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Original Article

Evidence for secondary ciliary dyskinesia in patients with cystic fibrosis

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ABSTRACT

Background: Mucociliary clearance (MCC) impairment can be due to mucus abnormalities or to a ciliary dysfunction, which can be innate, or secondary to infection and/or inflammation. In cystic fibrosis (CF), it is well documented that MCC is impaired due to mucus abnormalities, but little is known concerning ciliary beating. This study aimed to confirm that ciliary dyskinesia is present in CF, and if this might be innate or secondary to the chronic infection and/or inflammation.

Methods: Ciliated epithelial samples were obtained by nasal brushing from 51 CF patients, and from 30 healthy subjects. Ciliary beating was evaluated using digital high-speed videomicroscopy at 37 °C, allowing to evaluate ciliary beat frequency (CBF) and the percentage of abnormal beat pattern (CBP); this was repeated after air-liquid interface (ALI) cell culture.

Results: Ciliary dyskinesia was higher in CF patients than in healthy subjects, with a lower CBF and a higher percentage of abnormal CBP. Ciliary dyskinesia, already present in childhood, normalized after ALI cell culture. A chronic airway colonization did not worsen ciliary dyskinesia.

Conclusions: We showed that, in CF, a ciliary dyskinesia, present from childhood, might contribute to the impaired MCC. Our results also found that the abnormal ciliary beating was not associated with a chronic infection, and resolved after ALI cell culture, suggesting that ciliary dyskinesia in CF is not innate, and might be secondary to chronic inflammation.

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* (*CFTR*) gene [1]. The CFTR receptor is present in several organs, such as the lungs, pancreas, intestine, and liver ducts, which can be affected by the pathology [2]. While CF can occur in any ethnicity, the incidence is higher in the Caucasian population (between 2500–4000 [3]), being the most common severe genetic respiratory disease in this population. CF remains a life-limiting disease; however, continuous improvement of treatments, and newborn screening (NBS) allowing care optimization from birth, have greatly improved life expectancy [2].

Abbreviations: ALI, air-liquid interface; CBF, ciliary beat frequency; CBP, ciliary beat pattern; CF, cystic fibrosis; CFA, ciliary functional analysis; CFTR, cystic fibrosis transmembrane conductance regulator; DHSV, digital high-speed videomicroscopy; FEV_1, forced expiratory volume in the first second; HBSS, hanks balanced salt solution; Hz, hertz; M199, medium 199; MCC, mucociliary clearance; NBS, newborn-screening; PA, pseudomonas aeruginosa; PCD, primary ciliary dyskinesia; SD, standard deviation; SE, standard error; μOCT, micro optical coherence tomography.

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CFTR disease-causing mutations are categorized in 6 classes, depending on the molecular mechanism responsible for the damage at the protein level [4]. *CFTR* mutations cause an alteration of ions exchange through the CFTR receptor, leading to changes in the composition of mucus, and increased thickness and viscosity of the mucus layer [1]. This results in a progressive accumulation of mucus in the airways and in an abnormal mucociliary clearance (MCC), leading to chronic respiratory infection and inflammation, bronchial obstruction, and a progressive destruction of the lungs [5]. The mucus stasis prevents the clearance of bacteria and other pathogens from the airways, leading to chronic airway colonizations [6]. A chronic colonization by *Pseudomonas Aeruginosa* (*PA)* is associated with an accelerated decline in lung function and an increased mortality [3].

In CF, it is well documented that MCC, the first line of defense of the lungs, is impaired [7]. MCC results from an effective interaction between the mucus layer, the periciliary layer, and the cilia present on the surface of ciliated cells [8]. MCC is responsible for the removal from the lower respiratory tract, of the pathogens and particles entrapped in the mucus, by the coordinated ciliary beating [8]. Impaired MCC leads to recurrent sinopulmonary infections, mucus accumulation in the airways, and can lead to bronchiectasis [8]. MCC defects can be due to a ciliary dysfunction, or to an abnormal mucus layer composition or depth [8].

Cilia dysfunction is called ciliary dyskinesia, and is defined by an abnormal ciliary beat frequency (CBF) and/or beat pattern (CBP). Ciliary dyskinesia can be primary, due to mutations in genes coding for axonemal structure and/or functional components of motile cilia [8], such as in primary ciliary dyskinesia (PCD), or secondary to acute or chronic infection, inflammation, exposure to pollutants, tobacco smoking, etc. [8]. Moreover, it has been described that several inflammatory cytokines and pathogens had an impact on ciliary beating [6,9–11]. The exposition to these factors, but also the procedure for collecting respiratory cilia, might be responsible for secondary ciliary dyskinesia in both healthy subjects and in patients with chronic respiratory diseases.

Ciliary beating can be evaluated using digital high-speed videomicroscopy (DHSV) which allows a complete ciliary functional analysis (CFA), including CBF and CBP evaluation [12]. Repeating CFA after air-liquid interface (ALI) cell culture allows to differentiate primary from secondary ciliary dyskinesia [13]. MCC can be evaluated *in vitro* or *in vivo* using different techniques. Scintigraphy enables to study MCC throughout the entire lung, but also in certain targeted areas. The micro optical coherence tomography (μOCT) is a technique that allows to evaluate both mucociliary transport rate and CBF *in vitro* or *in vivo* [14,15].

Some previous studies have shown that MCC and CBF are impaired in CF. Scintigraphy was used to study MCC in CF: a study by Robinson et al. [16] showed that MCC was decreased in CF patients compared with healthy subjects. Scintigraphy is also useful to study the effect of specific therapies on secretion clearance in CF patients [16]. μOCT was used in a study by Leung et al. [14], showing that mucociliary transport rate and CBF, evaluated *in vivo*, were lower in CF patients than in healthy controls. Birket et al. [15] used μOCT to show that CBF was decreased in CF cell cultures of human bronchial epithelial cells derived from lung explants, and that mucociliary transport was delayed. However, few studies have performed a complete evaluation of ciliary beating in CF, using DHSV, and available data concern only the comparison between fresh nasal samples from healthy and from CF children, and are contradictory, describing, in CF children, either a higher CBF without evaluation of CBP [17], or a lower CBF with an increased abnormal beat pattern [18,19].

To our knowledge, no data have investigated whether ciliary dyskinesia, evaluated by both CBF and CBP, using DHSV, persisted after ALI cell culture in CF.

We aimed to confirm that ciliary dyskinesia, evaluated by a complete CFA, and on a higher number of subjects, is present in CF, and if this might be innate or secondary to chronic infection and/or inflammation. We also aimed to identify the factors that might modify ciliary beating in CF.

2. Materials and methods

2.1. Patients

This was a single center, cross-sectional study, on a series of 28 pediatric (<18 years old) and 23 adult (≥18 years old) CF patients. Patients were recruited in the Liege center for functional rehabilitation for CF between January 2021 and February 2022 in the context of routine visit.

Inclusion criteria were a diagnosis of CF, defined as a positive sweat test, with a sweat chloride level ≥60 mmol/L, and/or a genetic analysis positive for 2 CF-causing *CFTR* mutations. Exclusion criteria included patients treated by a CFTR modulator therapy (reimbursed in Belgium in April 2021, allowing to include patients before the initiation of the treatment), a pulmonary transplantation, a respiratory infection, a systemic corticosteroids therapy within 4 weeks prior the visit, and a Sars-Cov-2 infection with respiratory symptoms within 6 months before the visit. Patients were asked not to take any inhaled or topical nasal medication 24 hours before the visit.

Patients underwent a complete clinical evaluation during the inclusion visit, including a spirometry and a nasal brushing to obtain respiratory ciliated epithelial samples. Clinical data collected by questionnaires and medical chart review included *CFTR* genetic analysis, NBS results, reported diagnosis of nasal polyps by an ear, nose and throat specialist, the presence of bronchiectasis on chest computed tomography scan, and microbiology. A chronic colonization by *PA* was defined by the European Consensus [20] as: “the presence of *PA* in the bronchial tree for at least 6 months, based on at least 3 positive cultures with at least one month interval between them without direct (inflammation, fever etc.) or in direct (specific antibody response) signs of infection and tissue damage” [20].

A nasal brushing was also obtained from 13 pediatric and 17 adult healthy subjects, with exclusion criteria being a chronic respiratory disease, a familial history of PCD or CF, a respiratory infection within 4 weeks prior to the brushing, regular use of chronic nasal or inhaled medication, and active smoking.

This study was approved by the ethics committee of the University Hospital of Liege (2020–373, 2020–174, 2021–369) and by the ethics committee of the Regional Hospital Center of Liege (JL/bl/1909 - 2020–373-MHC-B4122021000004). All subjects provided informed consent to participate in the study.

2.2. Ciliary functional analysis

Ciliary beating was evaluated using DHSV as previously described [12]. Briefly, respiratory cells were obtained by brushing the middle nasal turbinate with a cytology brush, without anaesthesia [12,21,22] . Ciliated cells were placed in Medium 199 (M199)(ThermoFisher, USA) supplemented with antibiotic solution (1% of streptomycin/penicillin) and antifungal solution (1% of amphotericin B)(ThermoFisher, USA) [12]. The cell suspension was then placed in a visualisation chamber, and the video sequences of beating cilia were recorded within 9 hours after sampling [23]. Strips of ciliated epithelium were visualised using an inverted light microscope (Axio Vert.A1, Zeiss, Germany) with a 100x oil-immersion lens. Ciliary beating was observed at 37 °C and recorded at 500 images/second using a high-speed video camera (CrashCam Mini 1510, IDT Innovation in motion, USA) [12].

A minimum of 300μm of respiratory ciliated epithelium with cilia beating in a sideways profile were recorded and analysed for each subject. Only normal strips or strips with minor projections [24], and at least 50μm in length, were used to perform a CFA, assessed by CBF and by the percentage of abnormal CBP. A minimum of 6 strips of epithelium were analysed for all the participants, and the strips selected were of the highest quality [24], and allowing the higher number of CBF and CBP

evaluations along the strips. To calculate CBF, ciliated strips were divided into 5 adjacent areas of 10µm, and CBF was evaluated using the number of frames required to complete 5 beat cycles [12]. For each cilium or group of cilia used for a CBF measurement, the movement of the cilium or the group of cilia was compared to a normal CBP observed using DHSV. Then, a normal or abnormal CBP was attributed [12]. The categories of abnormal CBP are the following: stiff CBP (reduced amplitude and/or failure to bend along the axoneme), immotile CBP (lack of movement), circular CBP (circular movement of the cilia) and dyskinetic CBP (all types of abnormal movements not corresponding to the previous categories) [22,25]. A minimum of 4 CBF and CBP evaluations were done in each ciliated strip [12]. For each subject, the mean CBF of all recorded CBF (including static cilia: CBF=0Hertz (Hz)), the percentage of abnormal CBP, and the percentage of each category of abnormal CBP, were calculated.

2.3. ALI cell culture

After recording ciliary beating using DHSV, the remaining samples were used for ALI cell culture (protocol adapted from Coles et al. [26]). Briefly, ciliated samples were washed with 2ml of Hanks Balanced Salt Solution (HBSS(ThermoFisher, USA)) without calcium and magnesium [26]. After a centrifugation of 7 minutes at 400x g, the cell pellet was re-suspended in 3ml of PneumaCult Ex-plus (StemCell, Canada), supplemented with 0.1% hydrocortisone (StemCell, Canada), 1% penicillin/streptomycin and 0.002% nystatin (ThermoFisher, USA), and seeded in collagen-coated T12.5cm flask [26]. At 80% of confluence, cells were passaged with 0.25% trypsin EDTA (ThermoFisher, USA) and centrifuged twice at 400x g for 5 minutes with 12ml of HBSS. Then, 150,000 cells were placed on 12mm Transwell® with 0.4µm pore polyester membrane insert (Greiner, Austria) in a 12-well plate. Cells were submerged in 250µl of PneumaCult Ex-plus, on the apical side, and 650µl of PneumaCult Ex-plus were placed on the basal side. When cells reached confluence, the PneumaCult Ex-plus was removed. On the apical side, cells were directly in contact with air, and 650µl of PneumaCult ALI (StemCell, Canada) supplemented with 1% penicillin/streptomycin, 0.002% nystatin, 0.5% hydrocortisone and 0.2% heparin (StemCell, Canada) were placed on the basal side [26]. Cells were stocked in an incubator maintained at 37 °C and 5% CO_2, and 3 times per week, all cell media were replaced and the apical sides of the cells were washed with HBSS to remove mucus [26].

After ciliogenesis process, and a complete ciliation, cells were mechanically detached from the insert with a tip (after 6 weeks of ALI cell culture process), and placed in 1ml of M199. Cultured cell suspensions were centrifuged 5 minutes at 400x g, and the cell pellets were re-suspended in 2ml of M199. A complete CFA was re-assessed as described previously.

2.4. Statistical analysis

Data were expressed as mean and standard deviation (±SD) for continuous symmetric variables, and median (interquartile range) for variables with skewed distributions. All CBF and percentage of abnormal CBP were described as mean±SD.

The effect of CF on ciliary beating has been evaluated using a linear model, adjusted for age on the day of the nasal brushing. The linear model was performed on the total (children and adults) healthy and CF population, and on subgroups of healthy and CF children and adults, separately. The impact of different clinical factors on CBF and CBP has been evaluated using a linear model, adjusted for age on the day of the nasal brushing. A linear model was run for each factor. As no factor had an impact on ciliary beating, a multivariate linear model was not performed.

Results were considered statistically significant at the 95% confidence level ($p<.05$). Analyzes were performed on the highest number of available data; missing data were not replaced. Statistical analysis were performed using SAS version 9.4 (SAS Institute, Cary, NC, USA) and R version 4.2.0 (R Core Team, Vienna, Austria).

3. Results

3.1. Demographic characteristics

We recruited 51 CF patients (Table 1) and 30 healthy subjects (mean age: 20.7±10.8 years; 30% of males), matched for age ($P=.15$). Among them, 28 patients and 13 healthy subjects (mean age: 10.4±2.9 years; 46% of males) were pediatrics and matched for age ($P=.29$), and 23 patients and 17 healthy subjects (mean age: 28.5±7.2 years; 18% of males) were adults and matched for age ($P=.48$) (Table 1). The demographics, functional and clinical characteristics of CF patients are presented in Table 1.

3.2. Ciliary dyskinesia in CF

Ciliary beating evaluation was feasible in 51/51 patients, and 13 nasal brushing samples allowed a complete CFA after ALI cell culture. In

Table 1
Demographic, functional, and clinical characteristics of patients with CF.

	Patients with CF		
	Total population (N=51)	Children (N=28)	Adults (N=23)
Demographics			
Sex (males), n (%)	30 (58.8)	16 (57.1)	14 (60.9)
Age, years	18.3 ± 14.5	8.6 ± 5.4	30.1 ± 13.2
Age at diagnosis, months	(N=46) 0.0 (0.0 – 4.6)	(N=28) 0.0 (0.0 – 0.0)	(N=18) 3.5 (0.8 – 66.0)
BMI, kg/m^2	(N=49) 19.0 (15.2 – 21.1)	(N=26) 15.3 (15.0 – 18.2)	(N=23) 21.0 (19.0 – 23.9)
Lung function			
FEV_1, % pred	(N=42) 90.8 ± 22.4	(N=21) 99.5 ± 15.6	(N=21) 82.1 ± 25.0
Bronchiectasis, n (%)	(N=44) 33 (75.0)	(N=21) 12 (57.1)	(N=23) 21 (91.3)
Diagnosis			
Diagnose by newborn screening, n (%)	26 (51.0)	22 (78.6)	4 (17.4)
Mutations ♦	(N=49)	(N=27)	(N=22)
F508del/F508del, n (%)	28 (57.1)	15 (55.6)	13 (59.1)
F508del/other, n (%)	12 (24.5)	8 (28.6)	5 (22.7)
Other mutations, n (%)	9 (18.4)	5 (17.8)	4 (18.2)
Microbiology			
Chronic colonization, n (%)	32 (62.7)	12 (42.9)	20 (87.0)
Chronic colonization by *Pseudomonas Aeruginosa,* n (%)	10 (19.6)	3 (10.7)	7 (30.4)
Chronic colonization by pathogens other than *Pseudomonas Aeruginosa*, n (%)	22 (43.1)	9 (32.1)	13 (56.5)

Data are expressed as mean ± SD for continuous variables or median (interquartile range) for variable with a skewed distribution. BMI: body mass index, CF: cystic fibrosis, FEV_1: forced expiratory volume in the first second. ♦ In the group of patients heterozygous for the F508del mutation (F508del/other), patients had the following variants combinations: F508del/Q652X, F508del/5T; TG13, F508del/R553X, F508del/N1303K (in 2 patients), F508del/3849+10kbC>T (in 3 patients), F508del/2118del4, F508del/2183AA->G, F508del/G542X (in 2 patients). In the group of patients with two other mutations, patients had the following variants combinations: 1677delTA/1677delTA, S1251N-N133K, 1774delCT/R347P, 593insT/R764X, G542X/D1152H, 330A>TCT/330A>TCT, G542X/1497delGG, N1303K/5T; 13TG, G542X/1497delGG.

healthy subjects, 30/30 ciliary beating evaluations were feasible, with 23 CFA achievable after ALI cell culture.

The evaluation of CBFs and of the percentage of abnormal CBPs measurements in healthy subjects and in CF patients, before and after ALI cell culture, are presented in Table 2.

Before ALI cell culture, the results showed a significantly higher ciliary dyskinesia in our total (children and adults) population of CF patients than in the total population of healthy subjects. Indeed, the results of the linear model, adjusted for age, showed that CF patients had a lower CBF (coefficient±SE: -1.59±0.53Hz; P=.003) (Fig. 1 a), and a higher percentage of abnormal CBP (coefficient±SE: 13.47±3.22%; P<.001) (Fig. 1 b) compared with healthy subjects. Sub-analysis were performed, comparing on one hand CF children and healthy children, and on the other hand CF adults and healthy adults. We found that, when comparing CF children with healthy children, CBF was lower (coefficient±SE: -1.95±0.83Hz; P=.024) (Fig. 1 c), and the percentage of abnormal CBP was higher (coefficient±SE: 14.80±4.86%; P=.004) (Fig. 1 d). In CF adults, the percentage of abnormal CBP was significantly higher than in healthy adults (coefficient±SE: 11.43±4.46%; P=.015) (Fig. 1 d), but there was no difference in CBF (P=.085) (Fig. 1 c). Furthermore, we found no difference in ciliary beating between healthy children and healthy adults, and between CF children and CF adults (Fig. 1 c,d).

When comparing ciliary beating after ALI cell culture between the total (children and adults) CF patients and healthy subjects populations, the results of the linear model, adjusted for age, showed that there was no difference in CBF (P=.63) (Fig. 2 a), nor in the percentage of abnormal CBP (P=.96) (Fig. 2 b). Furthermore, there was no difference in ciliary beating after ALI cell culture between healthy and CF children, and between healthy and CF adults (Fig. 2 c,d).

The main distinct abnormal CBP found in our total population (children and adults) of CF patients was stiff (24.3%), and this persisted after ALI cell culture (9.8%).

3.3. Factors modifying ciliary beating

When evaluating the factors that might modify ciliary beating in our total (children and adults) population of CF patients, the results of the linear model, adjusted for age, showed that none of the factors had an effect on CBF (P>.05), and only one factor (nasal polyps) had a minor effect on the percentage of abnormal CBP (P=.047) (Table 3).

4. Discussion

Evidences of a MCC decrease have been well described in CF [7]. Our study aimed to confirm whether an abnormal ciliary beating is present in CF, and if this might be innate, or secondary to chronic infection and/or inflammation. Our results showed that ciliary dyskinesia was higher in CF patients than in healthy subjects, but normalized after ALI cell culture, and that ciliary dyskinesia started in childhood.

Previous results evaluating ciliary beating in CF were sparse and conflicting, and concerned only small cohorts of CF children. A study by Alikadic et al. showed that CBF was significantly higher in 11 non-transplanted CF children (mean age: 8.9±4.5years) than in healthy children (mean age: 11.5±4.7years) [17]. Another study compared ciliary beating between 9 lung transplanted CF children (mean age: 14.1years) and healthy children, and found a decreased CBF, and a higher dyskinesia index [18].

In this study, we aimed to evaluate ciliary beating in a larger cohort of non-transplanted CF children and adults. We performed linear model, adjusted for age, as it has been described that age has an influence on ciliary function [21,27]. Our results showed an abnormal ciliary beating in CF, with both a higher percentage of abnormal CBP, and a decreased CBF, compared with healthy subjects. In our CF cohort, the total percentage of abnormal CBP was 26.9%, and the main abnormal CBP observed was a stiff CBP (24.3%), with 3.4% of immotile CBP and 1.7% of dyskinetic CBP. This is not specific to CF; this proportion of specific abnormal CBPs is similar to the findings in our healthy subjects population (stiff CBP: 15.4%, immotile CBP: 0.8%, dyskinetic CBP: 0.2%) and in non-PCD patients with secondary dyskinesia (patients referred to our PCD diagnostic centre, unpublished data). On the contrary, in PCD, some genetic mutations or ultrastructural defects have been associated with specific CBPs [28].

We also aimed to understand the origin of ciliary dyskinesia in CF respiratory epithelium, and one hypothesis is that the abnormal ciliary beating in CF might be innate, linked to an intracellular ions concentration in CF airway epithelial cells, which might alters ciliary beating. Indeed, in healthy subjects, it has been described that CBF increases with the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [29], and that a decreased $[Cl^-]_i$ induces an increased ciliary beat distance [30]. In CF airway epithelial cells, *CFTR* mutations are responsible for an alteration of ions exchange [1], with an increased absorption of Na^+, and a decreased Cl^- secretion [31]. An increased $[Ca^{2+}]_i$ has also been described in CF [31]. However, the modifications of intracellular ion concentrations linked with *CFTR* mutations probably do not explain the abnormal ciliary beating in CF, as we found a decreased CBF and an increased abnormal stiff beat pattern(cilia with a reduced amplitude and/or unable to bend along their axoneme [22]). Furthermore, it seems unlikely that ciliary dyskinesia is innate in CF, as ciliary beating normalized in our CF cultured samples.

Another hypothesis is that ciliary dyskinesia in CF might be secondary to chronic respiratory infection and/or inflammation, rather than linked to *CFTR* mutations. This hypothesis is supported by our results, showing a normalization of ciliary beating after ALI cell culture.

It has been described that a viral [10], bacterial [6], or fungal [9] infection induces a disruption of the respiratory ciliated epithelium and a ciliary dyskinesia. We did not evaluate the effect of an acute infection on ciliary beating in CF, but our results showed that chronic airway bacterial or fungal colonization did not worsen ciliary beating in CF. We then hypothesized that an airway colonization by *PA* might induce a

Table 2
Descriptive statistics of ciliary beat frequency and of the percentage of abnormal ciliary beat pattern in healthy subjects and CF patients.

	Healthy subjects		CF patients		Healthy children		CF children		Healthy adults		CF adults	
Variable	N	Mean±SD	N	Mean ±SD	N	Mean ±SD	N	Mean ±SD	N	Mean ±SD	N	Mean ±SD
CBF before ALI cell culture (Hz)	30	14.9±2.1	51	13.4±2.6	13	15.7 ±1.6	28	13.9±2.7	17	14.2±2.3	23	12.9±2.3
CBF after ALI cell culture (Hz)	17	15.6±1.9	13	16.0±2.1	11	16.2 ±1.4	7	15.7±2.3	6	14.6±2.4	6	16.4±2.0
Percentage of abnormal CBP before ALI cell culture (%)	30	16.5±9.5	51	29.4 ±16.3	13	13.1 ±6.8	28	28.1 ±16.3	17	19.0 ±10.5	23	31.0 ±16.5
Percentage of abnormal CBP after ALI cell culture (%)	17	11.8±7.6	13	12.1±8.1	11	12.0 ±7.4	7	14.1 ±10.4	6	11.6±8.7	6	9.8±3.7

Data are expressed as mean ± SD. ALI: air-liquid interface, CBF: ciliary beat frequency, CBP: ciliary beat pattern, CF: cystic fibrosis, Hz: Hertz, SD: standard deviation.

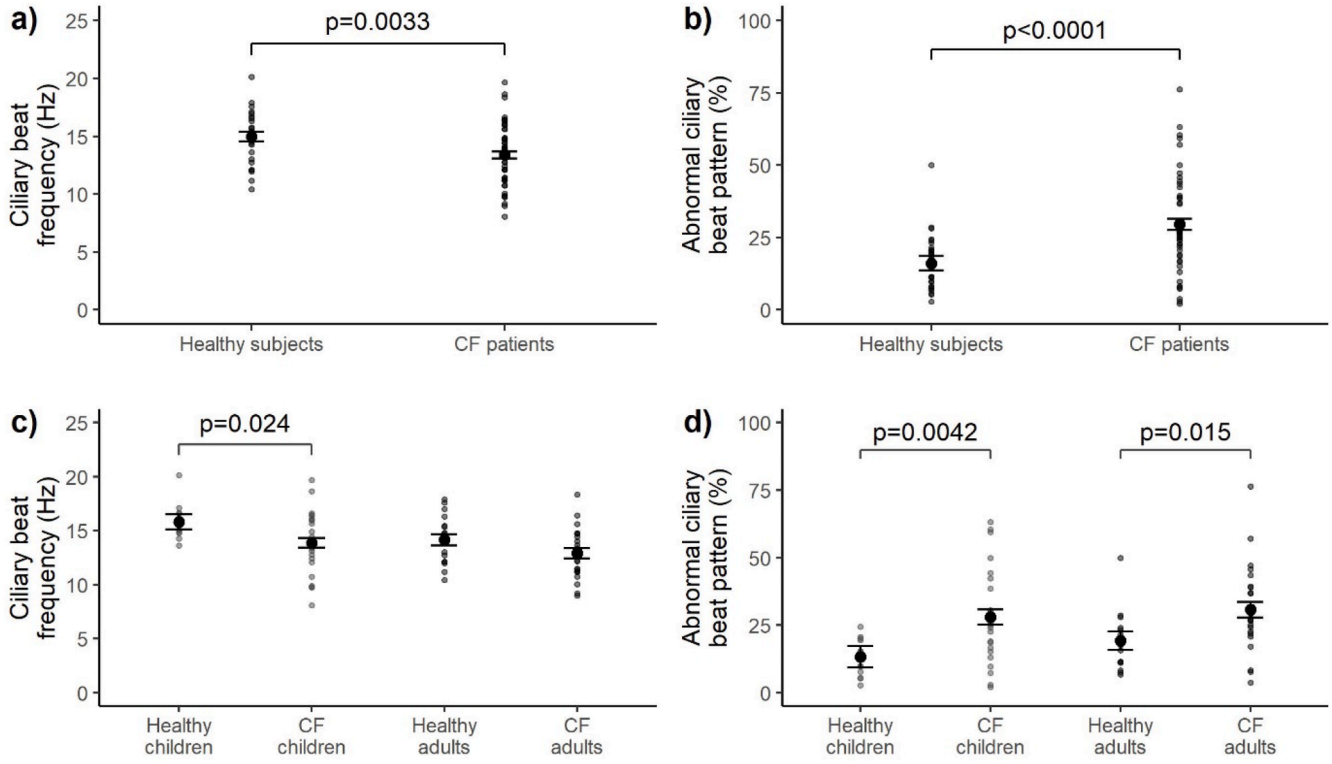

Fig. 1. Graphs showing the comparisons of ciliary beating between healthy subjects and CF patients, before ALI cell culture. (a) CBF in healthy subjects and in CF patients; (b) percentage of abnormal CBP in healthy subjects and in CF patients; (c) CBF in healthy and CF children, and healthy and CF adults; (d) the percentage of abnormal CBP in healthy and CF children, and healthy and CF adults. CF patients: N=51 (children: N=28, adults: N=23); healthy subjects: N=30 (children: N=13, adults: N=17). ALI, air-liquid interface; CBF, ciliary beat frequency; CBP, ciliary beat pattern; CF, cystic fibrosis; Hz, hertz.

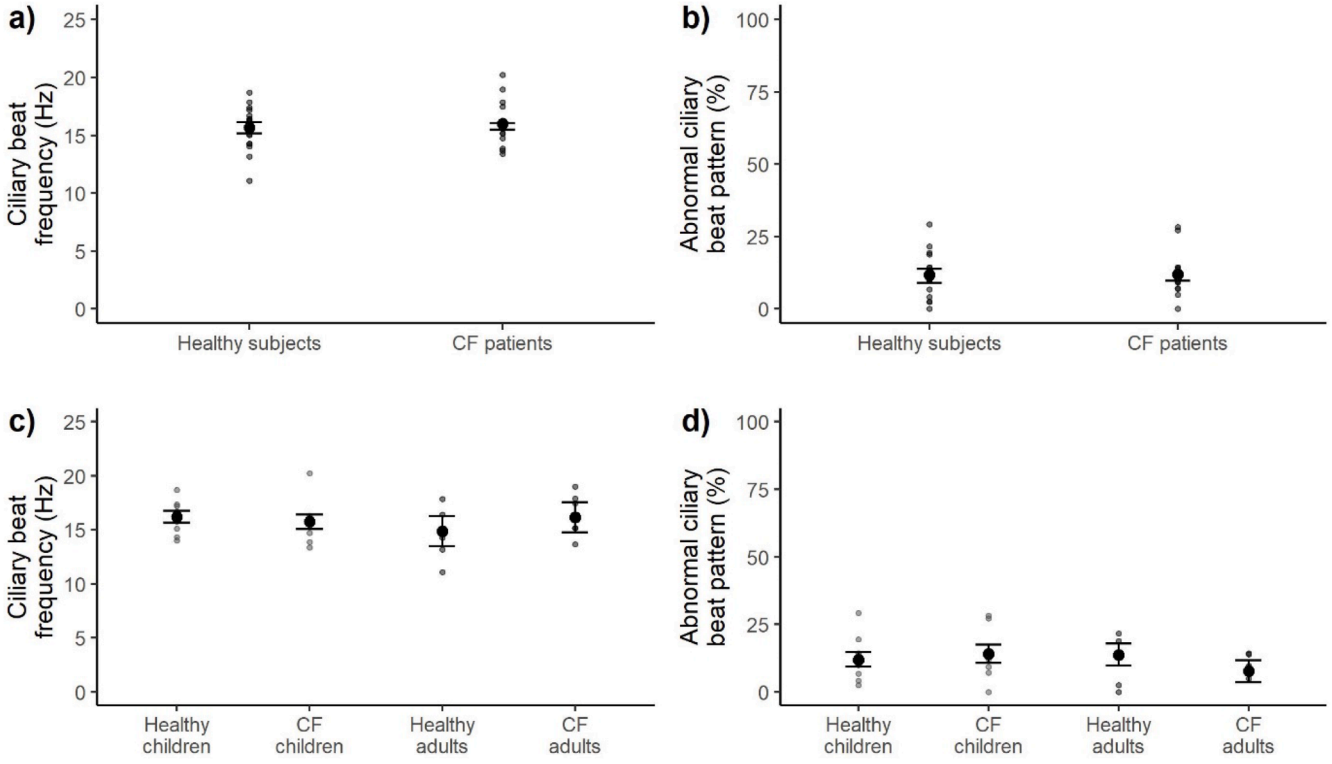

Fig. 2. Graphs showing the comparisons of ciliary beating between healthy subjects and CF patients, after ALI cell culture. (a) CBF in healthy subjects and CF patients; (b) percentage of abnormal CBP in healthy subjects and CF patients; (c) CBF in healthy and CF children, and healthy and CF adults; (d) the percentage of abnormal CBP in healthy and CF children, and healthy and CF adults. CF patients: N=13 (children: N=7, adults: N=6); healthy subjects: N=23 (children: N=12, adults: N=11). ALI, air-liquid interface; CBF, ciliary beat frequency; CBP, ciliary beat pattern; CF, cystic fibrosis; Hz, hertz.

Table 3
Evaluation of the influence of functional and clinical characteristics of the CF population on ciliary beating.

				Ciliary beat frequency (Hz)		Abnormal ciliary beat pattern (%)	
Variable	Category	N total	N (%)	Coefficient±SE	*P*-value	Coefficient±SE	*P*-value
Homozygous for F508del (ref.=No)	Yes	49	28 (57.1)	-0.40±0.74	.59	8.56±4.31	.053
Newborn-screened (ref.=No)	Yes	49	26 (53.1)	0.94±0.88	.29	-4.43±5.77	.45
FEV_1 (% pred, ref.=≥ 90)	<90	42	18 (42.9)	-0.61±0.87	.49	2.59±5.71	.65
Bronchiectasis (ref.=No)	Yes	44	33 (75.0)	-1.17±0.90	.20	-5.33±5.69	.35
Chronic bronchorrhea (ref.=No)	Yes	48	19 (39.6)	-0.47±0.84	.58	2.95±5.49	.59
Chronic cough (ref.=No)	Yes	50	23 (46.0)	-0.11±0.91	.90	2.92±5.84	.62
Chronic rhinitis (ref.=No)	Yes	47	20 (42.6)	-0.10±0.80	.90	-6.95±5.12	.18
Nasal polyps (ref.=No)	Yes	49	13 (26.5)	1.03±0.81	.21	-10.45±5.12	**.047**
Exocrine pancreatic insufficiency (ref.=No)	Yes	45	33 (73.3)	-0.69±0.80	.39	2.07±5.09	.69
Exacerbation in the past year (ref.=≤1)	>1	46	8 (17.4)	0.32±1.02	.75	5.91±6.56	.37
Active or passive smoking (ref.=No)	Yes	50	11 (22.0)	1.41±0.84	.10	5.05±0.056	.17
Chronic colonization by pathogens other than *PA* (ref.=No)	Yes	51	32 (62.7)	-0.79±0.77	.31	-4.55±4.97	.36
Chronic *PA* colonization (ref.=No)	Yes	51	10 (19.6)	-1.62±0.88	.072	2.52±5.90	.67

Data are expressed as number (N) and coefficient ± SE. Significant p-values ($P<.05$) are in boldface font. CF: cystic fibrosis; FEV_1: forced expiratory volume in the first second; Ref, reference; SE, standard error. Linear models were performed, and were adjusted by age on the day of the nasal brushing.

ciliary dyskinesia. Indeed, Nair et al. [6] described that the *in vitro* addition of cyanide (a virulence factor secreted by *PA*) to ALI cell culture from healthy subjects and from CF patients, led to a CBF decrease [6]. This study did not evaluate the effect of cyanide on CBP. However, we found that chronic *PA* colonization, compared with no chronic colonization or chronic colonization by other pathogens, had no effect on ciliary beating in our CF population. These conflicting results might be explained as, in our study, we assessed the effect on ciliary beating of chronic *PA* colonization in CF patients, and not of the isolated effect of cyanide.

In different chronic lung diseases, a chronic airway inflammation is associated with impaired MCC and with ciliary beating and ultrastructural abnormalities [32]. In CF, a chronic neutrophilic lung inflammation is described, with the presence of increased neutrophils and neutrophilic inflammatory mediators within CF airways [33]. Previous studies showed that several cytokines, mainly involved in eosinophilic inflammation, have an impact on CBF [11,34]. However, data concerning the effect of neutrophilic cytokines on ciliary beating are limited. Neutrophil elastase, an inflammatory protease released by neutrophils, is involved lung inflammation in CF, and is also responsible for an alteration of mucociliary function and for premature epithelial senescence in CF [35].

In this study, we also aimed to evaluate if the clinical factors associated with increased respiratory inflammation and increased lung disease severity in CF might be associated with a higher ciliary dyskinesia. We found no links between being homozygous for the F508del mutation, a FEV_1<90% predicted, the presence of bronchiectasis, or a pancreatic insufficiency, indicators of increased lung disease severity and/or inflammation in CF, and a worsened ciliary beating. We then wondered if NBS might improve ciliary beating, as it has been shown that optimal treatments, started early in life, reduce chronic respiratory inflammation in CF [36]. However, we found no difference in ciliary beating between patients diagnosed by NBS or diagnosed later in life. Moreover, it has been described that nasal polyps, linked with a chronic nasal inflammation, have been associated with eosinophilic, but also neutrophilic nasal infiltrate in CF [37], and that nasal polyps are responsible for a motile cilia impairment in CF [37]. Therefore, we expected to observe a worse ciliary dyskinesia in CF patients with nasal polyps, but, interestingly, we rather found an association with a slightly decreased abnormal CBP in these patients. Finally, whereas it is well known that cigarette smoke impairs ciliary beating and induces airway inflammation [38,39], we found no difference in ciliary beating between non-smokers, and passive and active smokers. However, this has been evaluated in a low number of subjects (only 2/50 active smokers and 9/50 passive smokers in our CF population).

A limitation of the study was the low number of ALI cell cultures obtained from CF patients. Indeed, while all nasal brushing samples were cultured, only 13/51 ALI cell cultures were successful and allowed a CFA. The success rate for ALI cell culture was lower in CF patients (26%) than in healthy subjects (77%), but similar between CF children and adults. Despite the addition of antibiotic and antifungal solutions in the cell culture medium, most cell cultures from CF patients did not succeed due to cell culture contamination. Moreover, contamination of ALI cell cultures from healthy subjects was higher than expected, but it might be explained by the fact that they were conducted at the same time that ALI cell culture of CF patients.

Furthermore, in each group, all subjects were anonymized, but the analyses of the videos of beating cilia were not blinded between the healthy subjects and the CF patients groups. To reduce the selection bias, the method to select strips of epithelium included in the ciliary beating analysis was the same in both groups. Indeed, the same quality criteria were used to select ciliated strips of epithelium obtained from CF patients and from healthy subjects: only normal strips or strips with minor projections [24] were analyzed for each subject of each group, and the strips with the higher quality and that allowed the higher number of CBF and CBP evaluation were selected. But to ensure that the results obtained in this study are not biased, the study should be repeated using videos of beating cilia blinded between healthy subjects and CF patients.

In conclusion, we showed that an abnormal ciliary beating is present in CF patients from childhood, and that this ciliary dyskinesia is not innate, as it resolves after ALI cell culture. No clinical factors were associated with a worse ciliary dyskinesia in CF, except for the absence of nasal polyps. Particularly, no links were found between a chronic airway colonization and an increased abnormal ciliary beating.

These results suggest that the intrinsic abnormalities associated with CF are not responsible for the abnormal observed respiratory ciliary beating, and our hypothesis is that ciliary dyskinesia in CF might be secondary to chronic airway inflammation. Indeed, previous data suggest a link between eosinophilic, but also neutrophilic inflammation, and ciliary beating modifications. To study this hypothesis, and to understand the effects of CF lungs inflammation on ciliary beating, two approaches could be used. The first could be to model inflammation in CF on ALI cell cultures, in order to evaluate the effects of neutrophilic cytokines on ciliary beating, while the second could be to study the correlation between ciliary beating and CF airways inflammatory profile (including sputum cellular composition and the presence of pro-inflammatory mediators in the sputum supernatant).

Symptomatic CF lungs disease management aims to treat or prevent respiratory infection, to decrease chronic inflammation, and, but also to improve MCC. Indeed, MCC abnormality is a major component of CF respiratory disease, and our results suggest that an abnormal ciliary beating might contribute to the impaired MCC. Studies evaluating MCC in parallel with ciliary beating might confirm this hypothesis, and, in the future, new treatment strategies could target ciliary beating, which

might improve MCC and bronchial drainage.

CRediT authorship contribution statement

Romane Bonhiver: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Visualization, Project administration. **Noemie Bricmont:** Conceptualization, Methodology, Investigation, Writing – review & editing, Visualization, Funding acquisition. **Maud Pirotte:** Investigation, Resources, Data curation. **Marc-Antoine Wuidart:** Investigation, Resources. **Justine Monseur:** Methodology, Formal analysis, Writing – review & editing, Visualization. **Lionel Benchimol:** Investigation, Writing – review & editing. **Anne-Lise Poirrier:** Conceptualization, Investigation, Writing – review & editing. **Catherine Moermans:** Conceptualization, Resources, Writing – review & editing. **Doriane Calmés:** Conceptualization, Investigation, Writing – review & editing. **Florence Schleich:** Conceptualization, Writing – review & editing. **Renaud Louis:** Conceptualization, Writing – review & editing. **Marie-Christine Seghaye:** Conceptualization, Writing – review & editing. **Céline Kempeneers:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anne-Lise Poirrier reports a relationship with GSK that includes: consulting or advisory and speaking and lecture fees. Anne-Lise Poirrier reports a relationship with Sanofi that includes: consulting or advisory and speaking and lecture fees. Renaud Louis reports a relationship with GSK that includes: consulting or advisory, funding grants, and speaking and lecture fees. Renaud Louis reports a relationship with AstraZeneca that includes: consulting or advisory, funding grants, and speaking and lecture fees. Renaud Louis reports a relationship with Chiesi that includes: funding grants and speaking and lecture fees. Florence Schleich reports a relationship with GSK that includes: consulting or advisory, funding grants. Florence Schleich reports a relationship with AstraZeneca that includes: consulting or advisory, funding grants. Florence Schleich reports a relationship with ALK that includes: consulting or advisory. Florence Schleich reports a relationship with Novartis that includes: consulting or advisory. Celine Kempeneers reports a relationship with Sanofi that includes: consulting or advisory.

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