3.0 within R studio version 0.99.902. Non-metric multidimensional scaling was performed using the *metaMDS* function (vegan R-package). To determine whether the microbiota composition in NP-/BAL-samples was significantly different from gauzes and DNA isolation blanks, we used a permutational multivariate analysis of variance (PERMANOVA) test (vegan package, *adonis* function, 1,000 permutations). Both samples and controls were subjected to average linkage hierarchical clustering based on the Bray-Curtis dissimilarity matrix, which was visualized by a dendrogram combined with a heatmap. The optimal number contained in the data was determined by calculating the Silhouette and Calinski-Harabasz indices.

SUPPLEMENTAL RESULTS

Bacterial density

To avoid interference from background bacterial DNA, environmental contamination, we selected only respiratory samples with sufficient overall bacterial loads, defined as NP, OP and BAL samples with a bacterial density of ≥0.3 pg/µl above environmental controls (including DNA isolation controls and gauze-controls), for further sequencing. Only less than 5% of all our samples, from which this study is a subset, did not meet this condition. Fortunately, within this sub study all samples did meet the quality control standards, so all are included in the analyses.

Phylum-level microbial composition

We observed most frequently Firmicutes (43.9%, 57.9% and 51.8%), Proteobacteria (38.2%, 14.2% and 30.4%), Bacteroidetes (1.7%, 18.0% and 10.8%), Actinobacteria (16%, 8.7% and 6.1%) and Fusobacteria (0.1%, 1.1% and 0.9%) in NP, OP and BAL samples respectively (Figure S2), in total accounting for 99.9% of all sequences. An average Goods coverage of 0.954 revealed that these libraries well represented the majority of bacterial communities present in each sample (median 0.955, range 0.903-0.997).

Culture-based microbial composition

S. aureus was detected in 24%, 41% and 60%, *P. aeruginosa* in 0%, 5% and 8%, *H. influenzae* in 12%, 18% and 32%, *M. catarrhalis* in 16%, 0%, and 11%, Gneg-o bacteria (defined as members of the family Enterobacteriaceae such as *E. Coli* and non-fermenting bacteria) in 8%, 18% and 21% of NP, OP and BAL cultures, respectively. When early and late sample sets were compared, we detected lower *S. aureus* culture rates in all three niches at the later sample set. Since our culture results were negative for the potential respiratory pathogen *S. pneumoniae* we hypothesized that our most abundant Streptococcus OTU represented other streptococcal species. This was confirmed by culturing, optochin testing, LytA-qPCR and MALDI-TOF of a representative subset of samples, demonstrating the presence of *S. mitis* only (Figure S9).

Post-hoc microbiota analysis of our lowest density samples and controls to assess environmental cross-contamination

Bacterial density. We observed a statistically significant difference in bacterial density between samples and controls (Mann-Whitney U-test, median[IQR] 0.88 [0.52-4.49] vs 0.05 [0.04-0.07] pg/ul in samples and controls, respectively, $p=1.2 * 10^{-12}$), but no differences between either NP- and BAL-samples, or gauze-controls and DNA isolation blanks (Mann-Whitney U-test, p=0.508 and p=0.215, respectively).

Descriptives. The 15 highest ranking OTUs in both patient samples and controls were plotted in boxplots, demonstrating that their compositionality strongly differs, with high abundance of *Streptococcus*, *Staphylococcus*, *Haemophilus*, *Neisseria*, *Gemella*, *Prevotella* and *Veillonella* in the NP/BAL-samples, compared to enrichment of contaminating species *Sphingomonas*, *Rhizobium*, *Caulobacteriaceae* and *Pelomonas spp*. in gauze-controls and DNA isolation blanks (Figure S10; box-plots).

Non-metric multidimensional scaling samples vs controls. We performed a non-metric multidimensional scaling to visualize the difference in overall microbiota composition between samples and controls. We observed a clear discrimination between our lowest density NP- and BAL samples on the one hand and gauze-controls and DNA isolation blanks on the on the other (Figure S11; nMDS). This observation was confirmed by a PERMANOVA test, which revealed that indeed a substantial proportion of the variance contained in the data was driven by the separation between controls and samples (R^2 37.3%, $p<0.001$).

Clustering. Apart from supervised analyses, we additionally subjected both samples and controls to an unsupervised clustering analysis. Clustering indices suggested the existence of 4 clusters, which we visualized in a dendrogram (Figure S12; dendrogram/heatmap). We observed two larger clusters, 1) driven by enrichment for *Sphingomonas*, consisting of 26 controls and 1 NP-sample and 2) typified by high abundance of *Streptococcus* and *Staphylococcus*, comprising 19 samples and 1 gauze-control. The two small clusters each consisted of one NP-sample, characterised by either predominance of *Haemophilus* and *Dolosigranulum*.

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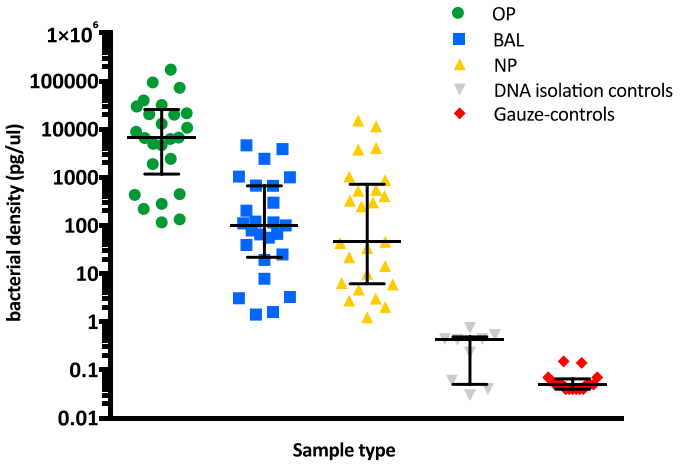
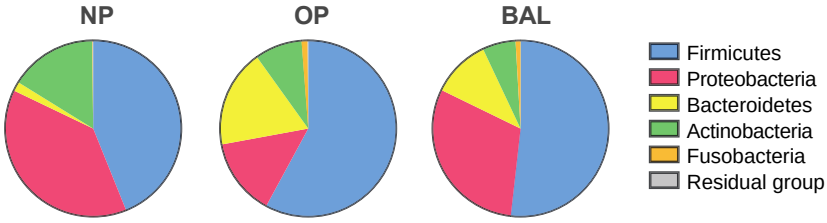
SUPPLEMENTAL FIGURES

Figure S1. **Bacterial density of different samples types.**



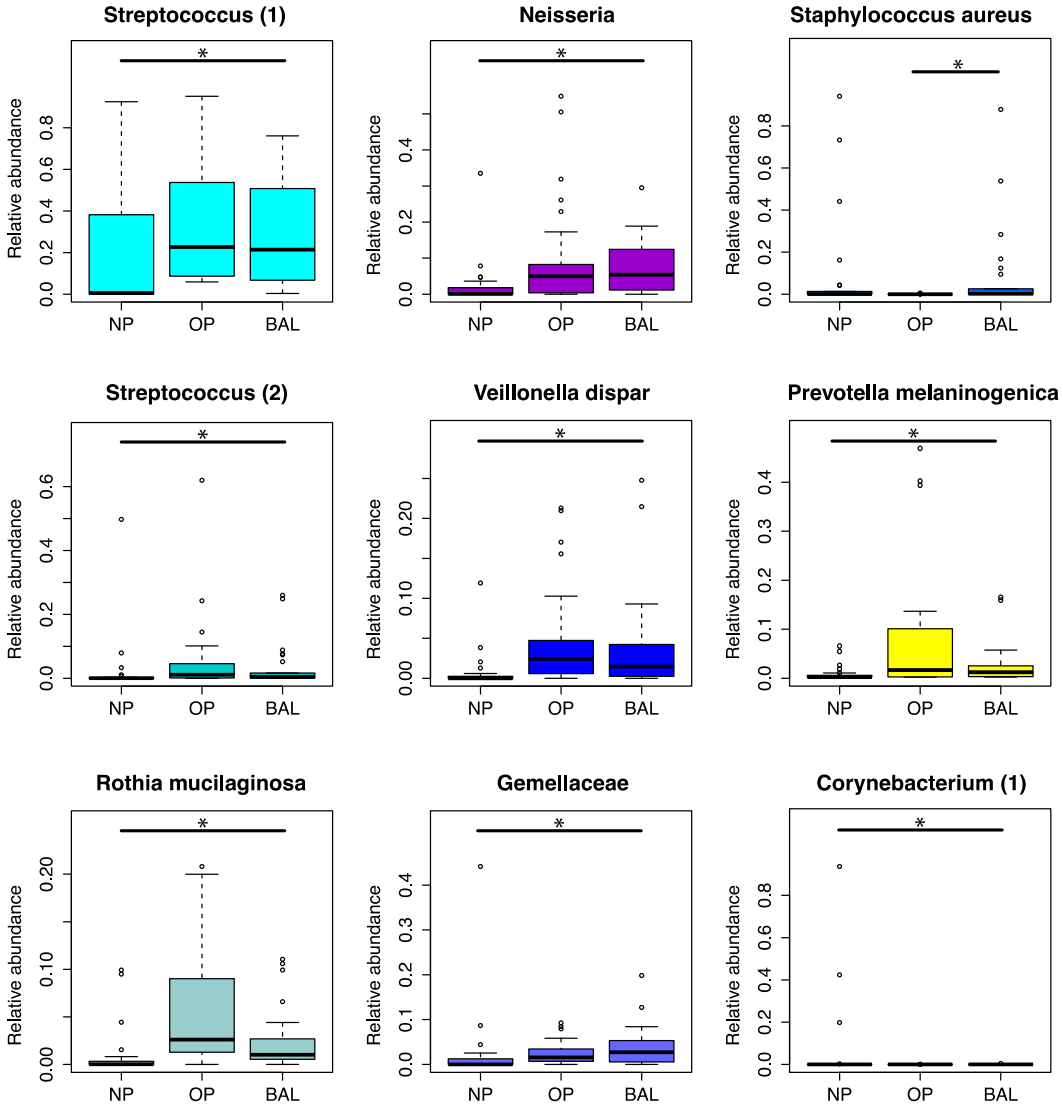
This scatter plot depicts the bacterial density of different respiratory niches and environmental background control samples, including DNA isolation controls and gauze-controls. Abbreviations: NP= nasopharynx, OP = oropharynx, BAL = bronchoalveolar lavage.

Figure S2. **Differences between niches in overall microbial composition**



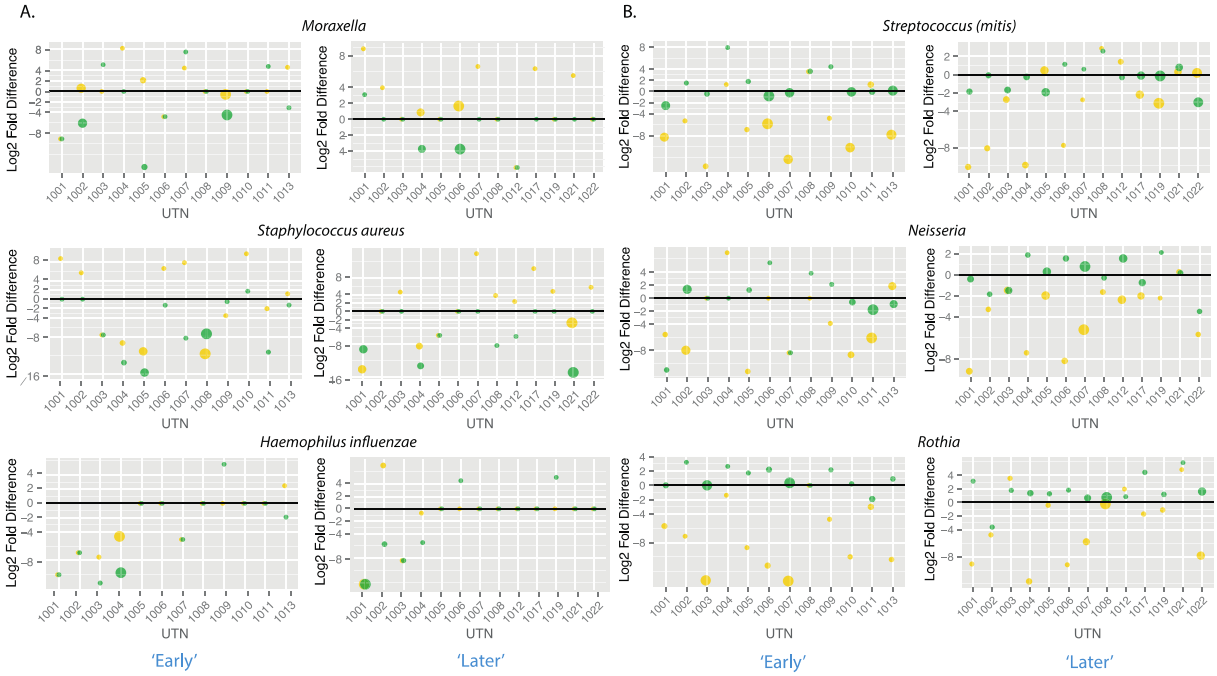
These three circle diagrams represent the microbial community composition on phylum level of the NP, OP and BAL samples (n=25, each). Abbreviations: NP= nasopharynx, OP = oropharynx, BAL = bronchoalveolar lavage.

Figure S3. **Differences in microbial community composition between niches**.



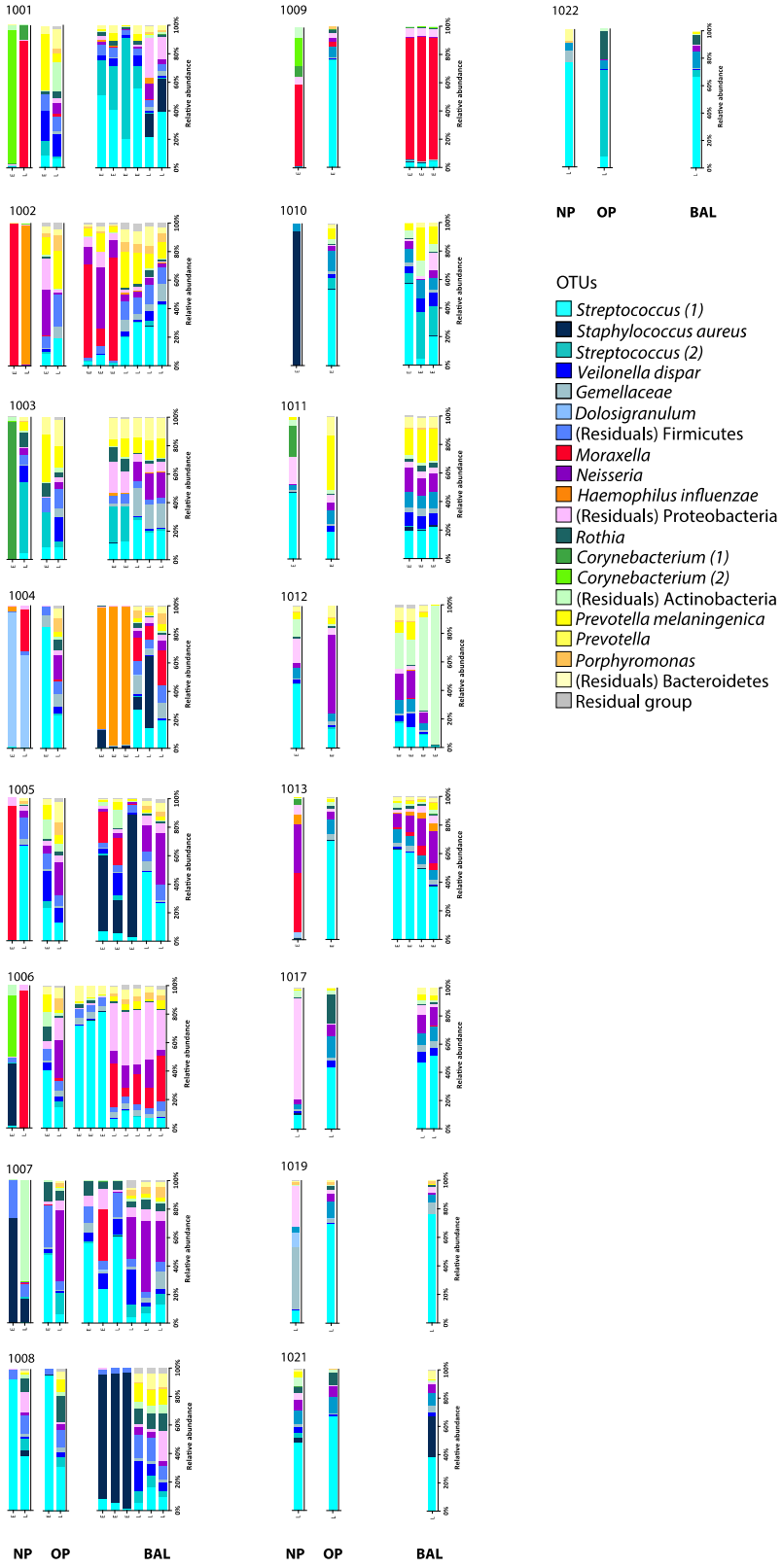
This figure depicts the top 9 most abundant OTUs of our 100 highest ranked OTUs within the n=75 NP OP BAL sample-set; presence of all these 9 OTUs is significantly different between the three niches (NP, OP and BAL), calculated by Kruskal-Wallis, with Benjamini-Hochberg correction (q-value ≤0.02) (not depicted). Nemenyi post-hoc tests results, considering NP-BAL and OP-BAL comparisons are visualized; black lines (*) represent significant differences (p ≤0.05) between two niches. Abbreviations: NP= nasopharynx, OP = oropharynx, BAL = bronchoalveolar lavage.

Figure S4. **Differences in microbial community composition within individuals**



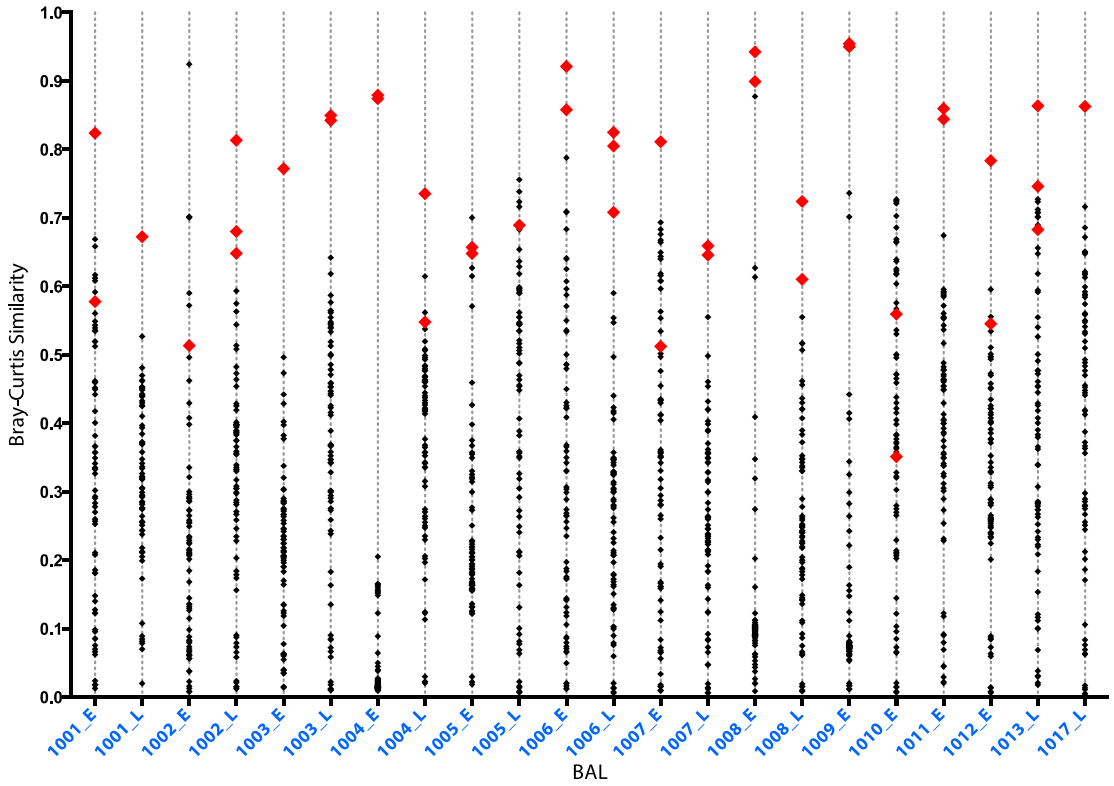
Log_2-transformed relative abundance ratios of potential pathogens (A) and most abundant commensal species/genera (B) within subjects. NP/BAL and OP/BAL ratios are represented in yellow and green, respectively. The diameter of each circle is related to the relative abundance of the corresponding species in the BAL. The circles on, below and above the horizontal line at y=0 represent individuals with similar, lower, higher abundances of corresponding microbiota in NP or OP, versus BAL, respectively. Early= <9 mo., Later ≥9 mo.. Abbreviations: UTN = unique trial number (Patient ID number).

Figure S5. **Individual microbiota profiles; all paired sets of NP, OP and BAL**



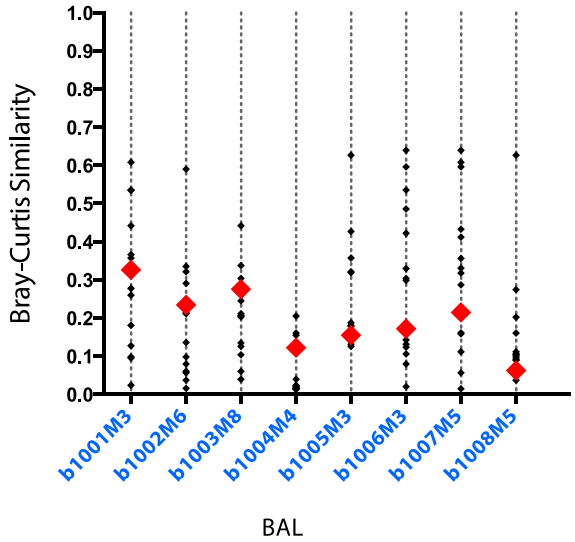
The numbers above each set of the barcharts are patient ID numbers. The number following the OTU names represent the hierarchical number of that OTU within its genus based on average relative abundance over all samples (e.g. *Streptococcus* (2) is the second dominant *Streptococcus* observed). Abbreviations: E (early)= <9 mo., L (later) ≥9 mo., NP = nasopharynx, OP = oropharynx, BAL = bronchoalveolar lavage.

Figure S6. **High intra-individual concordance between different lavage locations.**



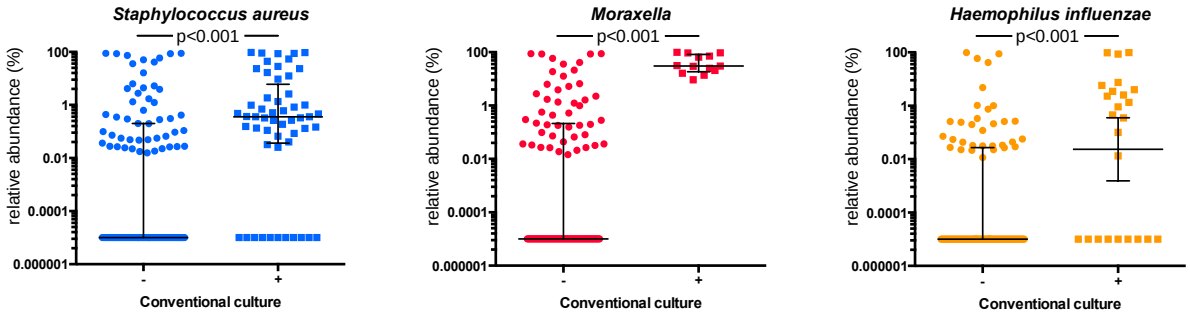
Each column represents a separate BAL procedure; each diamond represents the bacterial community similarity between lavage location A (lingula) and another location within subject (intra-individual) (red) or between-subjects (inter-individual) (grey). Abbreviatons: E (early)= <9 mo., L (later) ≥ 9 mo.

Figure S7. **LRT microbiota community composition changes over time**



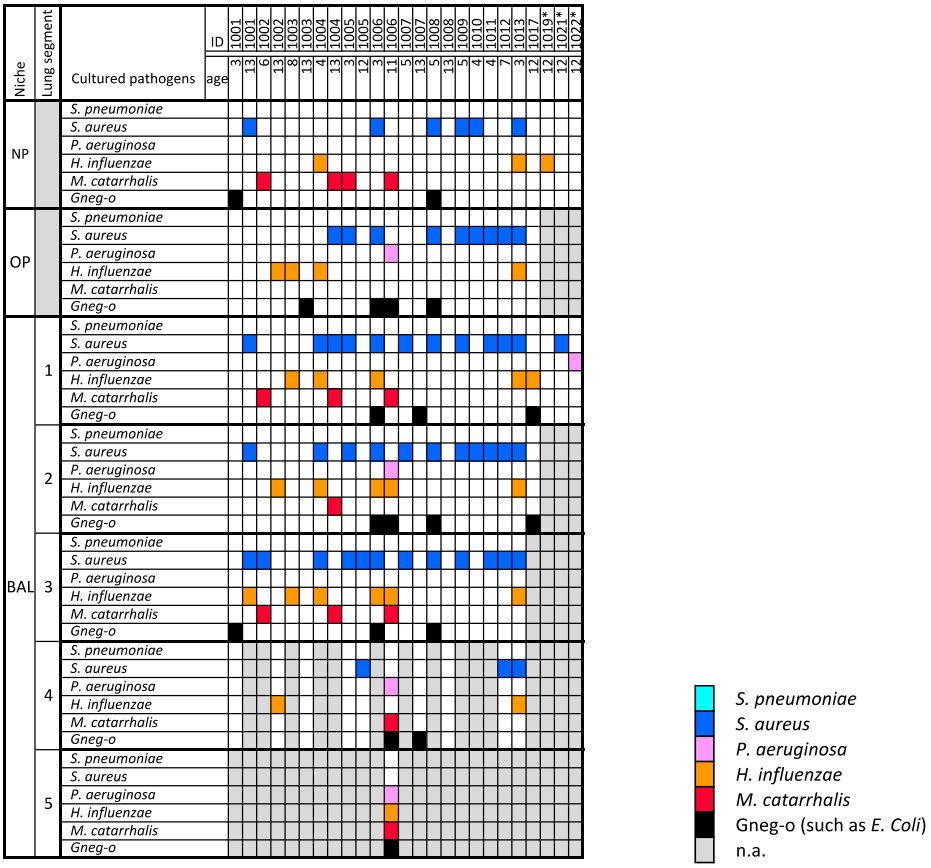
For each BAL procedure each diamond represents the similarity between one "early" lavage location (reference; lingula if available) and "later" corresponding location of the same individual (red), and of the other infants (grey). Abbreviations: b = bronchoalveolar lavage.

Figure S8. **Scatterplots of cultured bacterial species and relative abundance of the most abundant corresponding OTU.**



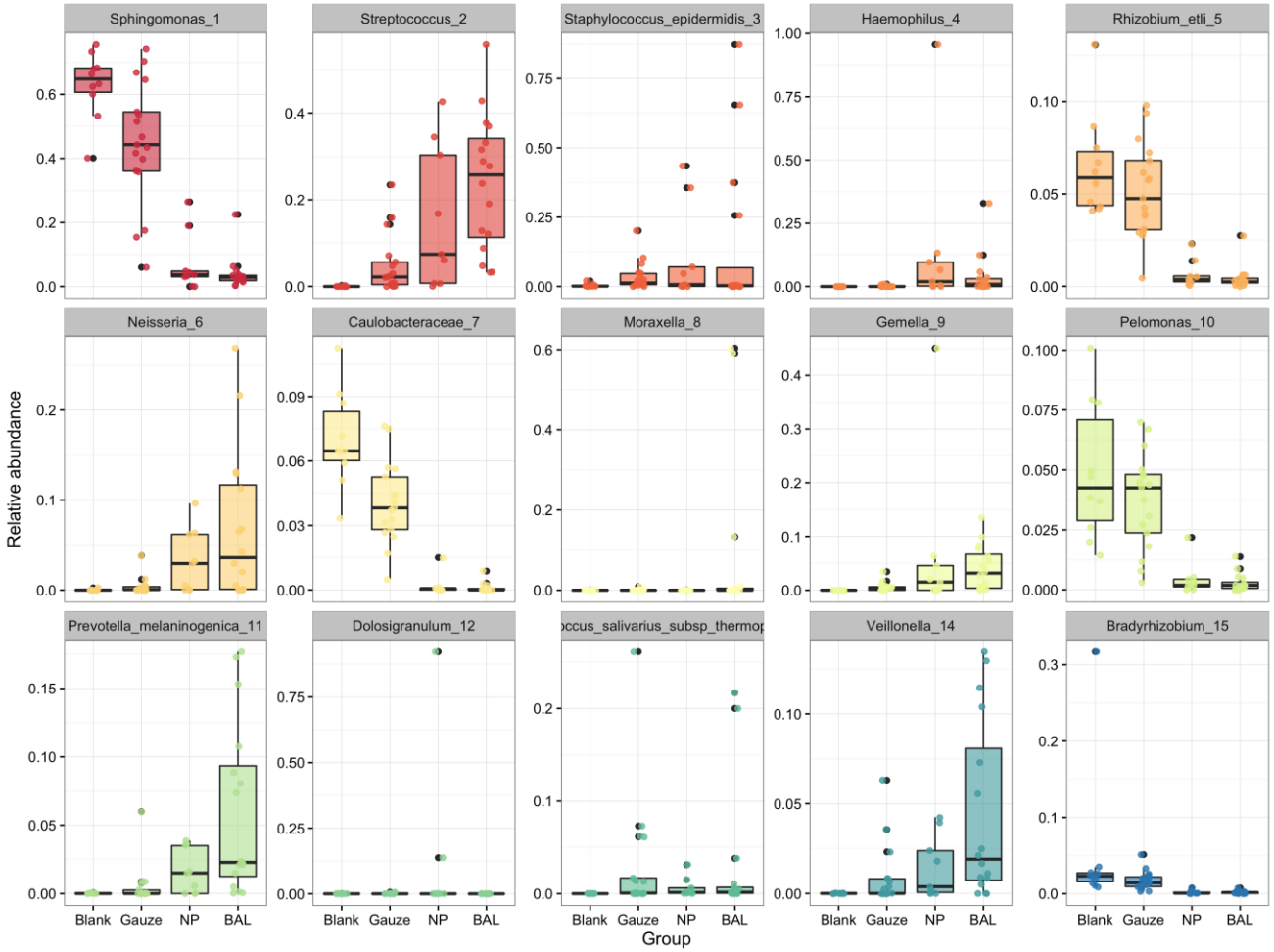
We depicted scatterplots, representing sequence versus culture data. Relative abundance of OTUs, assessed by Mann- Whitney U tests, was statistically different between corresponding culture positive (+) and negative (-) respiratory samples (n=121) and the most abundant OTU for the respective genera. To illustrate; the *S. aureus* OTU included in this figure is the most abundant one in our dataset and is strongly but not exclusively related to *S. aureus* culture results. In some culture positive, OTU negative samples other less common staphylococcal OTUs seems to correlate with the culture results, which is likely a consequence of genetic variation. Per group, the median with interquartile range is plotted. To avoid taking the log of zero we added 0.000010 to a relative abundance of zero.

Figure S9. **Bacterial colonization within paired NP, OP and BAL samples of CF infants**



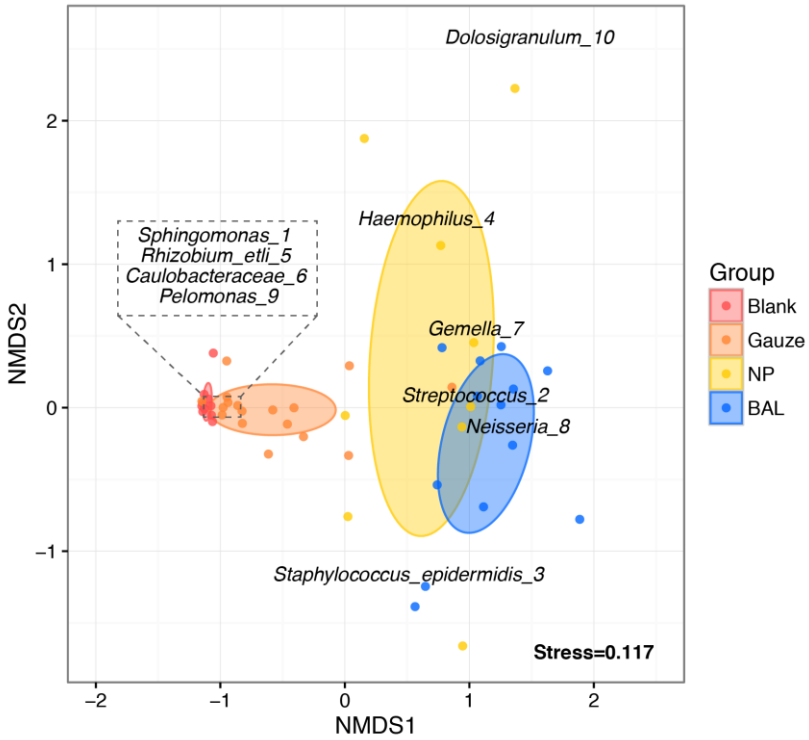
Abbreviations: NP = nasopharynx, OP = oropharynx, BAL = bronchoalveolar lavage, Gneg-o(ther) = *E. Coli* was most frequently cultured, n.a. = not applicable. * BAL fluids from different locations were pooled into one vial.

Figures S10. **15 Highest ranking OTUs in DNA isolation and gauze-controls, and low bacterial density NP and BAL-samples.**



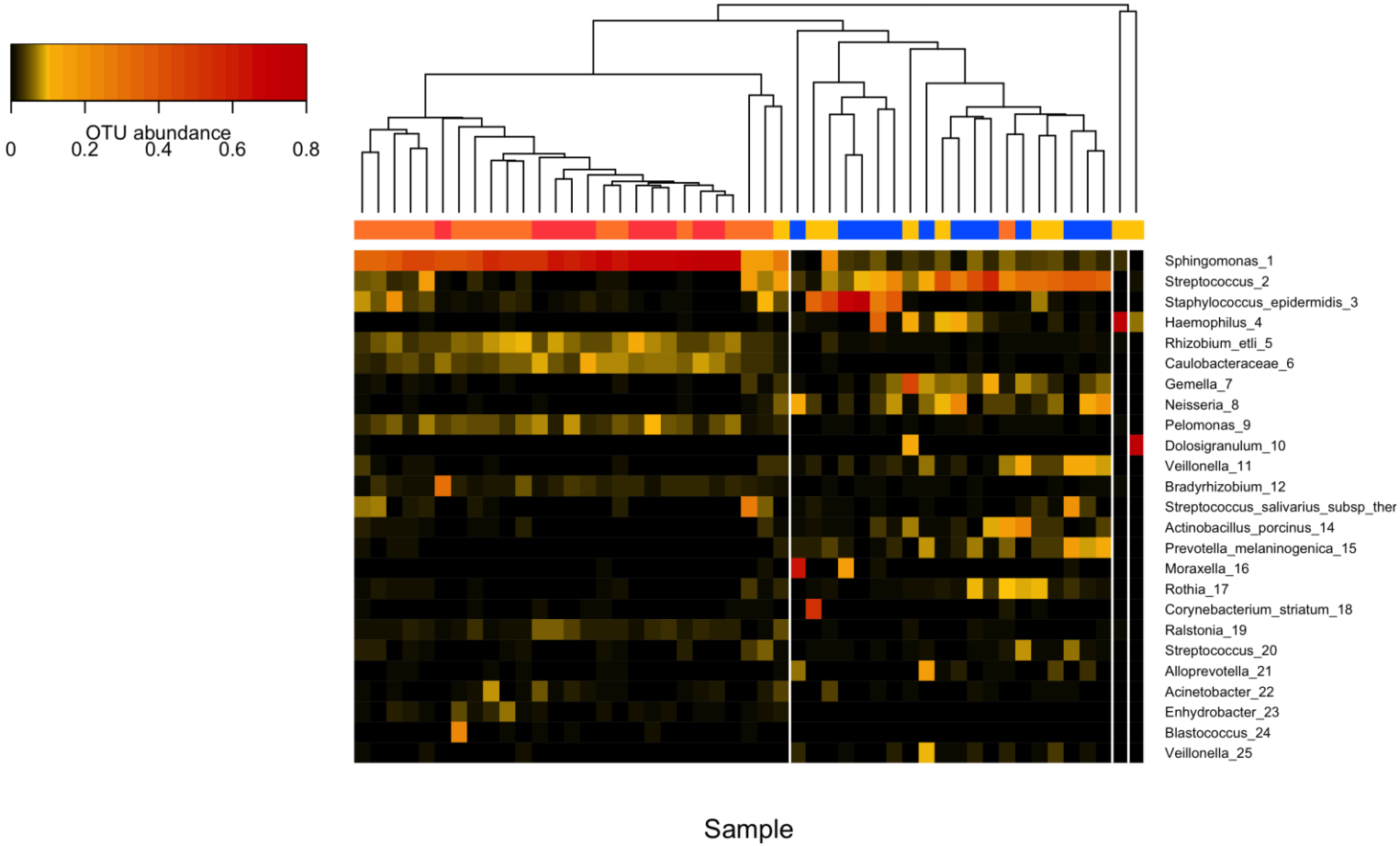
The 15 highest ranking OTUs in both patient samples and controls were plotted in boxplots. Abbreviations: Blank=DNA isolation control, Gauze=gauze-control, NP= nasopharynx, BAL= broncho-alveolar lavage.

Figure S11. **Bacterial community composition in DNA isolation and gauze-controls, and low bacterial density NP and BAL-samples.**



A non-metric multidimensional scaling (nMDS) plot based on the Bray-Curtis dissimilarity matrix was used to simultaneously visualize microbiota profiles (dots) originating from nasopharynx (NP; yellow), broncho-alveolar lavage (BAL; blue), DNA isolation controls (Blank; red) and 'tip-of-the-scope'- gauzes (Gauze; orange) and the 10 most abundant bacterial species among the four groups (based on the n=49 cohort). The standard deviations around the geometric mean of samples stratified by group are shown by the ellipses. As the OTU names of *Sphingomonas*, *Rhizobium*, *Caulobacteriaceae* and *Pelomonas* were projected on top of one another, we depicted them in a large, dotted lined box, indicating their original location by a smaller box. The numbers behind the OTU names represent the hierarchical number of that OTU within the top 10 highest-ranked OTUs.

Figure S12. **Hierarchical Clustering of DNA isolation and gauze-controls, and low bacterial density NP and BAL-samples.**



In this combined dendogram and heatmap we visualized the hierarchical clustering of the NP (yellow), BAL (blue) samples and the DNA isolation controls (red) and 'tip-of-the-scope' gauzes (orange; horizontal panel adjacent to dendrogram branch ends). The numbers behind the OTU names represent the order of the OTU in the top 25 highest-ranked OTUs. The colours in the heatmap correspond with the relative abundance. We identified 4 clusters discerned by vertical white lines: 1) typified by contaminating species (i.e. *Sphingomonas*, *Rhizobium*, Caulobacteriaceae and *Pelomonas*), including 1 NP-sample; 2) characterised by *Streptococcus*, *Staphylococcus*, *Haemophilus*, *Gemella*, *Neisseria*, *Veillonella*, *Actinobacillus*, *Prevotella* and *Rothia*, including 1 gauze-sample; 3) 1 NP-sample with high abundance of *Haemophilus* and 4) 1 NP-sample strongly enriched for *Dolosigranulum*.

AREST-CF follow-up protocol for 0-5 years

Rationale and execution protocol new patients with CF

This is a monitoring protocol for all patients who are diagnosed with Cystic Fibrosis by newborn screening (NBS). CF is included in the national neonatal screenings programme since May 1st 2011. This monitoring protocol is followed by the paediatric CF-clinics of Wilhelmina Children's Hospital/UMC Utrecht, Sophia Children's Hospital/Erasmus MC Rotterdam and Juliana Children's Hospital/Haga Hospital the Hague.
The purpose of this monitoring protocol is to standardize and intensify the follow-up of children with CF in the mentioned 3 centres of the 'CF-Centrale', in order to detect, prevent and treat early damage due to CF. The protocol is made according to the model of the monitoring protocol used in Australia, the AREST-CF program (Australian Respiratory Early Surveillance Team for Cystic Fibrosis).
The standardized protocol is also a good basis for participation in large multi-centre trials, both national and international.

An overview of the monitoring protocol is shown in the table at the end of the protocol. Each item is explained below with the corresponding number.

1. Informed Consent
All data collected in this monitoring protocol will be stored in a coded fashion after informed consent from the patient's parent/legal guardian. Informed consent from the participant's parent/legal guardian will be asked during one of the first visits. Information about the diagnostic protocol will be given. Data will be stored in a local and national database. Informed consent for storage and use of rest-materials for research will be asked separately.

2. Demographic History
Demographic history should be taken to obtain the participant's date of birth, sex, and age at diagnosis.

3. DNA
The positive heel prick screening result will be confirmed by DNA analysis or if necessary by extended gene analysis (in case of incomplete CF mutation analysis). This is part of the routine procedure of the newborn screening.

4. Sweattest
A sweat test is performed on the day of the first visit to confirm the results of the new-born screening.
Pilocarpine iontophoresis is the preferred method of sweat stimulation. Sweat should be collected for 30 minutes onto preweighed gauze or filter paper low in sodium chloride.
A minimum sweat rate of 1 g/m2 body surface area/min is required; thus a sweat volume of 50–100 ml is adequate. Sweat chloride concentrations will be measured in a reference lab of a CF centre (De Boeck et al, Thorax 2006).

5. Out-patient clinic visit

The GP will receive the result of the positive NBS. The first visit at the outpatient clinic will be within 48 hours after the probable diagnosis is told by the GP to the parents. At the first visit, a sweat test is performed to confirm the diagnosis found in the heel prick blood. There will be a consultation with the paediatric pulmonologist, who will explain the diagnosis, treatment and prognosis of CF. There will also be a contact with the CF-nurse who will explain many aspects related to CF, logistics of the CF team, hospital visits, life with CF, etc. The second visit will be one week later. This will be a consultation with the paediatric pulmonologist, gastro-enterologist or the dietician and the CF-nurse. After that the outpatient clinic visits will be every month till the age of six months, and if necessary more often. If the child is stable the visits will be every 2 months from the age of 6-12 months and every 3 months from the age of 1 year. At the annual check-ups other disciplines such as the dietician, physiotherapist, psychologist and social worker will see the patients, and more often on indication.

5a: Paediatric Pulmonologist/CF nurse
Each out patient clinic visit will be with the paediatric pulmonologists, and with the CF-nurse.

5b: Gastro-enterologist/dietician
The second visit, the gastro-enterologist and dietician are introduced. From then, the dietician and/or gastro-enterologist will see the patient at month 2,3 6, 10 and 12, and after that 1 to 4 times yearly depending on the problems. At month 10 the gastro-enterologist informs parents about the assessment and possible risks of the ICM at 12 months.

6. Medical History/Review of symptoms

A comprehensive general medical history will be taken by the CF doctor to determine significant previous and current medical conditions, diseases, procedures and surgeries. This should also include information regarding the participant's CF disease history, treatment and procedures.
At each visit the presence of symptoms associated with CF will be recorded using a standard form.

7. Physical Examination

A physical examination should be performed at Visit 1 by a CF doctor. The examination should include respiratory, cardiovascular and abdominal organs. At each visit, any changes or new physical examination findings since the previous visit should be recorded.

8. Height & weight & HC

Height, HC and weight should be measured to the nearest 0.1cm and 0.1kg respectively. Patients must be weighed in their underwear, until the age of 2 years old the length must be measured horizontally.

9. Symptoms Diary Card Review

Following the first visit, diary cards will be dispensed to parents at each visit to record respiratory symptoms such as cough, wheeze and shortness of breath, and general health indicators, including appetite, energy levels and the parent/legal guardian's impression of general wellbeing at times the child has respiratory symptoms. Diary cards will be collected at each clinic visit.

10. Sputum culture or throat swab & nasopharyngeal swab

When possible a sputum specimen will be collected for microbial culture (see SOP).

If it's not possible to collect sputum, a throat swab, for detecting colonisation in the oropharynx, will be collected for microbial culture by rubbing the posterior oropharynx using separate cotton-tipped swabs (Copan Swabs 484CE, during BAL 482CE) and placed in Amies transport medium. Respiratory specimens will be cultured onto appropriate media.
Nasopharyngeal samples will be performed using a small flexible cotton wool swab (Copan Swabs 484CE, during BAL 482CE) by a trained nurse/doctor. This swab will be placed immediately in liquid Amies transport.

11. Faecal elastase

To assess directly exocrine pancreatic function, Faecal Elastase-1 will be measured in a portion of stool using a sensitive enzyme-linked immunoabsorbent assay with monoclonal antibodies. In case of a pancreatic sufficient CF patient, faecal elastase will be yearly monitored.

12. Faecal fat balance

Faecal fat quantities will be measured in a 72 hours collection of stool to assess the degree of fat resorption, each year at the annual check-up until the age of 12.
Exceptions are: not yet continent for faeces, good growth and no complaints of steatorroea; breastfeeding; already on maximal dosage of pancreatic enzymes and protonpompinhibitors.
Fat intake will be noted in a nutrition diary during 72 hours prior to the collection of stool. A resorption coefficient can be measured and will be used for adjustment of dosage of pancreatic enzymes.

13. Blood

Blood samples will be collected under general anaesthesia at the times of BAL (at the age of 3, 12, 36 and 60 months) and at the age of 24 and 48 months, for routine clinical tests. All laboratory tests must be analysed at the selected laboratories for each site, and adhere to approved protocols and age-referenced range of values. The following tests should be completed:
At 3 months:

- Vitamine A/E/25-OHD
- PT
- Hb, Ht, trombo, leuco
- ALAT en γGT
- Alkaline phosphatase
- Glucose
- Serum for storage
- Urine: Natrium

From 1 till 5 years:

- Vitamine A/E/25-OHD
- PT
- Hb, Ht, trombo, leuco
- ALAT en γGT
- Alkaline phosphatase

Glucose
Serum for storage
Only at the age of 1 yr.: Urine Natrium

From 6 years:
Vitamine A/E/25-OHD
PT
Hb, Ht, trombo, leuco diff
ALAT en GGT
Albumine
AF
IgG
IgE, asp serology (=IgE asp fum and IgG asp fum)
Glucose
Serum for storage

Only on indication:
Bile acids, Factor V, ASAT, bilirubin, Na, K, Chloride, Creatinine

14. Urine

In urine samples collected at the age of 3 and 12 months concentrations of Sodium (Na) will be detected the rest will be stored (see SOP) for immunology. A urine sample at the age of 36 months will also be stored for immunology (anti-inflammatory mechanisms in CF).

15. Bronchoalveolar Lavage (BAL) & Bronchial Brushing (BAL at 3 months and brushings in Utrecht)

Most infants with CF are asymptomatic and have no clinically-apparent lung disease. A recent study in which 57 infants (median age 3.6 months) with CF underwent BAL and chest CT using a 3-slice inspiratory and expiratory protocol demonstrated that pulmonary inflammation was common at this age (Sly et al, Am J Respir Crit Care Med 2009). Despite the absence of respiratory symptoms in 48 (84.2%), a substantial proportion (20-25%) of infants had lung disease with: bacterial infection, neutrophilic inflammation and presence of pro-inflammatory cytokines.

A bronchoscopy with bronchial brushing and bronchoalveolar lavage will be completed at the age of 3, 12, 36 and 60 months. Immediately following the CT scan a bronchoscopy is undertaken via a laryngeal mask airway (see SOP). The bronchoscope is wedged in the right middle lobe and 3 aliquots of normal saline (each 1 ml/kg to a maximum 20ml) are lavaged and retrieved by low-pressure suction. The bronchoscope is then moved to either the most diseased lobe (seen on CT) or the lingula and a single aliquot (1ml/kg) is lavaged and retrieved. The first aliquot from each site is sent from microbiological analysis and the second and third aliquot are kept on ice until transport to the local laboratory for processing (culture and cytology).

To receive airway epithelial cells a brush will be pushed out of the bronchoscope and directed down to the trachea until resistance is felt. After five gentle back and forth motions with the brush, is will be brought back to just beyond the tip of the bronchoscope, both bronchoscope and brush will be brought out together. The brush tip will be cut off into 5 mL of collection media and transported to the local laboratory, on ice, for processing (culture, cytology, CFTR function). See the SOP for all details.

16. Chest X-ray

At the age of 3 months a chest X-ray will be performed to exclude major pulmonary abnormalities, or earlier on indication. Subsequent chest X-rays will be made on age 2 and every 2 years after.

17. Chest Computed Tomography Scan

In 57 asymptomatic infants with CF radiological evidence of structural lung disease was common with 46 (80.7%) having an abnormal CT; 11 (18.6%) had bronchial dilatation, 27 (45.0%) had bronchial wall thickening and 40 (66.7%) had gas trapping (Sly et al, Am J Respir Crit Care Med 2009).

The chest CT scan is to be conducted immediately prior to the bronchoscopy under general anaesthesia following the protocol. At approximately 3 months of age a limited (3-slice) scan will be taken in both inspiration (25 cmH_2O) and expiration (0 cmH_2O) following the protocol. At 12 and 36 months of age a volumetric scan will be taken in inspiration (25 cmH_2O) and at end-expiration (0 cmH_2O). From the age of 5 years (60 months) a spirometry controlled maximal both in- and expiratory CT-scan will be performed.

18. SOT (Utrecht)/LCI (Rotterdam)

Passive respiratory mechanics can be assessed with SOT. While infants are sleeping and spontaneously breathing, respiratory compliance (Crs), respiratory resistance (Rrs) and time constant (τrs) can be measured non-invasively (Katier et al, Chest 2005). Guidelines for equipment and methodology have been published by the ERS/ATS Task force on standards for infant respiratory function testing.

Multiple-breath washout detects abnormal lung function in children with CF more readily than plethysmography or spirometry (Aurora, Am J Respir Crit Care Med 2005).
A method for analysing an MBW is to study the progression of the end-tidal marker gas concentration, plotted against time, breath number or an index of cumulative expired volume. From the last of these plots, it is possible to calculate the lung clearance index (LCI) which is the cumulative expired volume required to clear this inert gas from the lungs to 1/ 40th of the starting concentration, divided by the functional residual capacity (FRC) (Beydon et al, ATS/ERS taskforce on pulmonary function testing in preschool children 2007).

19. Spirometry

Pulmonary function will be assessed as forced expiratory volume in 1 s percentage predicted for height and sex (FEV1%) and forced vital capacity percentage predicted for height and sex (FVC%) after inhalation of 800 mg salbutamol using reference data of Zapletal and Samenek.

20. CFQ-R

The validated disease-specific CF Quality of Life Questionnaire Revised (CFQ-R) will be administered to all parents with CF children at the age of 60 months.

21. ICM

The abnormalities in epithelial ion transport characteristic of CF are also expressed throughout the intestinal tract. Measurements can be performed on rectal or jejuna mucosa. There is a clear difference between ICM measurement in classic CF and in normal individuals. There are differences between and within genotypes of CF mutations. With the coming new drugs for CF, which are correcting or potentiating CFTR function, the CFTR function should be known before the drugs can be used.
For methodology and reference values of ICM measurements, see the study of Derichs et al, Thorax 2010. ICM is done at age of 1 year under general anaesthesia, combined with the BAL and blood sample. If rest activity of CFTR is found, the test will be repeated at age 5.

AREST-CF protocol. New patients with CF.

(months of age)	0	1	2	3	4	5	6	7	8	9	10	11	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
1. Informed consent	x																												
2. Demographic History	x																												
3. DNA	x																												
4. Sweattest	x																												
5. Out-patient clinic visits/contacts	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
a. Pediatric pulmonogist/CF nurse	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
b. Gastro-enterologist/dietician		x	x°	x			x				x°		x°				x°				x°				x°				x°
6. Medical history/review symptoms	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
7. Physical examination	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
8. Length, weight, head circumference	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
9. Symptom diary	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
10. Throat swab/sputum culture, nasopharyngeal swab (culture)	x	x	x	x	x	x	x		x		x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
11. Faecal elastase	x												X#				X#				X#				X#				X#
12. Faecal fat balance																									x				x
13. Blood				x									x				x				x				x				x
14. Urine				x*									x*								x								
15. Bronchoscopy and BAL				x									x								x								x
16. Chest X-ray				x													x								x				
17. Chest CT scan													x								x								x
18. SOT				x																									
19. Spirometry																									x	x	x	x	x
20. CFQ-R																													x
21. ICM													x																x^

X* for Na and storage (immunology), at 36 months only for storage (immunology)
X# only in pancreatic sufficient patient
X° both GI specialist and dietician, other months only dietician, X^ in case of rest activity measured at 12 months