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RESEARCH ARTICLE

Chlamydia pneumoniae and *Mycoplasma pneumoniae* in children with cystic fibrosis: impact on bacterial respiratory microbiota diversity

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One sentence summary: This study documented *Chlamydia pneumoniae* in 4.9% of children with cystic fibrosis, stressing the need for routine screening, and investigated its impact on airway microbiota by 16S amplicon-based metagenomics.

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ABSTRACT

Objectives: The contribution of intracellular and fastidious bacteria in Cystic fibrosis (CF) pulmonary exacerbations, and progressive lung function decline remains unknown. This project aimed to explore their impact on bacterial microbiota

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diversity over time in CF children. *Methods:* Sixty-one children enrolled in the MUCOVIB multicentre prospective cohort provided 746 samples, mostly nasopharyngeal swabs, throat swabs and sputa which were analysed using culture, specific real-time qPCRs and *16S rRNA* amplicon metagenomics. *Results: Chlamydia pneumoniae* ($n = 3$) and *Mycoplasma pneumoniae* ($n = 1$) were prospectively documented in 6.6% of CF children. Microbiota alpha-diversity in children with a documented *C. pneumoniae* was highly variable, similarly to children infected with *Staphylococcus aureus* or *Pseudomonas aeruginosa*. The transition from routine follow-up visits to pulmonary exacerbation ($n = 17$) yielded variable changes in diversity indexes with some extreme loss of diversity. *Conclusions:* The high rate of *C. pneumoniae* detection supports the need for regular screenings in CF patients. A minor impact of *C. pneumoniae* on the microbial community structure was documented. Although detected in a single patient, *M. pneumoniae* should also be considered as a possible aetiology of lung infection in CF subjects.

Keywords: cystic fibrosis; pulmonary exacerbation; intracellular bacteria; pneumonia; metagenomics; *Chlamydiales*

INTRODUCTION

Cystic fibrosis (CF) is a common genetic disorder, characterized by chronic inflammation and repeated bacterial pulmonary infections leading to pulmonary exacerbations (PEs). Despite recent advances in the understanding of the role of the respiratory microbiota constituents on disease progression (O'Toole 2018), the prevalence and impact of respiratory viruses, intracellular bacteria (such as *Chlamydia pneumoniae*) and fastidious organisms (such as *Mycoplasma pneumoniae*) remain poorly documented.

While earlier studies essentially focused on pyogenic pathogenic bacteria, the development of metagenomic analyses emphasized the role of complex ecological interactions within the microbiota (O'Toole 2018). CF airways typically harbor early formed complex microbial communities with large interpatient variability (Huang and LiPuma 2016). Yet, a complete understanding of the impact of individual pathogens on respiratory bacterial microbiota is currently lacking. New models must take into account polymicrobial infections in biofilm-like structures that may better represent the airway microbiota in CF (O'Toole 2018). These new models must consider dominant bacterial species, but also viruses and *M. pneumoniae* or *C. pneumoniae,* which could affect the host directly or indirectly by interacting with other pathogens of the microbial community (Hajishengallis *et al.* 2011; de Dios Caballero *et al.* 2017).

The impact of *M. pneumoniae* and *C. pneumoniae* has already been reported in a wide range of respiratory syndromes, including community-acquired pneumonia and asthma exacerbations (Emre *et al.* 1995; Welti *et al.* 2003; Lamoth and Greub 2010; Senn *et al.* 2011; Asner *et al.* 2014; Parrott, Kinjo and Fujita 2016; Webley and Hahn 2017). Limited publications reported on the prevalence of *C. pneumoniae* and *M. pneumoniae* in CF patients (Petersen *et al.* 1981; Emre *et al.* 1996), likely as a result of small pediatric cohorts in individual centers and the use of culture and serology rather than molecular assays. In addition, screening for these microorganisms by molecular assays is often only considered when respiratory tract infections lead to severe complications such as pericarditis rather than routinely (Esposito *et al.* 2006). The use of metagenomics-based assays might offer more insights on the incidence and the prevalence of these bacteria in CF.

The aim of this MUCOVIB sub-study was to report on the prevalence of *C. pneumoniae* and *M. pneumoniae* and their impact on the respiratory microbiota of children with CF*,* by using a specific multiplex quantitative PCR and *16S* amplicon-based metagenomics. Their potential impact on overall bacterial diversity before PE, during exacerbation events, and during routine follow-up was also investigated.

METHODS

The MUCOVIB cohort and sampling

The MUCOVIB project is a longitudinal multicentre prospective cohort study involving all children under 18 years with CF followed at University Hospitals of Lausanne and Geneva, Switzerland. Respiratory samples, including throat swabs (TS), nasopharyngeal swabs (NPS) and sputa (EXP; when able to expectorate), were collected during routine visits and PEs requiring hospital admission from April 2016 to June 2018. PE were defined according to Rosenfeld *et al.*'s definition (Rosenfeld *et al.* 2001). Relevant clinical and laboratory data were also collected at each visit.

Bacterial culture and quantitative PCRs

Standard bacterial cultures were performed on both TS and EXP samples, when available. NPS were tested by qPCR for the detection of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae,* as previously described (Welti *et al.* 2003; Senn *et al.* 2011; Greub *et al.* 2016). Briefly, DNA was extracted with a MagNA Pure system (Roche Life Science). A multiplex quantitative real-time TaqMan PCR (qPCR) allowed to quantify *M. pneumoniae* and *C. pneumoniae* DNA in copies/ml using a calibrated positive plasmid control. In patients with a positive NPS *Chlamydia* or *Mycoplasma* qPCR, all other samples available (TS and/or EXP) were subsequently tested using the same qPCR described above.

16S rRNA metagenomics analyses

Libraries of *16S rRNA* V3-V4 amplicons were prepared according to the *16S* Metagenomic Sequencing Library Preparation (Part. # 15044223 Rev. B) protocol and sequenced at the Institute of Microbiology of the University of Lausanne using a MiSeq (Illumina, San Diego, USA). Positive (ATCC-2002 mock community) and negative controls for DNA extraction and library preparation using DNA free water were included in each sequencing run. Samples with less than 5 nM DNA after PCR amplification or less than 20,000 reads were not used for further analyses. Sequences were attributed to Amplicon Sequence Variant (ASVs) by a home-made pipeline (v.0.9.11) based on DADA2 (1.12.1) (Callahan *et al.* 2016) and taxonomically assigned using the Ribosomal Database Project classifier (Wang *et al.* 2007) in Qiime 1.9.1 (Caporaso *et al.* 2010) against the EzBioCloud reference database (05.2018) (Yoon *et al.* 2017). Bacterial alpha-diversity was measured after rarefying to 20 000 sequences per sample. Shannon

index, which accounts for both species richness (number of species) and evenness (relative abundance of species) and the Chao1 index, which only accounts for species richness, were calculated with Phyloseq R package (1.28.0) (McMurdie and Holmes 2013). All statistical analyses and visualizations were carried out in the R environment (v. 3.3.3) (R Core Team 2019). Raw sequencing reads were deposited in the European Nucleotide Archive (ENA) under the project number PRJEB41059.

RESULTS

Bacterial distribution within the MUCOVIB cohort

From April 2016 to June 2018, 61 patients were included with a median age of 7.4 years old (interquartile range (IQR): 3.7–12.2). During follow-up, 20 patients (33%) presented at least one documented PE resulting in a total of 32 PE events. During 1 to 9 visits (IQR: 5–8), a total of 366 samples, including 309 TS, 52 sputa samples and 5 NPS were successfully analysed by *16S rRNA* metagenomics and 363 NPS by qPCRs. From the 30 TS and EXP collected during 24 PE events, 21 samples were analyzed by bacterial culture. Bacterial pathogens were detected from 15 samples including *Staphylococcus aureus* ($n = 4$), *H. influenzae* ($n = 2$), and mixed bacteria corresponding to any combination of *S. aureus, P. aeruginosa*, *H. influenzae* and/or other remaining pathogens, which essentially included *Streptococci* ($n = 9$). A total of 293 TS and EXP collected during 315 follow-up visits were analysed by bacterial culture, of which 167 were tested positive for *S. aureus* ($n = 99$), mixed bacteria ($n = 37$), single other bacteria ($n = 19$), *P. aeruginosa* ($n = 9$) and *H. influenzae* ($n = 3$).

During the study period, 4 patients presented with an intracellular bacteria or fastidious organism infection, none of them receiving any prophylactic antibiotic. *C. pneumoniae* was documented from 3 patients by qPCR, in higher quantity from NPS compared to TS (Table 1 and Fig. 1C), albeit the highest quantity of *C. pneumoniae* DNA was reported from a sputum sample collected from the single patient able to expectorate.

Descriptive characteristics and outcomes of study subjects with *C. pneumoniae* and *M. pneumoniae* documented from their respiratory samples

Table 1 summarizes clinical symptoms and outcomes in addition to viral and bacterial pathogens identified in samples from children with documented *C. pneumoniae* and *M. pneumoniae*. The first patient was co-infected by *C. pneumoniae* and a rhinovirus, while being asymptomatic. The second symptomatic patient had a documented *C. pneumoniae* infection with no other bacterial or viral pathogen identified. His lung clearance index increased from 7 to 11, thereby suggesting an acute deterioration of his lung function albeit without criteria for a PE. He was treated with clarithromycin. The third patient presented with pharyngitis and a productive cough, with high loads of *C. pneumoniae* documented by qPCR from EXP, NPS and TS, still detectable 2 months later, despite being treated with a macrolide. He also presented an increase in his lung clearance index from 7 to 10.1. A co-infection with rhinovirus, *S. aureus* and *H. influenzae* was reported from his initial EXP sample, while only low load (10^2 copies/ml) of *S. aureus* was documented from his 2-month follow-up EXP sample. *M. pneumoniae* was documented from only one symptomatic child in co-infection with coronavirus OC43 and human metapneumovirus. Moreover, a moderate load of methicillin-sensitive *S. aureus* was recovered from his TS. His symptoms resolved after being treated with co-amoxicillin and clarithromycin (Table 1).

Measures of microbial diversity in children documented with *C. pneumoniae* and *M. pneumoniae*

To assess whether *C. pneumoniae* or *M. pneumoniae* impact on microbial diversity, two alpha-diversity indexes, namely, Chao1 index, which reflects species richness, and Shannon index, which accounts for both species richness and evenness, were calculated based on ASV counts in TS. Figure 1 A/B displays Shannon and Chao1 indexes according to the documentation of *S. aureus, P. aeruginosa* or other bacteria by bacterial cultures or *C. pneumoniae* and *M. pneumoniae* identified by qPCR during routine follow-up visits (grey dots) or PEs (red dots). To avoid the over-representation of patients with multiple follow-up visits, only one TS per patient in a routine follow-up visit, and one in a PE event was randomly selected for each category of pathogen. No difference in alpha diversity indexes was documented from TS collected from patients in whom *C. pneumoniae* or *M. pneumoniae* were reported compared to those collected from children infected with *S. aureus* or *P. aeruginosa*. The transition from routine follow-up visits to PE ($n = 17$ documented) yielded both increased or decreased diversity depending on the patient and visit, with some extreme loss of diversity (maximum Shannon and Chao1 change of −1.73 and −177.3, respectively). Shannon mean change in transitions to PE was −0.14 (IQR − 0.66 to 0.29) whereas Chao1 mean change was of −18.6 (IQR −28.8 to 8.5). Slightly decreased alpha indexes measures were documented during *S. aureus* PE (with or without *P. aeruginosa*), albeit not statistically significantly, likely as a result of the small number of events. Chao1 and Shannon indexes were similar during PE events as during routine follow-up visits for mixed infections and other bacteria.

Next, the ability of *16S rRNA* metagenomics assays to detect *C. pneumoniae* and *M. pneumoniae* was evaluated (Fig. 1C). In metagenomics analyses, up to 30% of 16S rRNA sequences were assigned to *C. pneumoniae* in NPS ($n = 4$). However, TS ($n = 5$) and EXP ($n = 1$) samples positive by qPCR reported lowly abundant *C. pneumoniae* and *M. pneumoniae* sequences, ranging from 0.01% to 3.1% and 0.035% of *16S rRNA* sequences, respectively (Fig. 1C). In one TS sample, *C. pneumoniae* reads were identified only using operational taxonomic unit (OTU) clustering (97% identity), data not shown) but not using ASVs, even without rarefaction. These results highlight the small proportion of *C. pneumoniae* and *M. pneumoniae* species in throat swabs. Both *C. pneumoniae* and *M. pneumoniae* only encode for one copy of the *16S rRNA* gene targeted by the PCR used for the present metagenomics analyses, which might contribute to their weak detection by 16S rRNA metagenomics.

The low abundance of sequencing reads attributed to *C. pneumoniae* and *M. pneumoniae* contrasted with a subset of *S. aureus* induced PE in which *S. aureus* was documented as the predominant bacterial species during a PE (Fig. 1E). In this case, the decreased proportion of various microbial species and increased amount of *S. aureus* was documented 3 months prior to exacerbation (Fig. 1E), thus questioning whether the use of metagenomics could allow to anticipate episodes of PE. In contrast, other children with *P. aeruginosa* and/or *S. aureus* PE documented by bacterial culture exhibited low abundance of sequences attributed to their respective pathogens at time of exacerbation (Fig. 1F), with a rather conserved microbial diversity.

Table 1. Descriptive characteristics and clinical outcomes of children with documented *C. pneumoniae* or *M. pneumoniae* from their respiratory samples.

Patient	Symptoms	*C. pneumoniae*/*M. pneumoniae*	Sample type	Copies/ml	Other microorganisms documented	Treatment
1	Asymptomatic	*C. pneumoniae*	NPS	11 220 000	RV	None
			TS	675 000		
2	Rhinorrea,dry cough	*C. pneumoniae*	NPS	45 500	None	Clarithromycin
			TS	280 000		
3	Pharyngitis, productive cough	*C. pneumoniae*	EXP	82 270 000	RV	Clarithromycin
			NPS	1 891 000	10^4 *S. aureus* bacteria/ml	
			TS	46 000	10^4 *H. influenzae* bacteria/ml	
4	Rhinorrhea, dry cough	*M. pneumoniae*	NPS	2370	OC43; hMPV	Co-amoxicillin, clarithromycin
			TS	N/A	*S. aureus* 10^2 cp/ml	

Legend: Rhinovirus (RV); Coronavirus OC43 (OC43), human metapneumovirus (hMPV); nasopharyngeal swabs (NPS); throat swabs (TS); Sputa samples (EXP).

DISCUSSION

As part of the MUCOVIB study, we documented *C. pneumoniae* or *M. pneumoniae* infections in 6.6% of all children with CF of whom 75% presented with upper respiratory tract (URT) symptoms despite not meeting criteria for a PE. The 4.9% (3/61) rate of *C. pneumoniae* documented in CF is significantly higher than the 0.13% prevalence of *C. pneumoniae* positive PCRs previously reported from all patients using the same PCR (Senn *et al.* 2011), but lower than the 12.5% (4/32) rate previously reported in CF children with PE using culture and serology (Emre *et al.* 1996). Furthermore, only one third (19/61) of our CF cohort was on a macrolide prophylaxis during the study period. Only one patient was detected positive for *M. pneumoniae,* resulting in a 1.6% prevalence, which was similar to that reported in a recent Swiss study of children with respiratory tract infections (Dierig *et al.* 2020), and a report from the 1980s in CF patients (Petersen *et al.* 1981). This high rate of *C. pneumoniae* detection in children with CF is in line with the previous reports (Petersen *et al.* 1981; Emre *et al.* 1996), and supports the need for a systematic detection during regular visits by using a chlamydia qPCR. As such, a better estimation of the prevalence of *C. pneumoniae* in CF patients coupled with a better understanding of their reciprocal inferences with CF respiratory microbiota might provide additional insight into the pathogenesis of CF chronic inflammation. As these obligate intracellular organisms are not susceptible to antibiotics usually prescribed during CF PE, they result in prolonged clinical symptoms requiring longer antibiotic treatments with macrolides (Hammerschlag *et al.* 1992). 'Traditional' antibiotics typically impact on bacterial microbiota diversity, which could change one's susceptibility to these not-targeted microorganisms. Furthermore, long-term prescription of macrolide among patients with CF has resulted in improvement of respiratory function and reduction of PE episodes, without fully understanding the exact underlying mechanisms (Southern *et al.* 2012). In this regard, documentation and treatment of occult intracellular bacteria might impact progressive lung function decline (Janahi *et al.* 2005). Given the higher loads of *C. pneumoniae* documented from NPS compared to TS, the use of NPS should be privileged.

Using a limited number of samples, no impact of *C. pneumoniae* or *M. pneumoniae* on Shannon or Chao1 diversity indexes as compared to *S. aureus* and/or *P. aeruginosa*-positive TS was documented in our cohort. Interestingly, changes in microbial diversity were generally observed between routine follow-up visits and PE. Whether these changes have an impact on the susceptibility to infections with *C. pneumoniae* and *M. pneumoniae* is unknown. Our observations suggest a minor impact of *C. pneumoniae* on the microbial community structure. However, the small number of samples and the high inter-individual diversity, limited our analyses to measures of alpha-diversity indexes only. Future larger datasets should also consider the evaluation of changes in bacterial composition and taxonomy by using beta-diversity indexes. Future study should also aim to evaluate to what extent changes in the structure of the respiratory microbial community, over time or due to antibiotic treatment, have an impact on the susceptibility to infections with *C. pneumoniae* and *M. pneumoniae*.

Metagenomics profiling might allow both the study of changes in the microbiota structure and the analysis of specific established human pathogen, including *M. pneumoniae* and *C. pneumoniae*. Yet, while the qPCR was sensitive enough to detect chlamydial DNA in all sample types (NPS, TS and EXP), only a few reads classified as *C. pneumoniae* were detected by *16S*-based metagenomics in EXP and TS. This suggests that a large proportion of other microbial species in the airway microbiome may potentially hide significant intracellular or fastidious pathogens in metagenomics analyses. Intracellular bacteria with particularly small genomes such as *C. pneumoniae* or *M. pneumoniae* only harbour one copy of the *16S rRNA* (Kalman *et al.* 1999; Klappenbach, Dunbar and Schmidt 2000) targeted for amplification and may thereby be more difficult to detect than other common pathogens. These observations support the need for sufficient sequencing coverage and suggest the risk of analyses on rarefied data, specifically when they are not coupled with documentation of pathogens by other methods. In addition, the low amount of chlamydia sequences highlights the importance of the *16S* PCR amplification prior to NGS analyses and questions the potential sensitivity of shotgun metagenomics for such cases.

As previously documented in CF patients (Zhao *et al.* 2012; Carmody *et al.* 2015), common pathogens such as *S. aureus* or *P. aeruginosa* have become a major component of the bacterial microbiota only in some cases (Fig. 1). Regular metagenomics profiling could allow to evaluate the overgrowth of a pathogen and potentially to anticipate PE. Microbial communities were to some extent resilient to PE and antibiotic treatment, but major, stable changes in composition were also documented. While

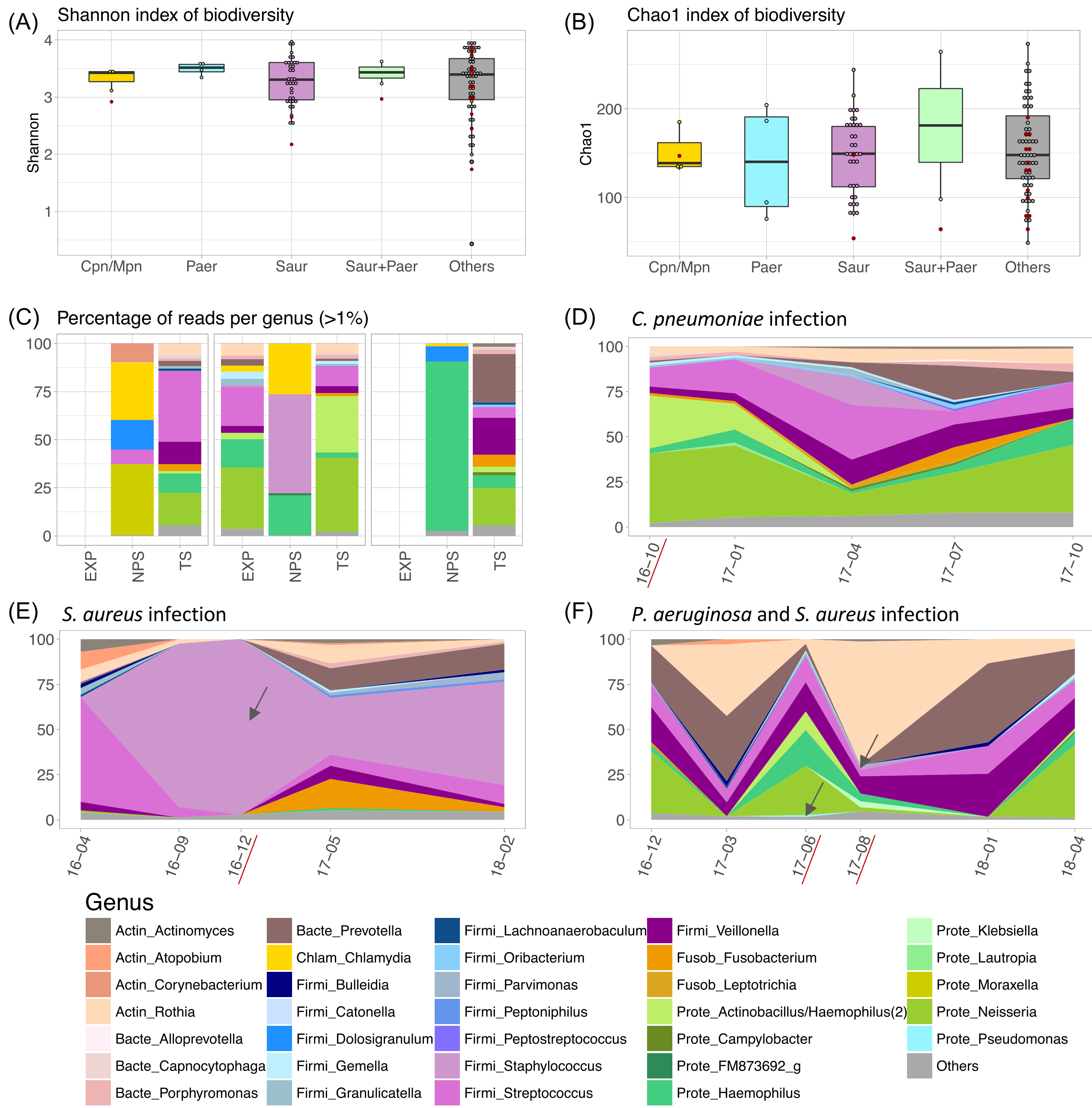

Figure 1. ***16S rRNA* amplicon metagenomics of CF patients. (A)**, Shannon index of diversity, taking into account both the number of amplicon sequence variants (ASVs) and their relative abundance, and **(B)** Chao1 index of diversity, taking into account number of ASVs, in throat swabs of follow-up visits according to the bacteria documented by qPCR (*Chlamydia pneumoniae* (Cpn) and *Mycoplasma pneumoniae* (Mpn)) or culture (*Pseudomonas aeruginosa* (Paer), *Staphylococcus aureus* (Saur), or both (Saur + Paer), as well as other pathogens or mixed infections of *P. aeruginosa* and *S. aureus* with other bacteria (Others)). The diversity index during episodes of documented PE is overlaid with red points. **(C)**, Relative abundance of bacterial genera present in expectorations (EXP), nasopharyngeal swabs (NPS) and throat swabs (TS) of the three patients with documented *C. pneumoniae*. **(D)**, Relative abundance of bacterial genera in TS along time for one patient with a documented symptomatic *C. pneumoniae* infection at visit V01 (middle patient in panel C). *Chlamydia* sequences remained lowly abundant (0.01%) in the TS and are hence not visible among other more prevalent (>1%) bacterial species. **(E)**, Relative abundance of bacterial genera in expectorations along time for one patient with a symptomatic infection due to *S. aureus*. The impressive increase in *S. aureus* proportion can be seen by metagenomics before symptoms arose. **(F)**, Relative abundance of bacterial genera in TS along time for one patient with a symptomatic infection to *P. aeruginosa* and *S. aureus*, that appeared in small proportion before decreasing in the follow-up visits. In panel D, E and F, episodes of PE are underlined in red on the x axis.

larger-scale studies are obviously needed to better model the effect of intracellular and fastidious bacteria, overall changes in diversity may be difficult to detect, as the baseline diversity varies among patients and decreases with time along disease progression.

In conclusion, while our sample size was too limited to infer any association between presence of *C. pneumoniae* or *M. pneumoniae* and changes in bacterial microbiota composition over time, the observed high rate of positivity for *C. pneumoniae* (4.9%) suggests that children with CF should be routinely screened

from NPS rather than TS and adequately treated for *C. pneumoniae*. In addition, further large-scale multicentre studies will better delineate the impact of these fastidious and intracellular pathogens on the bacterial alpha and beta-diversity over time, as well as the impact of the change in the bacterial diversity on the susceptibility to infections with *C. pneumoniae* and *M. pneumoniae*.

AUTHOR CONTRIBUTIONS

Study design and set-up: SAA, GG, KMPB, LK; Enrolment of patients: LFP, CM, IR, AM, SAA; Metagenomics analysis: CB, VS, GG; Molecular diagnostics: RB, KJ, GG; Literature search & reading: CB, VS, LFP, SAA, GG; Writing of the manuscript: CB, VS, LFP, SAA, GG; Preparation of figure: CB; Critical review of manuscript content: LFP, CB, VS, CM, IR, RB, AM, KJ, KMPB, SAA, GG.

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Conflicts of Interest. None declared.

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