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Unexpected associations between respiratory viruses and bacteria with Pulmonary Function Testing in children suffering from Cystic Fibrosis (MUCOVIB study)

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ABSTRACT

Background: Various bacterial and viral assemblages composing Cystic Fibrosis (CF) lung microbiota contribute to long-term lung function decline over time. Yet, the impact of individual microorganisms on pulmonary functions remains uncertain in children with CF.

Methods: As part of the 'Mucoviscidosis, respiratory VIruses, intracellular Bacteria and fastidious organisms" project, children with CF were longitudinally followed in a Swiss multicentric study. Respiratory samples included mainly throat swabs and sputa samples for bacterial culture and 16S rRNA metagenomics and nasopharyngeal swabs for respiratory virus detection by molecular assays. Percentage of predicted Forced Expiratory Volume in one second (FEV1%) and Lung Clearance Index (LCI) were recorded.

Results: Sixty-one children, of whom 20 (32.8%) presented with at least one pulmonary exacerbation, were included. Almost half of the 363 nasopharyngeal swabs tested by RT-PCR were positive for a respiratory virus, mainly rhinovirus (26.5%). From linear mixed-effects regression models, *P. aeruginosa* (-11.35, 95%CI [-17.90; -4.80], p = 0.001) was significantly associated with a decreased FEV1%, whereas rhinovirus was associated with a significantly higher FEV1% (+4.24 95%CI [1.67; 6.81], p = 0.001). Compared to conventional culture, 16S rRNA metagenomics showed a sensitivity and specificity of 80.0% and 85.4%, respectively for detection of typical CF pathogens. However, metagenomics detected a bacteria almost twice more often than culture.

Conclusions: As expected, *P. aeruginosa* impacted negatively on FEV1% while rhinovirus was surprisingly associated with better FEV1%. Culture-free assays identifie significantly more pathogens than standard culture, with disputable clinical correlation.

1. Introduction

Cystic fibrosis (CF) is a common inherited disease affecting over 70,000 people worldwide [1]. Despite progress in management, pulmonary exacerbations and resultant respiratory failure are still associated with significant morbidity and mortality [2].

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Childhood is critical for the settlement of the respiratory microbiota in CF. In infants, the respiratory microbiota is characterized by a rich diversity of bacteria, including pathogens such as *S. aureus* and *P. aeruginosa* [3,4]. Exposure to antibiotics and structural changes progressively favor colonization by opportunistic pathogens such as *P. aeruginosa* leading to an adult microbiota generally composed of few dominant bacteria [3,4]. Cumulative exposure to antimicrobials favors resistant strains selection and reduces microbial diversity [5,6], an attribute associated with worse lung functions [5].

Culture-free methods, including metagenomics and viral molecular assays, provided relevant information on the contribution of respiratory viruses and difficult-to-grow bacteria to the decline of respiratory microbiota diversity [7,8]. These microbes can be directly pathogenic but also modulate host immunity, as well as antagonize or synergize with typical bacterial pathogens [4,7-11]. Similar incidences of viral respiratory infections were reported in children with CF compared to healthy controls [12], with an increased morbidity for children with CF [8,11]. Yet, the individual impact of specific respiratory viruses is still debated [8].

This substudy of the Mucoviscidosis, Respiratory VIruses and Intracellular Bacteria (MUCOVIB) project primarily aimed to correlate results from respiratory viral Real-Time Polymerase Chain Reaction (RT-PCR) assays and bacterial cultures to pulmonary functions in children with CF. Moreover, this project intended to better delineate the contribution of culture-free methods (viral respiratory RT-PCR and 16S rRNA metagenomics) in the monitoring of respiratory colonization and infection.

2. Materials and methods

2.1. Participants and definitions

The MUCOVIB project is a prospective longitudinal multicenter cohort study investigating the impact of respiratory viruses and intracellular/fastidious bacteria on the respiratory microbiota of children with CF. Patients under 18 years of age were included from April 1st, 2016 to May 31st, 2018 at the respiratory clinics of Lausanne and Geneva University Hospitals, two large pediatric referral centers. Patients followed routinely by their pulmonologist or those meeting criteria for a pulmonary exacerbation requiring hospital admission, as defined by Rosenfeld et al. [13], were enrolled. Informed consent from the legal guardians or adolescents ≥14 years (and assent from child as appropriate) were mandatory for inclusion. This study was approved by the local ethics committees of both centers (PB_2016-00646 and GE15-157) and conducted in accordance with the Declaration of Helsinki, the standards of Good Clinical Practice and Swiss regulatory requirements.

2.2. Collection of clinical information and specimens

Information on patient demographics, CF-related comorbidities (pancreatic insufficiency, diabetes, allergic bronchopulmonary aspergillosis, liver disease or others), antibiotic prescriptions, the presence or absence of a pulmonary exacerbation as defined by Rosenfeld et al. [13] and pulmonary functions - including the percentage of predicted Forced Expiratory Volume in one second (FEV1%) and Lung Clearance Index (LCI) values as indicated by the age of the child - were collected. Patients were sampled upon inclusion and during subsequent trimestral visits as scheduled by their pulmonologist. Samples gathered for clinical purposes during all visits included throat swabs, sputa, bronchial aspirates and bronchoalveolar lavages. Nasopharyngeal swabs were also collected from symptomatic children as deemed clinically appropriate. Nasopharyngeal swabs performed during routine follow up visits from asymptomatic children were collected for research purposes only. Nasopharyngeal swabs (FLOQSwab, Copan, Brescia, Italy) preserved in universal transport medium (UTM, Copan) were collected at each visit for PCR-based detection of viruses and intracellular/fastidious bacteria [14,15]. Throat swab (ESwab, Copan) and/or, whenever possible, expectorated sputum were collected for routine bacterial culture and 16S rRNA metagenomics.

2.3. Sample processing and microbiological investigations

Collected samples were submitted to routine microbiological culture when requested by the clinician in charge. Recorded bacterial pathogens were *Haemophilus influenzae, Staphylococcus aureus, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Escherichia coli* and other coliforms, *Streptococcus pneumoniae, Streptococcus pyogenes* and *Achromobacter* spp. or “any other”. Nasopharyngeal swabs were tested by RT-PCR for viruses after nucleic acid extraction with the Qiasymphony instrument (Qiagen, Hilden, Germany) using the Virus/Pathogen kit (Qiagen). The FTD Respiratory pathogens 21 panel (FTD2-64 FastTrack Diagnostics, Esch-sur-Alzette, Luxembourg) was used on the Viia7 thermocycler (Life Technologies, Carlsbad, CA) to test for respiratory syncytial virus A/B, seasonal coronavirus (OC43/229E/NL63/HKU1), human metapneumovirus A/B, picornavirus, enterovirus, human parechovirus, parainfluenza viruses 1-4, influenza A/B/H1N1, human bocavirus and adenovirus A-F [15]. A CT below 40 was considered positive. Picornavirus positive but enterovirus negative samples were considered as positive for rhinoviruses, while samples positive or negative for a picornavirus but positive for an enterovirus were considered as positive for “other picornavirus”. Results for PCR testing for coronavirus, parainfluenza and influenza strains were grouped and considered positive if one of the corresponding PCR reactions was positive. Nasopharyngeal swabs were tested for *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* and results are reported elsewhere [16].

2.4. Bacterial 16S rRNA amplicon-based metagenomics

DNA was extracted from throat swab, sputum, bronchial aspirates and broncholaveolar lavage, as well as from reagents only negative controls and a standardized positive control (MSA-2002, ATCC, Manassas, VA) on MagNA Pure automated platform (Roche, Basel, Switzerland). Libraries were prepared following the “16S Metagenomic Sequencing Library Preparation” (Part. #15044223 Rev. B) protocol from Illumina (San Diego, CA) and quality controlled with the Fragment Analyzer, NGS Fragment Kit (AATI, Ankeny, IA). A no template control was included during each library preparation. All samples were normalized to 4 nM prior pooling. Pooled libraries were diluted at 10 pM and spiked with 10 % of PhiX before sequencing on an Illumina MiSeq with v3 reagents. Reads we processed into Amplicon Sequence Variants (ASVs) using a DADA2-based bioinformatic pipeline [17], as described in Supplementary Methods. Samples with less than 20,000 reads after processing were resequenced once. To limit the effect of contaminants occuring in low biomass samples, only samples with over 5nM 16S rRNA library yield and 20,000 exploitable sequences were considered for analysis. Library DNA and sequencing yields for each sample type are reported in Supplementary Table 1.

2.5. Sample selection

To limit bias, only one sample per visit was retained in the following preference order based on analysis methods: i) 16S rRNA metagenomics and culture, ii) metagenomics only and iii) culture only (Supplementary Fig. 1). When more than one sample had been processed by the same set of methods at a given visit, the

sample type already analyzed in the previous patient's visit was selected.

2.6. Comparison of performance between bacterial culture and 16S rRNA metagenomics

To compare bacteria detected by culture and 16S rRNA metagenomics, a list of correspondance between the taxonomic identifier in culture and in 16S rRNA metagenomics data was established (Supplementary Table 2). A heatmap comparing the two approaches was generated with the ComplexHeatmaps R package [18] based on the unfiltered cumulated relative abundance of sequences matching these taxa. Sensitivity and specificity considering both culture and 16S rRNA metagenomics as gold standard, as well as Kappa scores for inter-rater aggreement, were computed in R.

2.7. Statistical analyses

To analyze longitudinal data, linear mixed-effects regression models were used for the continuous outcomes (FEV1%, LCI), and mixed-effects logistic regression models were used for the occurrence of a pulmonary exacerbation [19,20]. Rare pathogens that were observed in less than 5 visits or 2 patients were regrouped into two "other viruses" and "other bacteria" categories. To consider serial correlation, an exchangeable dependence structure was assumed. The cross-sectional and longitudinal effects of age, as well as the impact of gender, presence of comorbidities, antibiotic treatment, seasonality and the presence of selected bacteria and viruses were assessed. An interaction variable was introduced into the model to take into account the synergetic effect of coinfection by a virus and bacteria. As regards the occurrence of pulmonary exacerbations, the presence of comorbidities could not be assessed as all individuals having an exacerbation had comorbidities (complete separation). Goodness of fit was assessed by residual analysis, Hosmer & Lemeshow test and area under the ROC curve for pulmonary exacerbations. We completed these analyses (for the FEV1% outcome) by a covariate-patterns analysis as described in Supplementary Methods. Analyses were conducted using the STATA statistical package version 15.1 (Stata Corporation, College Station, TX).

3. Results

3.1. Descriptive characteristics of study subjects and clinical outcomes

During the 2-year study period, 61 children were followed during a total of 379 visits corresponding to a median of 7 visits (InterQuartile Range, IQR [5;8]) (Table 1). Fifty-nine percent of the children were males with a median age at inclusion of 7.4 years (IQR [3.7; 12.2]) and most of them (90.2%) presented at least one CF-related comorbidity. Twenty patients (32.8%) presented at least one pulmonary exacerbation (32 events) during the study period, mainly treated with β-lactams (71.9%).

3.2. Viral and bacterial detection studies

A total of 744 samples were collected during 373 visits, of which 363 were nasopharyngeal swabs, 315 throat swabs, 57 sputa, 7 bronchoalveolar lavages and 2 bronchial aspirates (Supplementary Fig. 1). From the 366 specimens tested for respiratory viruses by RT-PCR, 164 (44.8%) collected from 56 patients tested positive for at least one respiratory virus. Rhinovirus (26.5%), seasonal coronavirus (8.7%), human metapneumovirus (5.7%), human bocavirus (2.2%), and adenovirus (1.6%) were most commonly documented.

Table 1
Baseline characteristics and clinical outcomes.

	n=61 (%)
Gender	
Female	25 (41.0%)
Male	36 (59.0%)
Age at inclusion (years), median [IQR]	7.4 [3.7, 12.2]
Comorbidities	55 (90.2%)
Pancreatic insufficiency	50 (82.0%)
Allergic bronchopulmonary aspergillosis	4 (6.6%)
Diabetes	6 (9.8%)
Liver disease	5 (8.2%)
Other	16 (26.2%)
Follow-up (days), median [IQR]	553 [456, 640]
Genotype	
Heterozygous for DeltaF508	24 (39.3%)
Homozygous for DeltaF508	28 (45.9%)
Other than DeltaF508	9 (14.8%)
Antibiotics at inclusion*	
Azithromycin	9 (14.8%)
Inhaled colistin	4 (6.6%)
Inhaled tobramycin	9 (14.8%)
Others§	5 (8.2%)
None	44 (72.1%)
Pulmonary exacerbation (N of patients with at least one event)	20 (32.8%)
Pulmonary exacerbation (N of events)	32
FEV1%, median [IQR]	
Follow-up visits (n=210)	91.0 [81.0, 99.0]
Pulmonary exacerbations (n=14)	82.0 [58.0, 97.5]
LCI, median [IQR]	
Follow-up visits (n=194)	9.3 [8.2, 11.1]
Pulmonary exacerbations (n=9)	9.7 [8.0, 12.8]

* 7 patients had 2 or 3 prescribed antibiotics
§ including ciprofloxacin, levofloxacin, co-amoxiclav and cefuroxime; IQR = Interquartile range; FEV1% = percentage of predicted Forced Expiratory Volume in one second; LCI = Lung Clearance Index

Coinfections with two or more viruses were documented in 38 visits (10.4 %). Routine microbiology cultures were performed on 269 throat swabs, 51 sputa and 7 other respiratory samples. Among the bacterial pathogens identified in 323 samples all representing a distinct visit, *S. aureus* was the most common (44.6%), followed by *P. aeruginosa* (6.8%), *S. pyogenes* (3.7%) and *H. influenzae* (3.4%). Two or more bacterial species were found in 15% of samples and no bacterial pathogen was detected in 41.2% of samples (Supplementary Fig. 2). Bacterial-viral coinfection was detected in 85 of the 317 visits (26.8%) for which both cultures and viral detection were available. *S. aureus* and rhinovirus represented the most frequent bacterial-viral coinfection (39 cases) (Supplementary Fig. 3).

The detection of any bacteria (OR=1.96, CI95% [1.06; 3.64], p=0.032) and rhinovirus (OR=2.12, CI95% [1.09; 4.10], p=0.026) were significantly increased during spring compared to winter (Supplementary Table 3). Younger children presented with significantly higher odds ratio for viral detection (odds ratio (OR) = 1.11; CI95% [1.03; 1.19], p = 0.005) and more specifically for rhinovirus detection (OR= 1.13; CI95% [1.04; 1.23], p = 0.005).

3.3. Predictors of pulmonary

The detection of any bacteria by culture was associated with a significant FEV1% reduction by -5.62 (95% Confidence Interval (95%CI) [-8.85; -2.40], p = 0.001) and a 0.83 (95%CI [0.13; 1.52], p = 0.020) increase in LCI value (Table 2). The documentation of any respiratory virus was associated with a FEV1% reduced by -3.84 (95%CI [-7.60; -0.08], p = 0.045) but had no impact on LCI values. However, the coinfection of a virus and a bacteria (interaction variable) was associated with a higher FEV1%, (interaction = +8.65 (95%CI [3.90; 13.41], p = <0.001) with again no effect on LCI val-

Table 2
Predictors of Pulmonary Function testing and of a Pulmonary Exacerbation.

	FEV1% (n=218)			LCI (n=200)			PE (n=310)		
	Coefficient	95% C.I.	p value	Coefficient	95% C.I.	p value	OR	95% C.I.	p value
Age upon inclusion	-0.32	[-1.19; 0.52]	0.473	+0.08	[-0.06; 0.21]	0.271	1.04	[0.93; 1.16]	0.487
Male	+0.75	[-7.26; 8.76]	0.854	-0.34	[-1.54; 0.87]	0.584	2.80	[0.87.; 9.07]	0.085
Presence of any comorbidity	+1.08	[-12.52; 14.67]	0.877	+0.22	[-1.68; 2.13]	0.820	-	-	-
Winter season	0			0			1		
Spring	+0.13	[-2.74; 3.01]	0.927	+0.48	[-0.13; 1.10]	0.125	1.73	[0.39; 7.61]	0.469
Summer	-1.50	[-4.48; 1.49]	0.325	+0.23	[-0.44; 0.89]	0.505	2.13	[0.45; 10.02]	0.337
Autumn	-0.01	[-2.96; 2.94]	0.995	-0.17	[-0.82; 0.47]	0.595	2.84	[0.62; 12.94]	0.177
Current antibiotics	-5.38	[-9.25; -1.52]	0.006	+0.83	[-0.01; 1.68]	0.053	3.62	[1.30; 10.03]	0.014
Detection of any respiratory virus	-3.84	[-7.60; -0.08]	0.045	+0.49	[-0.29; 1.27]	0.221	2.79	[0.42; 18.58]	0.290
Detection of any bacteria by culture	-5.62	[-8.85; -2.40]	0.001	+0.83	[0.13; 1.52]	0.020	3.25	[0.63; 16.87]	0.161
Interaction variable (respiratory virus + bacteria)	+8.65	[3.90; 13.41]	<0.001	-0.79	[-1.80; 0.22]	0.126	0.37	[0.04; 3.23]	0.366
Total balance of the effect of virus and bacteria*	-0.80	[-4.07; 2.46]	0.628	+0.53	[-0.17; 1.23]	0.138	1.20	[-0.45; 2.87]	0.155

FEV1% = % of the predicted Forced Expiratory Volume in one second; LCI = Lung Capacity Index; PE = Pulmonary Exacerbations; C.I. = confidence intervals; OR = odds ratio. Lower FEV1% but higher LCI values are associated with worse pulmonary functions. Number of observations (n) available for FEV1%, LCI and PE outcomes differed (Supplementary Fig. 1).
* Total balance of the effect of virus and bacteria is the sum of the coefficients for virus + bacteria + interaction

Table 3
Detailed analyses of predictors of pulmonary functions.

		FEV1% (N=218)			LCI (n=200)		
		Coefficient	95% C.I.	p value	Coefficient	95% C.I.	p value
	Age upon inclusion	-0.31	[-1.20; 0.58]	0.491	+0.73	[-0.58; 0.21]	0.271
	Male	+1.73	[-6.41; 9.86]	0.677	-0.47	[-1.64; 0.69]	0.424
	Presence of any comorbidity	+0.92	[-12.84; 14.67]	0.896	-0.05	[-1.78; 1.87]	0.959
	Current antibiotics	-3.21	[-7.07; 0.65]	0.103	+0.93	[-0.08; 1.79]	0.033
Viruses	Human metapneumovirus	+0.10	[-5.10; 5.90]	0.969	+0.26	[-0.79; 1.32]	0.622
	Respiratory syncytial virus	-4.80	[-11.39; 1.80]	0.154	-0.72	[-2.17; 0.73]	0.328
	Seasonal coronavirus	-3.70	[-7.71; 0.30]	0.070	-0.20	[-1.08; 0.73]	0.655
	Parainfluenza	+2.07	[-3.79; 7.92]	0.489	-0.33	[-1.65; 0.98]	0.640
	Influenza	+2.77	[-4.29; 9.83]	0.442	-1.28	[-2.82; 0.26]	0.103
	Rhinovirus	+4.24	[1.67; 6.81]	0.001	+0.13	[-0.42; 0.69]	0.636
	Other viruses	-1.49	[-8.31; 5.34]	0.669	+1.30	[-1.81; 2.78]	0.085
Bacteria (detected by culture)							
	H. influenzae	+2.44	[-3.41; 8.28]	0.414	-0.46	[-1.82, 0.90]	0.507
	S. aureus	-2.51	[-5.05; 0.24]	0.052	+0.36	[-0.18; 0.90]	0.190
	P. aeruginosa	-11.35	[-17.90; -4.80]	0.001	+1.89	[0.25; 3.53]	0.024
	S. maltophilia	-3.13	[-9.52; 3.26]	0.337	+1.55	[-0.03; 3.14]	0.055
	S. pyogenes	-6.78	[-13.97; 0.40]	0.064	+1.33	[-0.33; 2.99]	0.117
	Other bacteria	-0.55	[-8.30; 7.20;]	0.890	+1.06	[-0.73; 2.84]	0.246

FEV1% = % of the predicted Forced Expiratory Volume in one second; LCI = Lung Capacity Index; C.I. = confidence intervals.

ues. This interaction almost completely counterbalanced the negative individual effects of a bacteria or a virus on FEV1%. Seasons were not associated with changes in FEV1% nor LCI value. (Table 2).

When assessing bacteria (detected by culture) and viruses individually, *P. aeruginosa* (-11.35, 95%CI [-17.90; -4.80], p = 0.001) was significantly associated with a decreased FEV1%, whereas the documentation of a rhinovirus was associated with a significantly higher FEV1% (+4.24 95%CI [1.67; 6.81], p 0.001) but had no effect on LCI. *S. aureus, S. pyogenes, endemic coronavirus* and respiratory syncytial virus were associated with trends towards decreased FEV1%, albeit not reaching statistical significance. (Table 3).

To evaluate the contribution of individual pathogens alone or in combination on the FEV1%, a covariate-pattern analysis was performed. The documentation by culture of *P. aeruginosa* (FEV1% difference to the mean of -1.9, number of pattern observations (n) = 4), coronavirus (-1.7, n = 5) and *S. aureus* (-1.5, n = 54) individually were associated with decreased FEV1%. Rhinovirus alone, (+1.4, n = 12), or in combination with *S. aureus* (+3.7, n = 20) were associated with increased levels of FEV1% (Fig. 1).

3.4. Comparison between bacterial culture and 16S rRNA analyses

The detection of bacteria by 16S rRNA metagenomics and culture in 317 samples (Supplementary Fig. 4) showed a fair overall agreement (Kappa score of 0.40), with scores ranging from 0.04 for *S. pneumoniae* to 0.82 for *Achromobacter* (Table 4). However, 16S rRNA metagenomics detected pathogens over twice more often than culture (508 versus 210). For example, *P. aeruginosa* was detected by 16S rRNA metagenomics in 37 patients but was only found in 10 patients by culture (Supplementary Table 5). Considering conventional culture as gold standard, 16S rRNA metagenomics presented an overall sensitivity and specificity of 80.0% and 85.4%, respectively. Conversely when considering 16S rRNA metagenomics as gold standard, culture displayed a sensitivity of 33.1% but a specificity of 97.9%. In selected cases, positivity by 16S rRNA metagenomics preceded documentation by culture on subsequent samples (Supplementary Fig. 4 and examples in Fig. 2).

4. Discussion

In this prospective multicenter longitudinal study, the detection of *P. aeruginosa*, and to some extent *S. aureus, S. pyogenes*, endemic coronavirus and respiratory syncytial virus were associated with a decreased FEV1%. Surprisingly, rhinovirus detection was associated with an increased FEV1%. Documentation of bacterial pathogens by 16S rRNA metagenomics only partially agreed with conventional culture.

As previously reported [8,11], respiratory viruses, mostly rhinovirus, were detected in nearly half of the samples and the doc-

Table 4
Performance of 16S rRNA metagenomics compared to conventional culture.

		H. influenzae	*S. aureus*	*P. aeruginosa*	*S. maltophilia*	*E. coli*	*S. pneumoniae*	*S. pyogenes*	*Achromobacter spp.*	Tot.
Cult.	16S rRNA									
+	+	9	111	15	10	4	1	11	7	168
+	-	2	31	6	1	0	0	1	1	42
-	+	117	51	49	31	37	43	10	2	340
-	-	189	124	247	275	276	273	295	307	1986
16S rRNA sensitivity [1]		81.8	78.2	71.4	90.9	100.0	100.0	91.7	87.5	80.0
16S rRNA specificity [2]		61.8	70.9	83.4	89.9	88.2	86.4	96.7	99.4	85.4
Culture sensitivity [3]		7.1	68.5	23.4	24.4	9.8	2.3	52.4	77.8	33.1
Culture specificity [4]		99.0	80.0	97.6	99.6	100.0	100.0	99.7	99.7	97.9
Kappa score [5]		0.07	0.48	0.28	0.35	0.16	0.04	0.65	0.82	0.40

[1] sensitivity of 16S rRNA metagenomics, with conventional culture as reference
[2] specificity of 16S rRNA metagenomics with conventional culture as reference
[3] sensitivity of culture with 16S rRNA metagenomics as reference
[4] specificity of culture with 16S rRNA metagenomics as reference
[5] Cohen's Kappa score for inter-rater agreement. Kappa scores weight the observed agreement by chance only. A perfect agreement is scored as 1 and an agreement corresponding to what is expected by chance only is scored as 0.

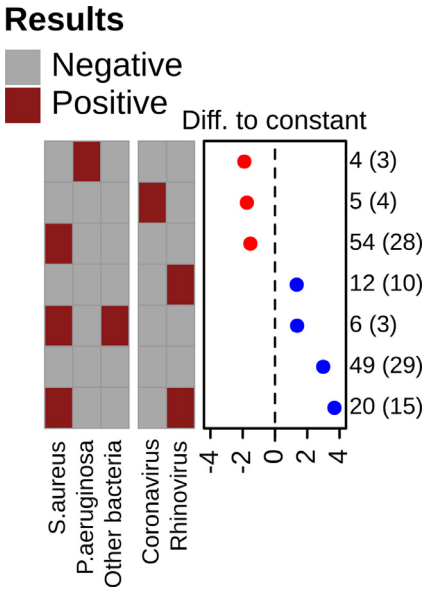

Fig. 1. Covariate pattern analysis
Heatmap of the microbial patterns and their associated FEV1% variations. Each pattern is represented by a row where pathogens are described as present (dark red) or absent (dark grey). Patterns associated with a FEV1% below average are represented by a red dot and the ones above by a blue dot. Rightmost numbers indicate the number of visits where this pattern was observed and the number of patients for whom the corresponding pattern was observed (in brackets). Only patterns observed at least 4 times and in at least 3 patients are represented. Complete results of covariate-pattern analysis are reported in Supplementary Table 4.

umentation of a virus was associated with lower FEV1% but had no effect on LCI values. In detailed analyses, the negative trend between respiratory syncytial virus and pulmonary function agreed with previous reports.[8], while no study so far correlated endemic coronaviruses to worse outcomes. To our best knowledge, this is the first report of an association between higher FEV1% and rhinovirus detection, although a protective effect of rhinovirus against influenza was reported [21]. Previous studies either reported no effect or a significant morbidity associated to rhinovirus in CF [8,11]. These contradicting findings may be explained by the inclusion of different age groups or outcomes as rhinovirus may be associated to upper respiratory symptoms considered in the diagnosis of pulmonary exacerbation, without significant effects on pulmonary functions [22]. Furthermore, the use of different primers

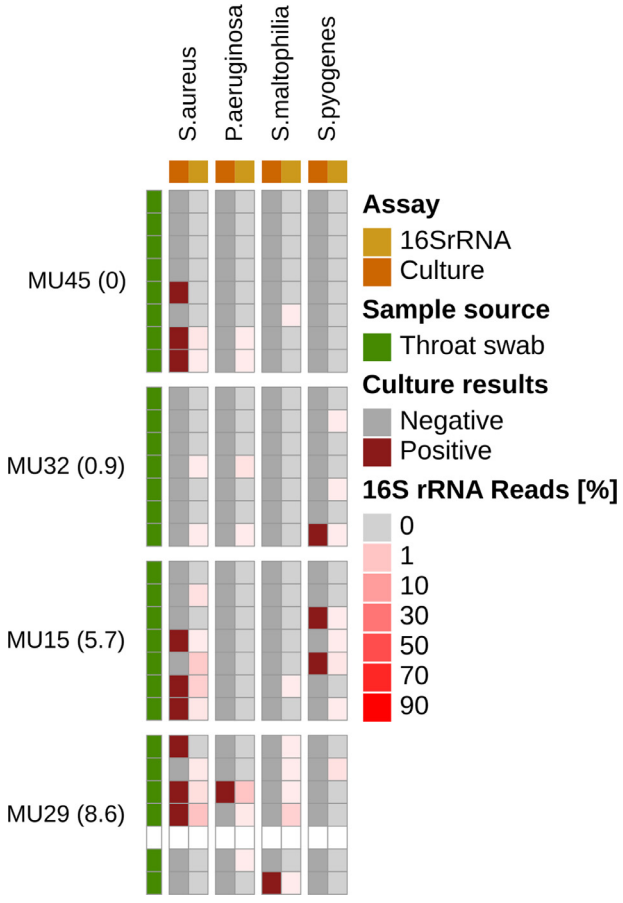

Fig. 2. Comparison of conventional culture and metagenomics for selected bacteria and patients
Heatmap comparing the results of conventional culture (Culture) and 16S rRNA metagenomics (16S rRNA) for bacterial detection. Each row represents a visit, each group or rows a patient (ordered by age at inclusion, in parentheses). The leftmost column represents the specimen type (throat swab). Each pathogen is represented by two columns: the left one indicates negativity (dark grey) or positivity (dark red) by culture and the right one indicates the matching proportion of reads (percent of reads, from 0% in grey to 100% in red). The visit missing a sample is filled in white. Four pathogens and 4 patients arbitrarily selected are represented as examples (full dataset in Supplementary Fig. 4). Interestingly, 16S rRNA metagenomics detected earlier or more systematically the presence of some pathogens (e.g. *S. aureus* in MU15, *S. pyogenes* in MU32 and MU15, *S. maltophilia* and *P. aeruginosa* in MU29). However, counterexamples in which culture detected pathogens earlier compared to metagenomics were also observed (e.g. *S. aureus* in MU45).

and probes in molecular assays for rhinovirus detection results in the detection of rhinovirus strains with distinct pathogenicity in CF [23]. Overall, our findings support that rhinovirus does not affect pulmonary functions as measured by FEV1%, unlike endemic coronaviruses and respiratory syncytial virus.

In addition to *P. aeruginosa*, *S. pyogenes* was associated with a trend for decreased FEV1%. *S. pyogenes* is common in children and has been associated to pulmonary exacerbations in adults with CF [24] without being considered as a typical pathogen in CF. Our results support that *S. aureus* probably often acts both as colonizer and as pathogen [25]. *S. aureus* detection presented a minimal impact on FEV1% in longitudinal analyses whereas it was associated with higher FEV1% when found along rhinovirus in covariate-pattern analysis. As such, bacterial-viral confection, mostly represented by *S. aureus* and rhinovirus, appeared to have a neutral effect of the FEV1% while bacteria and viruses were individually associated with significantly worse FEV1% Altogether, these results underline the importance of protecting young CF patients from early *P. aeruginosa* colonization, support to consider *S. pyogenes* as a pathogen and urge for the development of diagnostic tools discriminating bacterial colonization from infection.

Only a fair agreement was observed when comparing 16S rRNA metagenomics and conventional culture results for the detection of typical CF pathogens, with pathogen-specific differences, as reported elsewhere [26]. Antibiotic regiments might have impacted the detection of some bacteria by culture. Moreover, conventional culture followed by MALDI-TOF and 16S rRNA sequencing are known to sometimes differ in species-level classification of closely related strains [27], as exemplified by the fact that some species identified by culture corresponded to multiple 16S rRNA metagenomics taxonomic identifiers (Supplementary Table 2). Metagenomics based on short reads are also known to have an imperfect species-level resolution, which could lead to false positive results. These combined factors might explain some disagreements between culture and metagenomics results. As for *H. influenzae*, for which 36.9% of samples were positive by metagenomics but negative by culture, our results agree with previous reports [26] and point towards the established limited sensitivity of culture, even when using dedicated chocolate agar [28]. Culture false negatives could also occur when bacteria such as *P. aeruginosa* overgrow lowly abundant or fastidious bacteria [29]. The striking difference in the detection of *P. aeruginosa* by metagenomics compared to culture might result from a higher sensitivity but also from contaminations, especially when detected in low abundance and sporadically in a patient [30]. In contrast, sporadic and at low abundance detection of *S. pyogenes* and *S. pneumoniae* by metagenomics most likely resulted from transient carriages [24]. Overall, this comparison between the two methods highlights species-specific limitations when interpreting culture and 16S rRNA metagenomics results and the need for refinements of metagenomic protocols to enable interpretation of bacteria detected in low abundance.

Our findings illustrate the current debate regarding the use of culture-free assays in CF clinical settings. Metagenomics assays offer a broader overview of the bacterial respiratory microbiota and potentially a better sensitivity. Yet, the more frequent detection of bacteria such as *P. aeruginosa* by metagenomics might prompt unnecessary antibiotic prescription. In clinical settings, the detection of a respiratory virus, more specifically respiratory syncytial virus or influenza, can prevent antibiotic prescription. Yet, half of time viruses were detected along with a pathogenic bacteria, thereby limiting the withdrawal of antibiotics. Overall, our results highlight the complexity of establishing associations between viral respiratory infections and lung function decline [8,11] and question the clinical impact of documenting respiratory viruses by specific PCR in patients with CF.

Important strengths of our study include a prospective, longitudinal and multicenter design as well as the inclusion of children outside of the neonatal period, who present a dynamic microbiota and who are commonly exposed to respiratory viruses. Furthermore, the adjustment in mixed-effects logistic regression analyses for important covariates and the use of covariate-patterns analysis reinforced the association between viral and bacterial predictors and clinical outcomes.

Potential limitations are the observational nature of our study and the heterogeneity in recorded pulmonary function tests, which is inherent to the studied age groups. The use of different sample types is consistent with clinical practice and standardized recommendations [31–33]. However it is a limitation when addressing the question of a virus-bacteria coinfection. In addition, upper respiratory samples are not necessarily representative of lower tract microbiota [34]. Furthermore, the role of non-TB Mycobacteria was not addressed in this manuscript. Finally, increased FEV1% among rhinovirus positive children was rather unexpected, clinically insignificant (4.24) and was not seen for LCI. However, this discrepancy was not surprising as both outcomes were poorly correlated (Pearson's $r = -0.47$, $p < 0.001$) [35]. Furthermore, high baseline LCI median values possibly contributed to a decreased effect of respiratory virus, virus-bacteria co-infection and rhinovirus detection on LCI values. As such, the association between FEV1% and rhinovirus detection should certainly be evaluated in further cohorts. Moreover, pulmonary functions may present spontaneous variations over time and rhinovirus may be shed for a prolonged period, thus potentially reported among asymptomatic children, falsely suggesting a protective effect.

In conclusion, *P. aeruginosa* impacted negatively on FEV1% while rhinovirus was surprisingly associated with better FEV1%. Culture-free assays identifies a large number of microorganisms that would remain undetected by conventional culture, yet with disputable clinical correlation.

Declaration of Competing Interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2021.10.001.

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