Longitudinal Analysis of Respiratory and Intestinal Microbiome in Cystic Fibrosis

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Title of study:

Longitudinal Analysis of respiratory and intestinal microbiome in cystic fibrosis

Clinical project leader: Dr. med. Ingrid Bobis, CF unit of the Department of Pneumology, Clinic for Internal Medicine I, University Hospital Schleswig-Holstein (UKSH), campus Kiel, Germany

Scientific project leader: Prof. Dr. Andre Franke (Dipl.-Biol.), Institute of Clincial Molecular Biology (IKMB), Kiel University and UKSH, campus Kiel, Germany

Project team:

(in alphabetical order)

Prof. Dr. Burkhard Bewig, Department of Pneumology, Clinic for Internal Medicine I, UKSH, campus Kiel
Dr. Corinna Bang, IKMB, Kiel University and UKSH, campus Kiel
Prof. Dr. Andreas Claass, CF unit, Pediatric Department, Städtisches Krankenhaus Kiel
Prof. Dr. Andre Franke, IKMB, Kiel University and UKSH, campus Kiel
Prof. Philipp Rosenstiel, IKMB, Kiel University and UKSH, campus Kiel
Prof. Dr. Stefan Schreiber, Clinic for Internal Medicine I, UKSH, campus Kiel and IKMB, Kiel University and UKSH, campus Kiel
Dr. Christian Timke, CF unit, Pediatric department, Städtisches Krankenhaus
(all Kiel, Germany)

1. Background

1.1 Cystic fibrosis

Cystic fibrosis is caused by mutations in the *CFTR* gene with an autosomal recessive inheritance. It is a rare disease with a prevalence of 1-9/100,000 in Europe. The *CFTR* mutations result in a dysfunctional chloride channel that affects secretion from all exocrine glands.

The disease is chronic and generally progressive, with onset usually occurring during early childhood or, occasionally, at birth (meconium ileus). Virtually any internal organ may be involved but the principle manifestations concern the breathing apparatus (chronic bronchitis), pancreas (pancreatic insufficiency, adolescent diabetes and occasionally pancreatitis) and, more rarely, the intestine (stercoral obstruction) or liver (cirrhosis). The most common form of cystic fibrosis is associated with respiratory symptoms, digestive problems (steatorrhea and/or constipation) and staturoponderal growth anomalies. Mortality and morbidity depend on the extent of bronchopulmonary involvement. Male sterility is a constant feature. Late-onset forms, which are usually only mild or monosymptomatic, have also been reported.

Diagnosis is suspected on the basis of sweat test results (chloride concentration above 60 mmol/L) and is confirmed by identification of a *CFTR* mutation. Neonatal testing has been widely available since the end of 2002 and leads to diagnosis in 95% of cases.

Classical treatment of cystic fibrosis is mainly symptomatic, revolving around bronchial drainage, antibiotics for respiratory infections, pancreatic analysis and administration of vitamins and calorific supplements for digestive and nutritional problems. These cost-effective treatments have significantly improved the prognosis for cystic fibrosis patients: in the 1960's the majority of patients died before 5 years of age, whereas the current average life-span exceeds 35 years and life-expectancy is 40 years.

1.2 Microbial involvement in CF

The lung of CF patients is often affected by recurrent infections and colonized by *Pseudomonas aeruginosa* that form a viscous biofilm. Recent studies describe a complex microbiome in patients with cystic fibrosis, and common threads have emerged across studies in the relationships observed between features of the respiratory microbiome and clinical outcomes. CF airway microbial communities were shown to be relatively stable during periods of clinical stability, but differ substantially between patients [1]. See following Figure 1. Most studies to date have focused on bacterial members of the respiratory

microbiome, but some recent investigations have also begun to characterize fungal and viral communities in CF [2, 3].

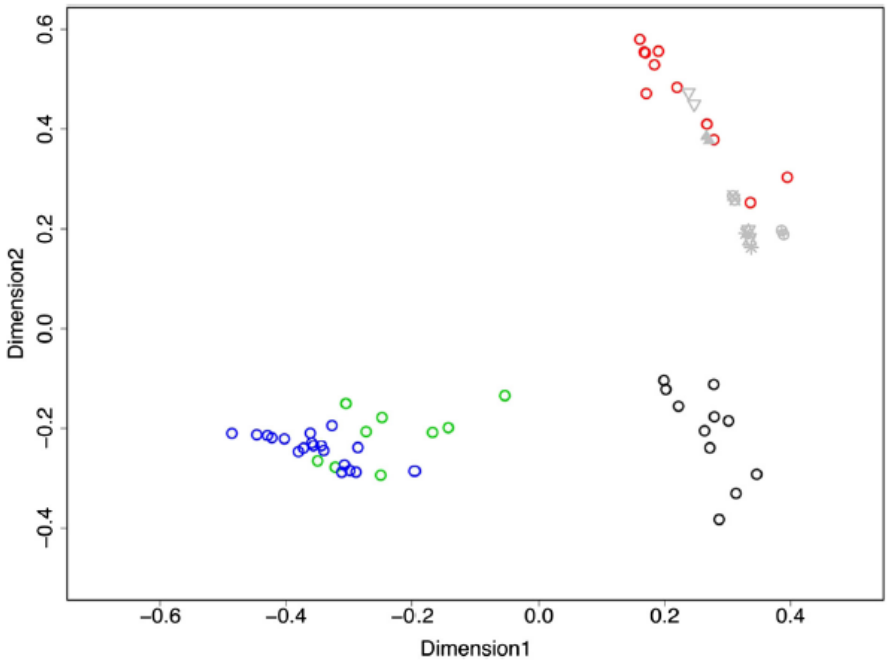


Figure 1 Baseline community structure. Bray-Curtis-based nonmetric multidimensional scaling plot showing daily sputum samples from four study subjects (subject A is red; subject B is green; subject C is black; subject D is blue), collected during clinically stable periods. Pairs of other symbols in gray are same sample replicate controls from subject A as described in Methods.

Taken from [1]

Although studies of respiratory microbiota have dominated CF microbiome studies, gastrointestinal complications of CF are a significant cause of disease morbidity, and problems beyond pancreatic insufficiency are often difficult to manage. Thus recently, investigations have also begun to characterize and analyze relationships between gastrointestinal bacterial microbiota, clinical disease markers, and profiles of airway bacterial community composition ([4, 5]; reviewed in [6]). Our knowledge on the relation of the intestinal and respiratory microbiome in CF is still very limited, but has great potential for biomarker discovery.

In CF, antibiotic therapy given in purpose to limit lung damage by bacterial colonization and infection, is a mainstay of therapy. Changes in respiratory and intestinal microbiome composition and diversity and the interrelationship between them are still to be elucidated.

1.3 Modern CF therapy

Since recently, medication is available that directly targets the impaired CFTR function. Depending on the type of mutation, the respective substances are called CFTR-potentiators or –correctors. In 2011, two large clinical studies (STRIVE und ENVISION) showed that the CFTR-potentiator Ivacaftor (trade name Kalydeco®) significantly improves clinical

parameters in patients with the rare G551D mutation [7, 8], and with to date eight other rare CFTR mutations.

Patients with the most common *CFTR* mutation Phe508del (= F508del) benefit from a therapy with a combination of the CFTR-corrector Lumacaftor with the *CFTR*-potentiator Ivacaftor, as shown in the TRANSPORT und TRAFFIC studies [9]. In Germany this combination is approved for CF since Nov 2015 (trade name **Orkambi**®). It improves CFTR function and thus influences secretion, e.g. in the lung and gut of patients.

For Ivacaftor alone, a small study comprising three patients described a modified respiratory microbiome with a trend towards higher diversity [10]. Based on this, we hypothesize that the treatment with **Kalydeco or Orkambi**® will influence the composition of the CF microbiome in eligible patients towards a healthy and stable high-diversity state.

2. Aims

Our study targets the influence of standard CF therapy on the CF microbiome. The respiratory and intestinal microbiome and their interrelationship will be characterized in a longitudinal study design. The observed changes will be correlated to functional (FEV1, body weight, laboratory parameters) und quality of life (CFQ-R, Kiel-Q) parameters We aim at the detection of significant changes in microbial parameters in the respiratory tract and in the gut following medical therapy (e.g. antibiotics, CFTR modulators). The microbial composition will further be correlated to treatment response, in order to discover potential microbial biomarkers.

3. Study design

3.1 Participants and clinical data

The study comprises analysis of clinical parameters and microbiome and 8 time points. Patients are recruited from the adult cf centre at the University Hospital of Schleswig Holstein, Campus Kiel, Innere Medizin I. Eligible patients are patients with a confirmed diagnosis of cystic fibrosis (sweat chloride >60mmol/l and /or 2 cf causing mutations in the CFTR gene) and the ability to give informed consent .

All participants receive a questionnaire at each time point of sampling that will either be filled in by themselves or by a study nurse via a telephone interview. Clinical data is collected by the medical study team from standard health records and via a questionnaire. From clinical data CF-genotype, microbial status concerning chronic infection with Pseudomonas

aeruginosa and other bacteria, height and weight, current medication, presence and frequency of exacerbations, lung function parameters (e.g.FEV1, FVC, MEF 25, MEF 75-25), full blood count, C reactive protein, liver function tests (AST, ALT, GGT, ALP, TBil), creatinkinase, calprotectin and elastase will be enrolled. The questionnaires contain i) basic items, like age and body weight, ii) items related to the CF phenotype and the clinical outcome, like quality-of-life, lung function and other medical complaints, and iii) items that relate to the microbiome, like food intake or other medication.

3.2 Recruitment and biosampling

Biosampling will be performed at each time point with a tolerance of three days. For stool sampling the participants will receive sampling tubes that will be sent back by mail. Respiratory samples will either be taken during the visit by the medical study team or by the participants at home. At each time point two stool samples, one sputum sample and one throat swab will be collected.

Biosampling is performed in an anonymized fashion. Sample preparation and the generation of molecular profiles is conducted at the IKMB and will be managed by Dr. Corinna Bang. The IKMB staff receives samples and phenotypes without any information that allows the identification of individual study participants, so that data protection policies will be fulfilled within the entire study.

3.3 Phases

The study course is subdivided in two phases. The first 3 samples collected at timepoints “0”, “2” and “4” will generate a baseline for analysis of the comparison with following samples.

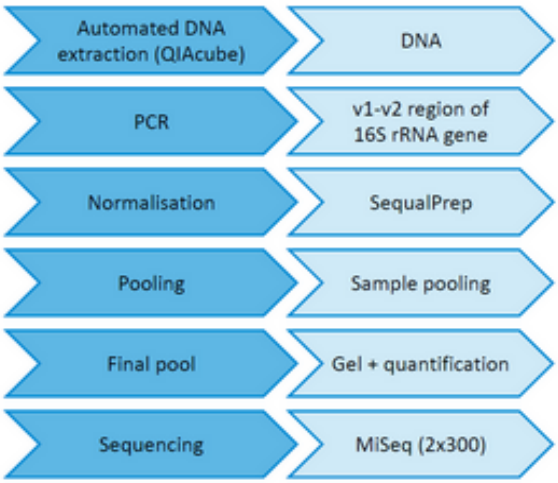
4. Molecular profiling and data analysis

4.1 Sample preparation

All collected samples will be processed at the IKMB according to established protocols. DNA will be isolated from stool and respiratory samples and used for the generation of microbial profiles. In addition, the isolated DNA samples can be used for all established sequencing application. A second stool sample will be used to measure the intestinal inflammatory marker Calprotectin and Elastase-1, in order to document intestinal and pancreatic involvement. Both methods are applied in the routine clinical setting, and are thus standardized and well established.

4.2 Microbial profiling by next generation sequencing (NGS)

Both bacterial and fungal profiles will analyzed within the study. The characterization of bacterial profiles is conducted by sequencing the *16S rRNA* gene, the so-called *16S DNA*, which is in parts highly variable and present in all bacterial and archaeal organisms. The 16S analysis by NGS is well established allows microbial profiling down to the genus level at a qualitative and quantitative level.



According to established protocols [11], the V1-V2 region of the *16S rDNA* of the extracted DNA is amplified in a sequence-specific PCR reaction. The PCR products are than normalized and a maximum of 384 samples are than pooled for sequencing with the Illumina MiSeq system.

For fungal profiling the highly variable *ITS1* and *ITS2* gene regions will be amplified and processed as described for bacterial profiling. For both bacteria and fungi the identification of the corresponding microorganisms is conducted by the comparison of the obtained sequences to public sequence databases.

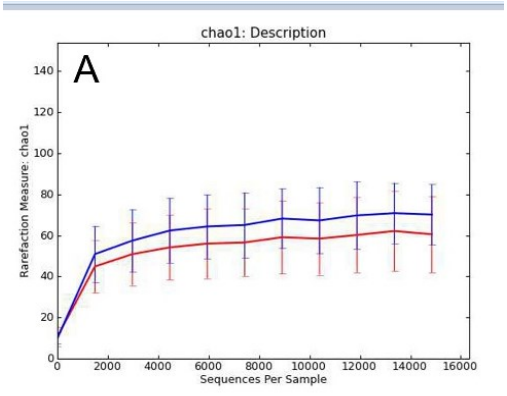
4.3 Statistical analysis of microbial profiles and modeling of microbial changes

The obtained sequences provide information of the composition of the microbial community in each sample at a qualitative and quantitative level. As the primary outcome the diversity within one sample (α-diversity) will be determined that can be expressed in various indices, e.g. Chao1 or Shannon index, and changes in α-diversity will be calculated (Δdiv). In addition, the specific composition of the microbiome profiles will the analyzed. Respiratory and intestinal profiles will first be investigated separately, and will be than be analyzed for a possible correlation. The following procedures and tests will be conducted in the given order:

1) Calculation of changes in α-diversity indices for each sample (Δdiv; "baseline" vs. "follow-up") and comparison of diversities correlated to treatment characteristics of the patients (e.g. antibiotic versus non-antibiotic, CFTR modified versus non-CFTR modified).
2) We hypothesize that the α-diversity in patient`s microbiome will change in response to external stimuli (E.g.: Decrease in response to antibiotic therapy? Increase in response to CFTR Modification?). In all patients indices of the three time points "baseline" will be summarized and used for the calculation of the α-diversity change. A non-parametric test will be applied to test, if there is are significant changes in α-diversity in response the initiation of antibiotic oder CFTR modifying treatment.
3) Fitting of a statistical model for the change in α-diversity in patient`s microbiome.
4) Correlation of the α-diversity with therapy response in as well as over the entire course of the study.
5) Analysis of the abundance of specific bacteria or fungi over the time course using a zero-inflated model
6) Comparison of the composition of the obtained microbial profiles (β-diversity) between timepoints and patient`s characteristic groups by PCoA (multiple scaling analysis)
7) Correlation of microbial parameters (changes in α-diversity or specific organisms) of respiratory and intestinal samples

4.4 Statistical power and confounding

For Ivacaftor alone, a small study comprising three patients described a modified respiratory microbiome with a trend towards higher diversity [10]. The study comprised only three participants, and sampling was



Red: Before treatment; *Blue*: After treatment [10]

performed at random, unequal intervals. Our study includes 45 patients and has thus an increased chance to detect significant changes in the microbial composition.

It is common sense that microbiome data are highly variable [12]. A number of parameters influence the microbial profile, e.g. age, food intake or medication. These known confounders will be included in the analysis. A possible technical bias will be minimized by using the same chemical reagents throughout the study and by performing NGS in patient-wise batches according to standardized protocols.

5. References

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