The nasal microbiota in infants with cystic fibrosis within the first year of life: a prospective cohort study

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ABSTRACT

Background: Respiratory tract infections and subsequent airway inflammation occur early in the life of infants with cystic fibrosis (CF). However, detailed information regarding the microbial composition of the respiratory tract in infants with CF is lacking. Here, we performed an in-depth characterization of the upper respiratory tract microbiota in infants with CF, who were followed throughout their first year of life.

Methods: Microbiota characterization was performed using 16S *rRNA* gene pyrosequencing and oligotyping of nasal swabs (n=461) collected biweekly from 30 infants with CF. Data was compared to an age-matched cohort of 47 healthy infants (n=872 samples). Additionally, the influence of antibiotic therapy on the microbiota of infants with CF was investigated.

Findings: The microbiota of infants with CF differed compositionally from the microbiota of healthy infants (permutational MANOVA, $P=0.001$). This difference was also found in exclusively antibiotic-naïve samples ($P=0.001$). The disordering was mainly, but not solely, due to an overall increase in the mean relative abundance of Staphylococcaceae, which persisted during antibiotic therapy. Oligotyping analysis allowed for a differentiation between *S. aureus* and coagulase-negative Staphylococci. While there was a trend of decreased *S. aureus* at antibiotic therapy, coagulase-negative Staphylococci, in contrast, increased.

Interpretation: This study describes compositional differences in the microbiota of CF infants compared to healthy controls, and the disordering of the microbiota upon antibiotic administration. Besides *S. aureus*, also coagulase-negative Staphylococci contributed to the disordering identified in infants with CF.

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INTRODUCTION

Cystic Fibrosis (CF) is an autosomal recessive disease, characterized by progressive lung disease contributing to morbidity and mortality in affected patients.¹ A mutation in the CF transmembrane conductance regulator was shown to cause a change in the airway microenvironment and subsequent changes in bacterial colonization.^{2, 3} This altered colonization of the respiratory tract is often characterized by an increased abundance of pathogens and is associated with disease progression, resulting in the frequent administration of antibiotics.⁴⁻⁶ However, little is currently known about the entire composition of the upper respiratory tract microbiota in infants with CF. Given that the presence of infections and inflammation in infants with CF plays a critical role in later disease progression, this knowledge would be both timely and relevant to our clinical approach with patients suffering from CF.⁷⁻⁹ Indeed, recent studies have revealed that airway inflammation¹⁰⁻¹² and diminished pulmonary function already occur within the first year of life.¹³ This suggests that the early detection of an imbalanced microbiota could inform subsequent treatment protocols, leading to better clinical outcomes.^{9, 14} The practical implementation of these changes to protocol, however, has not yet been determined

Here, we performed a longitudinal in-depth characterization of the nasal microbiota in infants with CF. We collected biweekly nasal swabs from 30 infants with CF during the first year of life and performed next-generation sequencing and oligotyping of the 16S *ribosomal RNA (rRNA)* gene. This data was compared to an age-matched cohort of 47 healthy infants with an identical study design.¹⁵ The primary objective of this study was to compare the nasal microbial composition of infants with CF to that of healthy controls during the first year of life. As a secondary objective, we examined

the effects of antibiotic treatment on the nasal microbiota in infants with CF, and whether or not alterations were transient or persistent.

METHODS

Study population

A total of 30 CF infants were enrolled between 2011 and 2014 from all CF study centers in Switzerland, after diagnosis of CF by newborn screening (Table 1, Figure 1 and Supplementary Table 1).¹⁶ The study was approved by the Ethics Committee of the Canton of Bern. The CF cohort was compared to a previously described, age-matched cohort of 47 healthy infants.¹⁵ Nasal swabs were collected biweekly by the parents of the study infants (see Supplementary Methods for details).

Study design

We first compared the microbiota of all CF infants to that of healthy infants. We then investigated the microbiota of only those samples from CF infants that were collected before the first administration of antibiotic (AB) therapy. Given that the number of CF infants not exposed to AB within the entire first year of life was low (N=6), we focused on AB-naïve samples in the first half year of life; these were then compared to the samples from healthy infants in the same time frame. A final step was to analyse the influence of AB therapy on the nasal microbiota by grouping all samples from CF infants into four groups (Figure 1): before first AB, at AB therapy and up to 10 days after therapy, after single AB therapy, and after more than one AB therapy. The lack of national guidelines for AB therapy for infants with CF means that it is largely determined based on i) respiratory symptoms, ii) the results from regularly-performed culture-based throat swabs, iii) the attitude of the parents toward antibiotics, or iv) the

attitude of tending physicians toward regular AB therapy in early CF disease.

Protocols for AB therapy also differ in Switzerland compared to other countries.¹⁴

There were only two samples of healthy infants at AB therapy. This did not allow us to analyse the effect of AB therapy in healthy infants.

16S *rRNA* 454 FLX Titanium sequencing analyses

DNA was extracted and PCR of the 16S *rRNA* (V3-V5) gene was performed as described.^{15, 17} Primer pair 341F/907R was used for amplification. Primers had a high *in silico* match for the bacterial families expected in the upper respiratory tract (Supplementary Table 2). The 454 FLX Titanium platform was used for sequencing and PyroTagger pipeline to analyse the sequence reads.¹⁸ Analysis included clustering based on 97% sequence identity (see Supplementary Methods for details). Detailed information on quality cut-offs of sequence reads, sequencing depth based on Chao estimates, and quality controls during sample processing (e.g. inclusion of negative controls), is found in the Supplementary Methods, and Supplementary Figures 1, 2 and 3. Sequence reads were submitted to the National Center for Biotechnology Information Sequence Read Archive (accession no. SRP063758). Analyses of bacterial density, alpha and beta diversity, relative abundance of bacterial families, and oligotyping were performed, as previously described, and/or are outlined in detail in the Supplementary Methods.^{15, 17}

Statistical analyses

Regression analyses were performed using a multivariable multilevel linear regression with random effect to correct for clustering on the individual level. We first compared healthy infants to CF infants, and adjusted for age, season, and AB therapy. We then analysed AB-naïve samples by stratifying by age and adjusting for

season. Last, we analysed the effect of AB therapy and adjusted for age and season. Depending on the analysis, outcome variables were PCR concentration, Shannon Diversity Index (SDI), the relative abundances of the most prevalent bacterial families (Streptococcaceae, Moraxellaceae, Staphylococcaceae, Corynebacteriaceae, Flavobacteriaceae, and Pasteurellaceae; all remaining bacterial families were grouped as “others”), and the relative abundances of the Staphylococcaceae oligotypes (OTs). As some of the outcome variables were not continuous with normally distributed errors, we additionally performed a multilevel Poisson regression analysis.

Role of the funding source

The sponsors of this study had no influence in study design, data collection, data analysis, data interpretation, or writing of the manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

RESULTS

Study cohort

Characteristics of the CF study cohort are shown in Table 1, Figure 1, and Supplementary Table 1. Most of the infants were either homo- or heterozygous for the $\Delta F508$ mutation. After exclusion of low quality samples, a total of 461 nasal swabs from 30 infants with CF were analysed (see Supplementary Methods for details). This corresponds to a mean of 15 (range 4-24) swabs per infant. In total, 24 infants with CF were treated with AB therapy at least once within the first year (Figure 1). The mean (SD) age of the first AB administration was 4.8 (95% Confidence Interval (CI) 3.6 to 6.0) months. The study cohort of healthy infants was previously described.¹⁵

The nasal microbiota in the first year of life: Bacterial composition differs between healthy and CF infants

In our first comparison, we included all samples (n=461) from infants with CF (N=30) and all samples (n=872) from healthy controls (N=47). Bacterial density differed between healthy and CF infants, probably due to more frequent AB use in the latter (mean 32.5 ng/ μ l (95% CI 30.7 to 34.5) and 24.7 ng/ μ l (95% CI 22.2 to 27.1), respectively). There was no significant difference in SDI between healthy infants and infants with CF (mean 1.2 (95% CI 1.2 to 1.3) and 1.4 (95% CI 1.3 to 1.5), respectively). Employing beta diversity analysis with non-metric multidimensional scaling (nMDS) ordination based on Operational Taxonomic Units (OTUs) with 97% sequence identity, we found, however, significant divergence in samples from CF infants versus those from their healthy counterparts (Figure 2A; permutational MANOVA, $P=0.001$). We then grouped the sequencing results according to bacterial

families and found a significantly higher abundance of Staphylococcaceae in infants with CF (Figures 2B and 2C; multivariable linear regression model adjusting for age, season, and AB therapy; $P<0.001$; Coefficient (Coef.) 10.8; 95% CI 5.8 to 15.9). In contrast, there was a significantly lower abundance of Corynebacteriaceae ($P=0.02$; Coef. -2.6; 95% CI -4.9 to -0.3) and Pasteurellaceae ($P=0.002$; Coef. -4.6; 95% CI -7.5 to -1.6) in infants with CF (Figures 2B and 2C). To account for the non-normal distribution of some bacterial families, a Poisson regression model was additionally performed (Supplementary Figure 4).

Knowing that *Staphylococcus aureus* is frequently isolated in infants with CF,¹⁰ we next investigated if this was also the case in our dataset and performed oligotyping of all Staphylococcaceae sequence reads. Oligotyping allows for the elucidation of diversity within final OTUs, based on the information that stems from the entropy analysis of variable sites in the sequences.¹⁹ The nucleotide variation at three positions in the sequence alignment allowed us to distinguish four different oligotypes (OTs; “T”, “AAT”, “AGAT”, “AGAC”, Supplementary Figure 5), one of which was identified as *S. aureus* (Figure 2D, OT “T”). Interestingly, we found that in addition to the *S. aureus*-OT, coagulase-negative Staphylococci were also more often found in the CF group (OT “AAT” and “AGAC”; Figure 2C and 2D; multivariable linear regression model adjusting for age, season, and AB therapy; OT “T”: $P<0.001$; Coef. 7.0; 95% CI 3.2 to 10.8, OT “AAT”: $P=0.02$; Coef. 2.8; 95% CI 0.5 to 5.2, and OT “AGAC”: $P<0.001$; Coef. 0.8; 95% CI 0.4 to 1.1, respectively. Corresponding Poisson regression in Supplementary Figure 4).

Alterations in the nasal microbiota of CF infants is detected early in life and independent of AB therapy

In order to investigate whether the different composition of the microbiota in infants with CF was rather due to the underlying disease or to the administration of AB, we performed permutational MANOVA tests of the whole bacterial community composition and found that AB therapy, indeed, significantly influenced the bacterial composition in our dataset (Supplementary Table 3). For the subsequent analyses, we focused on the first half year of life and investigated the CF samples prior to AB therapy (Figure 1; all yellow samples until six months).

Our initial results from analysing bacterial density and alpha diversity did not reveal any significant differences between the nasal microbiota of CF and healthy infants (Figure 3A and 3B). After performing nMDS analysis, however, we found that the CF samples clustered differently than the healthy samples at the ages of two, three, four, and five months. This would suggest that a disordering was already apparent in the second month of life (Figure 3C). Looking at the relative abundance of bacterial families, we found again that Staphylococcaceae were significantly increased in infants with CF from the second to the fourth months as compared to healthy infants (Figure 3D and Supplementary Table 4; multivariable linear regression model stratified by age and adjusted for season; second month: $P=0.04$; Coef. 16.2; 95% CI 0.6 to 31.9, third month: $P=0.02$; Coef. 17.9; 95% CI 3.3 to 32.5, and fourth month: $P=0.002$; Coef. 21.1; 95% CI 7.8 to 34.3, respectively). In contrast, Corynebacteriaceae were significantly decreased in the third and fourth months in infants with CF (Figure 3D and Supplementary Table 4; third month: $P=0.04$; Coef. -6.1; 95% CI -12.0 to -0.2 and fourth month: $P=0.03$; Coef. -6.8; 95% CI -12.8 to -0.8, respectively).

Changes of the nasal microbiota of infants with CF associated with AB therapy

Our next step was to investigate the influence of AB therapy on the nasal microbiota of CF infants. We found the SDI significantly increased at AB therapy compared to samples collected before first AB, indicating higher sample diversity upon AB administration probably due to transient colonizers (Figure 4A and Supplementary Table 5; multivariable linear regression model, adjusting for age and season; $P=0.02$; Coef. 0.4; 95% CI 0.1 to 0.7). A significant increase was noted for species richness, too (Supplementary Figure 6). There was no change detected in bacterial density upon AB administration (Supplementary Table 5). We revealed a decreased mean relative abundance of Moraxellaceae and an increase of “others” in samples collected at AB exposure (Figure 4B and Supplementary Table 5; Moraxellaceae: $P=0.03$; Coef. -12.7; 95% CI -23.9 to -1.6, and “others”: $P<0.001$; Coef. 22.3; 95% CI 10.9 to 33.7, respectively). Strikingly, the group of “others” remained significantly increased after 1x AB therapy (Figure 4B and Supplementary Table 5; $P=0.04$; Coef. 10.3; 95% CI 0.6 to 20.1). These findings indicate persistent changes in the microbiota upon AB administration. Investigating the group of “others” at AB therapy, we identified several different bacterial families (Supplementary Figure 7). However, no decrease of Streptococcaceae and Staphylococcaceae was observed, despite *S. aureus*-directed AB administration (Figure 4B and Supplementary Table 5). Investigating the family of Staphylococcaceae in more detail using oligotyping, we found a trend of decreased OT “T” (assigned as *S. aureus*) at and after 1x AB therapy, and in contrast, an increase of OT “AAT” (assigned as coagulase-negative Staphylococci *S. epidermidis/ caprae/ capitis*) (Figure 4C and Supplementary Table 5).

DISCUSSION

This is the first longitudinal study describing the nasal microbiota of infants with CF from such dense sampling in the first whole year of life. Our study design enabled us to compare CF infant data with an age-matched cohort of healthy infants, and to investigate the influence of AB therapy on the dynamics of CF infants' nasal microbiota. We found significant differences in bacterial composition between the two groups, which were mainly, but not solely, caused by an increased abundance of Staphylococcaceae in infants with CF. In order to investigate the family of Staphylococcaceae in more detail, we performed oligotyping, which allowed us to differentiate between *Staphylococcus aureus* and coagulase-negative Staphylococci.

The main finding of this study is the description of the changes within the microbiota of infants with CF as compared to healthy infants, which is somewhat in line with culture-based studies.¹⁰ However, microbiota analyses describe whole microbial community compositions rather than single bacterial species.^{15, 17, 20} In particular, we describe the absence of (potentially) beneficial bacteria (e.g. Corynebacteriaceae). A finding which is often overlooked in culture based studies, which are mostly focusing on potential pathogens. Uniquely, we also performed oligotyping, which has, to our knowledge, never been used for the molecular characterization of the respiratory tract microbiota. This method allows for an accurate species designation and, therefore, differs from the commonly used OTU-based clustering approach.^{19, 20}

Here, oligotyping differentiated species based on the V3-V5 region of the 16S *rRNA* gene among the family of Staphylococcaceae due to three different single-nucleotide polymorphisms, one of which we previously validated using a 16S *rRNA* Terminal Restriction Fragment Length Polymorphism (T-RFLP) protocol.²¹ In our study the *S. aureus*-oligotype was increased in infants with CF. This was expected given that

previous culture-based studies have shown more frequent *S. aureus* isolation from the airways at a very early age in CF.^{22, 23} In addition, there is a higher sodium chloride concentration in patients with CF, which may result in selective advantages for *S. aureus* in the nose.²⁴ We also found, quite unexpectedly, that coagulase-negative staphylococci were also increased in infants with CF.

However, even though a distinct disordering was found in the first analyses between healthy and CF infants, it was not clear if the observed changes were due to the effects of AB therapy or to the disease itself. In order to investigate this, we compared the nasal microbiota between the two groups prior to AB administration. Initially, we detected no difference in bacterial density or diversity. This was unexpected, since *Renwick et al.* showed a decreased SDI value in children with CF; the mean age of their CF cohort, however, was 3.95 years.²⁵ Therefore, the different observations in our data were probably due to the young age of our cohort, since *Cox et al.* showed a progressive age-dependent loss of diversity in older patients with CF.³ We would theorize that a decrease in bacterial diversity does occur later in life. In contrast, we found a significantly different bacterial community composition in infants with CF as compared to healthy infants; detectable as early as two months of age, due in part to an increase of Staphylococcaceae. This seems to illustrate that the disease progression, independent of AB therapy, causes compositional changes in the microbiota. A similar study recently published by *Prevaes et al.* also found increased Staphylococcaceae at an early age in infants with CF.²⁶ It is, however, difficult to compare the findings, as the study designs differ, and the patients in our study were more densely sampled.

Our study also identified several outcomes that indicate changes within the microbiota upon antibiotic therapy. Interestingly, and perhaps counter-intuitively, we

revealed higher sample diversity and richness upon AB administration. We hypothesize that AB therapy results in a vacant niche, which becomes subsequently colonized by various transient species, resulting in elevated diversity and richness. In addition, the bacterial composition from samples taken before and after >1x AB therapy was different; meaning, the microbial composition did not revert back to its pre-AB therapy composition. It was also surprising to observe that, despite AB therapy, the relative abundance of Staphylococcaceae did not decrease in CF infants. A recent study postulated that persistent *S. aureus* colonization may be due to biofilm building, which may also be the case in our study.²⁷ However, applying oligotyping in order to differentiate between *S. aureus* and coagulase-negative Staphylococci, showed that there was a slight reduction of *S. aureus* at AB therapy, but in contrast, an increase of coagulase-negative Staphylococci (*S. epidermidis/caprae/capitis*). This finding might confirm that *S. epidermidis* acts as a reservoir for resistance genes, as previously shown.²⁸

Our study has some major strengths. We performed a dense sampling of 30 infants with CF within the first year of life, resulting in more than 450 analysed samples. Such detailed longitudinal data is necessary for an in-depth characterization of the nasal microbiota of infants with CF. Furthermore, we compared the results with an age-matched control group of healthy infants in order to detect differences in the development of the microbiota within the first year of life, resulting in more than 1300 analysed samples. Furthermore, we were, to our knowledge, the first to perform oligotyping with human respiratory samples, which could differentiate between *S. aureus* and coagulase-negative Staphylococci. Finally, we believe that it is indeed a strength of our study to have included a large proportion of antibiotic-naïve swabs

from infants with CF. This is the most effective means to optimally assess the impact of AB therapy on the microbiome.

Our study has some limitations, too. We performed nasal sampling and considered nasal swabs as representative of the nasopharyngeal microbiota. However, both sites are very similar in infants, as it was recently shown.²⁹ In addition, the nasal swabs were taken by the parents of the study infants, and thus we cannot exclude a potential sampling bias due to inadequately obtained swabs, though it was recently reported, that the majority of parent-collected nasal swabs were adequate for analysis.³⁰ We also did not investigate how bacteria in the nasal passage of CF patients may reflect what is happening in the lungs, nor did we analyse samples from the oropharynx, which is also an important reservoir for lower airway colonization. In addition, the number of infants varied within the four chosen groups (before first AB, at AB therapy, after single AB therapy, and after more than one AB therapy).

However, we adjusted for this by applying statistical models and found the findings to be robust. Nevertheless, we are not able to clearly disentangle the exact influence of age, AB therapy, and clinical symptoms of underlying lung disease on the changes in the microbiota. As all of those factors are clearly interrelated, this will be possible with either a therapy-directed study design or a larger observational study with more detailed clinical information and alternative statistical modelling (e.g. structural equation modelling).

Finally, it is also important to recognize that the molecular methods do not distinguish viable organisms from DNA, which accumulates in mucosa. However, a recent study aimed at detecting potential respiratory pathogens in healthy adults using microbiological culture and PCR, revealed that there were 'no significant differences

in the detection of bacteria between culture and PCR methods', though the latter did not target the 16S *rRNA* and care should be taken as for the interpretation.³¹

To conclude, this study demonstrates the compositionally different nasal microbiota of CF and healthy infants in the first year of life. Additionally, it reports on the association of AB therapy with changes in the nasal microbiota, and reveals an unexpected trend of increased coagulase-negative *Staphylococci* during the course of AB therapy. These findings are of significant relevance given the critical role that bacterial pathogens play in the disease progression of CF in early life. Our findings should be used to inform future studies on the impact of AB therapy on the microbiota, and could greatly assist in the prevention of early disease progression in infants with CF.

Declaration of interests

Dr. Latzin reports personal fees from Vertex for grant review; from Roche for advisory board activities and from Gilead Sciences for lectures, all outside the submitted work.

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TABLE

Table 1: Characteristics of the CF study population, N=30 infants.

Characteristics	Summary Statistics
CF genotype Δ F508 homozygous, N (%)	13 (43.3)
CF genotype Δ F508 heterozygous, N (%)	15 (50)
CF genotype other than Δ F508, N (%)	2 (6.7)
Female, N (%)	17 (56.7)
Gestational age at birth (weeks), mean (SD)	39.1 (1.4)
Length at birth (cm), mean (SD)	49.0 (1.4)
Weight at birth (kg), mean (SD)	3.2 (0.4)
Spring born, N (%)	15 (50.0)
Summer born, N (%)	4 (13.3)
Fall born, N (%)	5 (16.7)
Winter born, N (%)	6 (20.0)
Breastfeeding, N (%)	25 (83.3)
Samples included, mean per infant (SD)	15.4 (3.9)

FIGURE LEGENDS

Figure 1: Overview of the study design. Indicated is the ID of the study infants (N=30 infants and n=461 samples), the CF genotype, and the number of samples per infant analysed in this study. Boxes represent samples. Subjects were ordered in increasing order of time until first antibiotic course and samples were grouped according to the age of the infant (in months or quarters of the first year of life; time axis below). Colours indicate the corresponding group based on AB treatment: yellow: before first AB therapy (n=221), red: at AB therapy (n=50), light blue: after 1x AB therapy (n=95), dark blue: after >1x AB therapy (n=95).

Figure 2: Comparison of the nasal microbiota of infants with CF and healthy infants within the first year of life. A) Significant difference in beta diversity of all samples from infants with CF as compared to all samples from healthy infants using nMDS (permutational MANOVA, $P=0.001$). B) Heat map of the relative abundance of bacterial families with >1.0% abundance per sample. Dendrogram based on hierarchical clustering. * Significantly higher abundance of Staphylococcaceae, but lower abundance of Corynebacteriaceae and Pasteurellaceae in infants with CF. C) Impact of CF on the relative abundance of bacterial families with relative abundance >3.0% and Staphylococcaceae oligotypes (OT). Data derived from multivariable linear regression models adjusting for age, season, and AB therapy. Indicated are the coefficients, the 95% Confidence Intervals (CIs), and the corresponding P -values. Staphylococcaceae, as well as OT “T”, “AAT”, and “AGAC” significantly increased in CF ($P<0.001$, $P<0.001$, $P=0.02$, and $P<0.001$, respectively). Corynebacteriaceae and Pasteurellaceae significantly decreased in CF ($P=0.02$, and $P=0.002$, respectively). D) OTs and corresponding taxonomic assignment in healthy infants and infants with CF. Mean and 95% CI indicated. OT “T”, “AAT”, and “AGAC”

significantly increased in CF (multivariable linear regression model adjusting for age, season, and AB therapy; $P<0.001$, $P=0.02$, and $P<0.001$, respectively).

Figure 3: Comparison of the nasal microbiota of infants with CF (before first AB) and healthy infants in the first half year of life without the influence of AB therapy. A) Bacterial density as measured by the concentration of the 16S *rRNA* PCR product, and B) SDI of healthy infants and infants with CF (before first AB) within the first 6 months of life. Mean and 95% CI indicated. There was no significant difference between healthy infants and infants with CF. C) nMDS ordination of healthy infants and infants with CF (before first AB) within the first six months of life. Arrows indicate clustering of samples by age. D) Heat map of bacterial families $>1.0\%$ mean relative abundance per sample. Dendrogram based on hierarchical clustering by age. * Significantly increased abundance of Staphylococcaceae, but decreased abundance of Corynebacteriaceae in infants with CF at age 2-4 and 3-4 months, respectively (Supplementary Table 4). H: healthy infants.

Figure 4: Nasal microbiota of infants with CF during AB therapy. AB therapy samples were grouped into samples at AB, after 1x AB, and after $>1x$ AB therapy, and compared to before first AB samples (see also Figure 1). A) SDI significantly increased at AB therapy (Supplementary Table 5). Mean and 95% CI indicated. B) Relative abundance of the most abundant bacterial families (Moraxellaceae, Streptococcaceae, Staphylococcaceae, and the remaining families grouped as “others”). Significantly decreased Moraxellaceae at AB therapy, while the group of “others” was significantly increased (Supplementary Table 5). Mean and 95% CI indicated. C) Relative abundance of OTs and the corresponding taxonomic assignments. Trend of decreased OT “T” and increased OTs “AAT”, and “AGAT” (Supplementary Table 5). Mean and 95% CI indicated.

Figure 1

Figure 3

Figure 4