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ORIGINAL ARTICLE

Intrapulmonary percussive ventilation improves lung function in cystic fibrosis patients chronically colonized with *Pseudomonas aeruginosa*: a pilot cross-over study

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Abstract

High levels of shear stress can prevent and disrupt *Pseudomonas aeruginosa* biofilm formation in vitro. Intrapulmonary percussive ventilation (IPV) could be used to introduce shear stress into the lungs of cystic fibrosis (CF) patients to disrupt biofilms in vivo. We performed a first-of-its-kind pilot clinical study to evaluate short-term IPV therapy at medium (200 bursts per minute, bpm) and high frequency (400 bpm) as compared to autogenic drainage (AD) on lung function and the behavior of *P. aeruginosa* in the CF lung in four patients who are chronically colonized by *P. aeruginosa*. A significant difference between the three treatment groups was observed for both the forced expiratory volume in 1 s (FEV1) and the forced vital capacity (FVC) ($p < 0.05$). More specifically, IPV at high frequency significantly increased FEV1 and FVC compared to AD ($p < 0.05$) and IPV at medium frequency ($p < 0.001$). IPV at high frequency enhanced the expression levels of *P. aeruginosa* planktonic marker genes, which was less pronounced with IPV at medium frequency or AD. In conclusion, IPV at high frequency could potentially alter the behavior of *P. aeruginosa* in the CF lung and improve lung function. Trial registration: The trail was retrospectively registered at the ISRCTN registry on 6 June 2013, under trial registration number ISRCTN75391385.

Keywords Cystic fibrosis · *Pseudomonas aeruginosa* · Shear stress · Intrapulmonary percussive ventilation

Background

The impaired mucociliary clearance in the cystic fibrosis (CF) lung leads to chronic colonization by various microorganisms, of which *Pseudomonas aeruginosa* is associated with the most severe disease outcome [1–3]. Genetic adaptation of *P. aeruginosa* to the CF environment results in the switch from a planktonic to a biofilm lifestyle that is characterized by an overproduction of the exopolysaccharide alginate and a down-regulation of genes involved in acute virulence [4–6]. The biofilm phenotype protects *P. aeruginosa* from antibiotics as well as from the immune system, thereby allowing this bacterium to persist in the hostile CF environment [7–9]. The continuous interactions between *P. aeruginosa*, as well as other CF microorganisms, and the host cause persistent inflammation, ultimately leading to lung dysfunction. Previous in vitro research demonstrated that high levels of fluid shear interfere with the formation of biofilms by the *P. aeruginosa* reference strain PAO1 [10, 11]. Additionally, we recently isolated a highly adapted, transmissible, *P. aeruginosa* strain from patients

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attending the Universitair Ziekenhuis Brussel (UZ Brussel) CF reference center, which is also unable to form robust alginate biofilms under high fluid shear conditions [12]. Hence, the introduction of fluid shear into the lung mucus of CF patients could offer novel therapeutic possibilities to prevent/disrupt pathogen biofilms and enhance susceptibility to antibiotic therapy. Indeed, biofilms that were disrupted by mechanical forces, such as high fluid shear levels, have previously been found to exert decreased tolerance to antibiotics [13, 14].

A possible way to increase the fluid shear levels in the CF lung mucus is by using intrapulmonary percussive ventilation (IPV). IPV is a mechanical device by which bursts of gas are introduced into the lungs via a mouthpiece at frequencies of 100–400 bursts per minute (bpm) and provides an addition to the conventional airway clearance techniques such as autogenic drainage (AD) [15, 16]. AD relies on breathing control via adjustment of the rate, depth, and location of respiration within the thoracic cavity to clear viscous pulmonary secretions [17–19]. Although a 2-day IPV treatment regimen did not enhance the clearance of viscous sputum from the lower airways in CF patients compared to wet inhalation of saline [20], its potential to influence biofilm formation by *P. aeruginosa* has not yet been studied.

In this study, we evaluated the effect of IPV on lung function, bacterial load in CF sputum, and *P. aeruginosa* gene expression by subjecting four CF patients to AD alone, and IPV at medium (200 bpm) and high (400 bpm) frequency in combination with AD during three consecutive hospital admissions, each patient being his own control.

Our results show that IPV at high frequency induces the expression of bacterial planktonic marker genes and improves lung function in CF patients that are chronically colonized with *P. aeruginosa*.

Materials and methods

Set-up of the clinical study and inclusion criteria

A randomized cross-over study was conducted, and an overview of the set-up of the clinical study is provided in Fig. 1, while the patient features—including clinical microbiology data at the start of the study—are listed in Table 1. Four CF patients (Table 1) were subjected to three different treatments (AD alone, IPV at a medium frequency of 200 bpm in combination with AD, and IPV at a high frequency of 400 bpm in combination with AD), during three consecutive hospital admissions for an intravenous antibiotic treatment for respiratory infection. A period of at least 3 months between each therapy was respected, during which the patients received AD as the standard airway clearance technique. Similarly, before the first treatment, AD was used as standard airway clearance technique for at least 3 months. Each treatment regimen lasted 5 to 10 days, and each day, patients received two times IPV or AD treatment during 30 consecutive minutes. Two sputum samples were taken at three different time points during the 5 to 10-day treatment: one that was used for determination of the bacterial load (storage at 4 °C for a maximum time of 6 h), while the other was immediately fixed with an equal volume of RNAlater (Qiagen) and was stored at − 20 °C for subsequent *P. aeruginosa* gene expression analysis. In addition, at each time point, lung function tests (LFT) were performed to determine the forced expiratory volume in 1 s (FEV1) and the forced vital capacity (FVC). Inclusion criteria for this study were a minimum age of 6 years old, the ability to expectorate sputum, the ability to perform LFT, clinical stability at the start of the study, and the ability to tolerate two IPV or standard sessions for airway clearance. Patients who received lung transplantation, exhibited massive hemoptysis, pneumothorax, received invasive ventilation, or were pregnant were excluded. Only CF patients who were chronically colonized by *P. aeruginosa* were included in the study. This study was performed blind for the laboratory and was not un-blinded before all data were obtained. All patients received antibiotics (listed in Table S1) before and during the different treatments.

Determination of lung functions

Lung function was determined using spirometry testing, via which the FEV1 and the FVC parameters were measured using a Jaeger Masterscreen (Acertys Healthcare, Belgium).

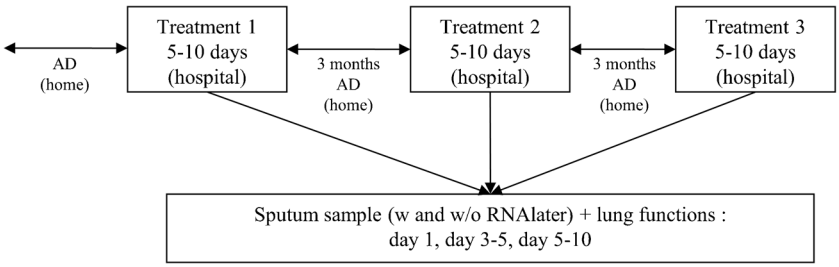

Fig. 1 Set-up of the clinical study. Patients were subjected to three different treatments, each separated by 3 months. During each treatment, lung functions were measured and sputum samples were collected at three different time points: a first time point at which the patient was hospitalized, a second time point in the middle of the hospitalization period (day 3–5), and a final time point at the day the patient left the hospital (day 5–10). All patients were minimally hospitalized for a period of 5 days. The study was performed blind. AD, autogenic drainage

Table 1 Patient characteristics

Patient	*CFTR* mutation	Age	Sex	FEV1 (%)[a]	FVC (%)[b]	Identified microorganisms[c]
02	F508del/F508del	28	F	60	75	*P. aeruginosa*, *S. aureus*, *S. mitis, Staphylococcus* spp. (non-*S. aureus*)
04	F508del/F508del	20	F	32.7	49	*P. aeruginosa*
07	F508del/1717-1G-A	34	M	24	58	*P. aeruginosa*
08	F508del/3849+10kbC-T	25	F	31	59	*P. aeruginosa*

1717-1G-A is a class I *CFTR* mutation. F508del is a class II *CFTR* mutation. 3849+10kbC-T is a class V mutation

P. aeruginosa Pseudomonas aeruginosa, S. aureus Staphylococcus aureus, S. mitis Streptococcus mitis

[a] FEV1 measured before the first treatment

[b] FVC measured before the first treatment. FEV1 and FVC are expressed as a percentage of their predicted values

[c] These bacteria were identified in this study by using either selective media (*S. aureus*), genotyping (*P. aeruginosa*) or 16S rRNA gene sequencing (*S. mitis*)

Collection of sputum samples

Sputum samples were obtained in the hospital within 30 min after receiving one of the three airway clearance treatments via assistance of an experienced physiotherapist, subsequently stored and preserved at 4 °C for up to 6 h during transport to the laboratory.

Determination of bacterial load and identification of microorganisms

Sputum samples were first diluted two-fold using PBS and vortexed to reduce viscosity. Next, ten-fold serial dilutions were plated on *Pseudomonas* P agar (Difco) supplemented with Irgasan (1 mg/l) (Sigma-Aldrich), mannitol salt agar (Difco), and lysogeny broth (LB) medium to detect *P. aeruginosa*, *Staphylococcus aureus*, and other CF microorganisms, respectively. Plates were incubated at 37 °C for 48 h. *P. aeruginosa* isolates were further genotyped using the multiplex amplification of ferripyoverdine siderophore receptor genes as described previously [21, 22]. *S. aureus* was identified by the formation of yellow colonies on mannitol salt agar. Other microorganisms (not showing a colony morphology characteristic of *P. aeruginosa* or *S. aureus*) that were identified on LB medium (or occasionally on *Pseudomonas* P agar), were identified via 16S rRNA gene sequencing. To do this, DNA was extracted from an overnight culture of the unknown microorganism grown in LB medium, using the DNeasy Blood & Tissue kit (Qiagen). The resulting genomic DNA was used as a template in a PCR reaction containing the universal 16S rRNA primers (27f 5′-AGAGTTTGATCMTGGCTC-3′ and 1492r 5′-GGYTACCTTGTTACGACTT-3′) [23]. Amplification was performed in a thermocycler (TC-412-Techne) using the following thermocycling conditions: 94 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 48 °C for 1 min, 2 min at 72 °C, and a final elongation step at 72 °C for 10 min. Finally, PCR products were purified and both strands were sequenced (VIB genetic service facility, Wilrijk, Belgium). Obtained sequences were assembled and analyzed using the nucleotide blast tool of the NCBI website at the species level.

RNA isolation and cDNA synthesis

Prior to RNA extraction, the sputum samples were treated following the protocol described by Son et al. [24] with minor modifications. Briefly, sputum samples were incubated with an equal volume of sputolysin reagent (Calbiochem, La Jolla, CA) and vortexed for 30 s. Next, samples were incubated with DNase I (Roche) for 20 min with intermittent mixing to remove extracellular chromosomal DNA. Finally, 30 μl of proteinase K (> 600 mAU/ml) (Qiagen, Hilden Germany) was added per 1 ml of sputum sample and samples were incubated at room temperature (RT) with intermittent mixing to remove proteins. To lyse eukaryotic cells and remove soluble cellular debris, sputum samples were washed twice with ice-cold sterile double-distilled water. Total RNA was purified from the pellets using the RNeasy mini kit (Qiagen, Hilden, Germany). Prior to RNA purification, bacterial cells inside the sputum were first lysed by following the enzymatic lysis of bacteria-protocol (Qiagen, Hilden Germany). To protect the RNA, it was fixed by adding 1 ml of RNAprotect Bacteria Reagent (Qiagen, Hilden Germany) to the bacterial culture. Next, samples were vortexed for 5 s and subsequently incubated at room temperature for 5 min. After a centrifugation step (5000 g, 10 min), the supernatant was decanted and 200 μl of Tris/EDTA-buffer (10 mM TrisCl, 1 mM EDTA, pH 8.0) containing 3 mg/ml lysozyme was added to the pellets. Before adding 350 μl of RLT buffer, the mixture was incubated for at least 20 min with vortexing for 10 s every 2 min. Finally, 500 μl of ethanol was used to allow binding of the RNA on the column membrane. After this preparatory work, we proceeded to the purification step following the protocol for total RNA purification of bacterial lysates with the RNeasy mini kit (Qiagen, Hilden, Germany). Prior to qRT-PCR, an additional Turbo

DNase (Ambion) treatment was performed via two 30 min incubation steps in the presence of 1 μl of (2 U/μl) Turbo DNase. In a next step, the Turbo DNase-treated RNA was purified and concentrated using the RNA clean and concentrator kit (Zymo Research). RNA quantity was determined using the Nanodrop ND-1000 spectrophotometer (NanoDrop technologies), while RNA quality was assessed via the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit (Agilent Technologies). In general, the RNA quality was significantly lower than that of RNA extracted from bacterial cultures grown in laboratory media. However, samples that exhibited extensive degradation were omitted for further processing. The removal of genomic DNA was verified via 35 cycles of PCR amplification (5 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 min at 55 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C) of the *oprI* gene of *P. aeruginosa* (forward 5′-ATGAACAACGTTCT GAAATTCTCTGCT-′3 and Reverse 5′-CTTG CGGCTGGCTTTTTCCAG-′3) [22].

cDNA was prepared using the iScript Select cDNA synthesis kit (Biorad), starting from 1 μg of DNA-free total RNA. The resulting cDNA was diluted 5× prior to use in qRT-PCR. To verify efficient conversion of RNA to cDNA, a PCR was performed using the *oprI*-specific primers and the PCR conditions described above.

Real-time PCR

Since the *P. aeruginosa* strains harbored by the CF patient are expected to be substantially different at the genotypic level due to their (often) environmental origin, we designed qRT-PCR primers that able to anneal to target genes in various *P. aeruginosa* strains without any mismatches. All primers used in qRT-PCR amplification were designed via Primer3 [25] and are listed in Table S2. Amplification was performed in a 96-well plate, in which each well contained 25 μl of volume consisting of 9.5 μl nuclease-free water, 1 μl of each primer (10 μM), 12.5 μl of the 2× iQ™ SYBR® Green Supermix (Biorad), and finally, 1 μl of template cDNA (5× diluted). The PCR amplification was performed via the iQ2 real-time PCR detection system (BioRad) using the following program: an initial cycle at 95 °C for 3 min for denaturation and enzyme activation, then 40 cycles of 95 °C for 10 s and 55 °C for 60 s. Finally, melting curves were determined to identify primer-dimer formation. qRT-PCR results were normalized to the expression of the *oprI* housekeeping gene encoding the major outer membrane lipoprotein I [22]. Fold changes were calculated using the Livak method [26]. The experiment was at least performed in technical duplicates.

Statistical analysis

A linear mixed model—using the lmer function as implemented in the R package lme4 (version 1.1.12) [27]—was used to assess whether the treatment had an effect on the parameters FEV1 and FVC, using the treatment (AD, IPV200 and IPV400) as a fixed factor and the patient as a random factor. Post hoc tests were performed using the glht function as implemented in the multcomp package (version 1.4.6) using the Holm method for multiple testing [28].

Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Results

Effect of IPV on lung function

Patients treated with IPV at a frequency of 400 bpm showed significantly increased lung function based on FEV1 and FVC parameters (7.7 ± 3.1% for FEV1 and 10.7 ± 3.2% for FVC) compared to both AD ($p < 0.05$) (4.8 ± 3.3% for FEV1 and 6.3 ± 1.0% for FVC) and IPV at 200 bpm ($p < 0.001$) (1.0 ± 2.6% for FEV1 and 0.7 ± 3.8% for FVC) (Table 2). It was also observed that the improvement in lung function of patients treated with AD was higher compared to treatment with IPV at a frequency of 200 bpm ($p < 0.05$), indicating that IPV frequency might influence the efficacy of this technique in improving lung function in CF patients chronically colonized with *P. aeruginosa*.

Table 2 Change in lung function during AD, IPV200, and IPV400 treatments

	FEV1			FVC		
Patient	AD	IPV200	IPV400	AD	IPV200	IPV400
02	8%	4%	11%	7%	5%	13%
04	1%	0%	5%	5%	− 1%	12%
07	3%	− 1%	N/A	6%	− 2%	N/A
08	7%	N/A	7%	7%	N/A	7%
Average	4.8%	1%	7.7%	6.3%	0.7%	10.7%
SD	3.3%	2.6%	3.1%	1%	3.8%	3.2%

The change in lung function was determined by calculating the difference between the lung function (FEV1 or FVC) measured at the end of the treatment and that measured at the start of the treatment ($\Delta FEV1 = FEV1_{end} - FEV1_{start}$; $\Delta FVC = FVC_{end} - FVC_{start}$)

N/A data not available, *SD* standard deviation

Change in bacterial load of CF sputum during AD and IPV treatment

All four patients in our study were chronically colonized with *P. aeruginosa* at the start of the first treatment (Table 1). Although to participate in the clinical study it was a prerequisite to be chronically colonized with *P. aeruginosa*, we verified if the same genotype remained present in the sputum of the CF patient during the different treatments by performing a multiplex PCR, amplifying fragments of the ferripyoverdine receptor genes [22]. No change in ferripyoverdine receptor gene profile was observed during any of the three treatments. Patient 02 was colonized by a *P. aeruginosa* harboring *fpvAIIb* and *fpvB*, sputum from patient 04 contained a *P. aeruginosa* harboring *fpvAIII* and *fpvB*, while patients 07 and 08 remained colonized by a *P. aeruginosa* strain containing the *fpvAI* and *fpvB* genes. Sequencing of the 16S rRNA genes revealed the presence of several other bacteria in the sputum. Besides colonization by *P. aeruginosa and Staphylococcus* spp., sputum from patient 02 also contained *Streptococcus mitis*, which has not been considered as a typical CF pathogen but has been isolated from CF sputum before [29, 30] (Table 1).

For patient 02, a strong decrease (10^8 to 10^6 CFU/ml) in *P. aeruginosa* cell numbers as well as in total bacterial count was observed during IPV at 400 bpm, which was not observed for the other treatments (Fig. 2a–c). For patient 04, *P. aeruginosa* cell numbers decreased during AD in contrast to the other treatments (Fig. 2d–f). For patient 07, bacterial counts were comparable during the different treatments (Fig. 2g–h). This overall variability might be elicited by the antibiotic treatment that the patients received before and during the hospitalization period. Although the antibiotic treatment was mainly conserved during the three treatments, the inclusion or exclusion of certain antibiotics may account for the observed drop in bacterial numbers when comparing one treatment to another (Table S1).

Effect of IPV on *P. aeruginosa* gene expression in the CF lung

Previously, we have identified several genes that could serve as marker genes for the planktonic and biofilm mode of growth, respectively [12]. Genes associated with planktonic growth are *PA1922* and *PA0121* (based on PAO1 nomenclature [31]), while the biofilm-associated genes are *norC* (denitrification), *algA* (alginate production), *glpD* (glycerol metabolism), and *mreB* (cell shape maintenance). The *pvdS* gene was included to study the effect of these treatments on the pyoverdine siderophore biosynthetic pathway. Since *pvdS* is an extracytoplasmic sigma factor (ECFσ) that regulates the expression of *toxA* (encoding exotoxin A), *prpL* (encoding an extracellular endoprotease), and the pyoverdine biosynthetic cluster, it may also be considered as a gene associated with the planktonic mode of growth. In addition, treatment of the CF lung by introducing gas bursts might also lead to a better aeration and increased levels of oxygen that favor iron in its

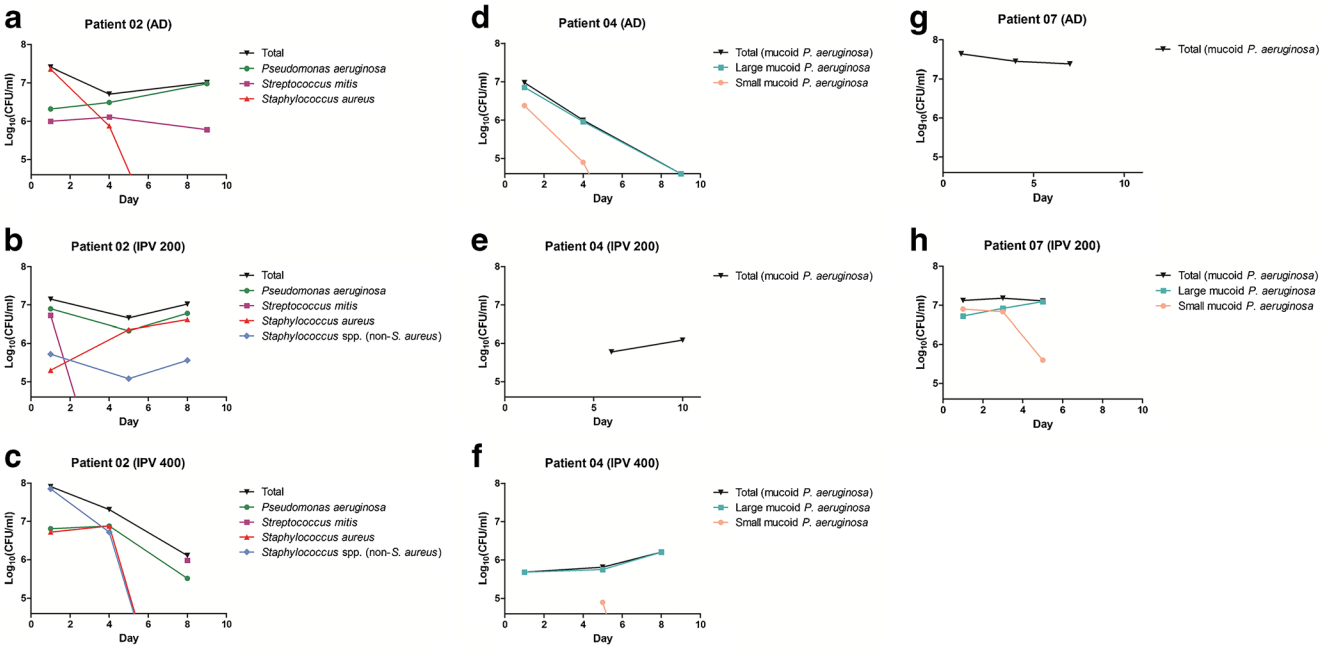

Fig. 2 Change in bacterial count during IPV treatment of patients 02 (**a**–**c**), 04 (**d**–**f**), and 07 (**g**–**h**). The $\log_{10}$ CFU/ml is presented in function of the time point in treatment. The limit of detection was reached at a $\log_{10}$ CFU/ml value of 4.6. D1 indicates the first day of hospitalization, before receiving treatment or airway clearance. In case of patient 04, the bacterial count was not determined during the first day of the second treatment. Patient 07 was only subjected to AD and IPV200

oxidized form [32], hence making it available for pyoverdine-mediated uptake.

An inherent problem of transcriptomic studies in CF sputum is the (partial) degradation of RNA [30, 33]. Therefore, in this study, due to the poor quality of the isolated RNA in some samples, *P. aeruginosa* gene expression analysis could only be performed in a limited number of sputum samples. Unfortunately, the changes in gene expression over time between all three treatments within the same patient could not be evaluated. Nevertheless, gene expression levels could be compared over time during two different treatments for three different patients that were chronically colonized with *P. aeruginosa* (patients 02, 07, and 08). Interestingly, a validation of the gene expression study could be performed using the RNA isolated from the sputum samples of patient 04, since this patient received IPV at 400 bpm twice. When evaluating *P. aeruginosa* gene expression profiles, a large interpatient variability was observed (Fig. 3), most likely reflecting the phenotypic and genotypic diversity in the CF lung [34, 35] as well as the singular nature of the host-pathogen relation and the complex regulatory networks in this pathogen [36]. For some patients, the gene expression levels in the middle (D3-D5) and late sampling points (D6-D8) were very similar (six out of seven genes were positively correlated in the case of the IPV200 sample of patient 02, and the IPV400 sample of patient 08) (Fig. 3). In contrast, the marker genes in the AD sample of patient 07 were inversely regulated when comparing different time points. Remarkably, in most samples, the planktonic *PA0121* gene, which is the second most downregulated gene under low fluid shear conditions [12], and *algA*, a biofilm marker gene, were inversely regulated (in nine of the 12 samples). In addition, for patient 02, both planktonic marker genes, as well as *pvdS*, were highly upregulated and *algA* was downregulated during IPV at 400 bpm, in contrast with IPV at 200 bpm. Similarly, in patient 08, the increase in expression of *PA1922* and *PA0121* was more pronounced in response to IPV at 400 bpm compared to AD. While in patient 07, the planktonic genes were downregulated during IPV at 200 bpm and *algA* upregulated, contrasting the expression of these genes in the AD sample. One of the subjects, patient 04, received the IPV treatment at 400 bpm twice. A similar expression pattern was observed in both IPV400 samples (5/7 genes), validating the reproducibility, and hence reliability, of the gene expression data set.

Discussion

There is a critical demand for novel approaches to treat chronic lung infections in CF patients [37]. The persistent biofilm phenotype of *P. aeruginosa* remains a major hurdle for successful clinical outcomes. To date, no pharmaceutical agent succeeds in completely eradicating biofilms formed by this microorganism [38–40]. Hence, using mechanical forces, such as fluid shear, may be an innovative and complementary approach to disrupt biofilms in situ and enhance their susceptibility to antibiotic treatments and the patient's immune system.

This is the first preliminary clinical study that assessed the influence of physical therapy on both patient outcome and bacterial behavior, and provides a framework for further

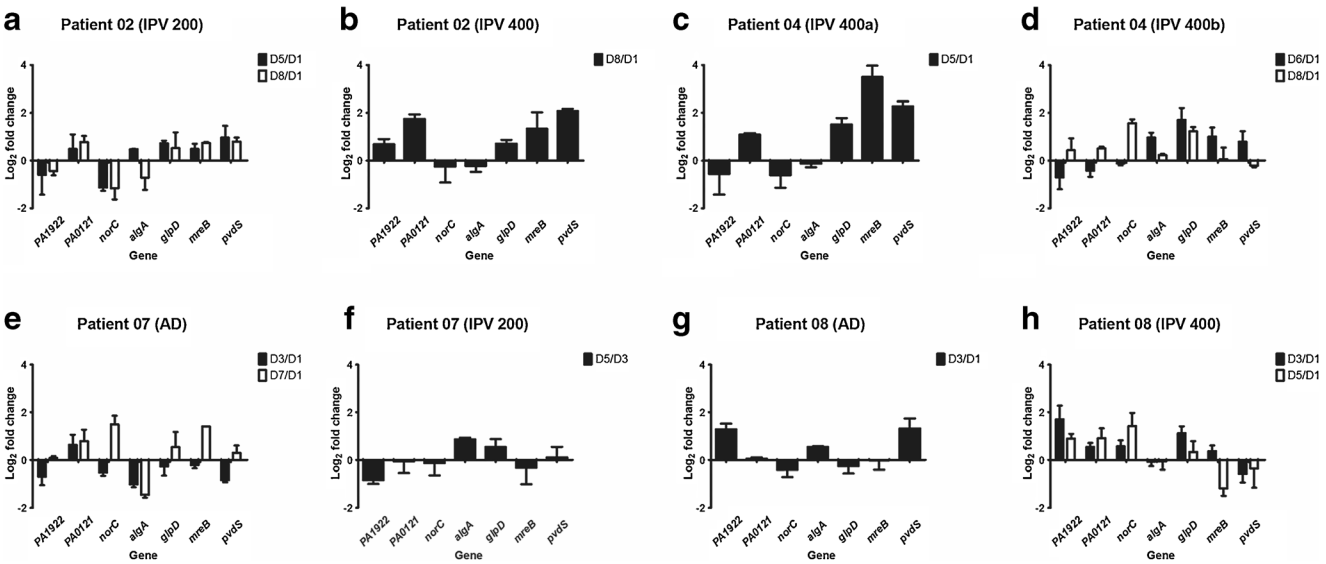

Fig. 3 Change in *P. aeruginosa* gene expression over time in function of the applied treatment for patients 02 (**a, b**), 04 (**c, d**), 07 (**e, f**) and 08 (**g, h**). All values are expressed as $\log_2$ fold changes. D1 indicates the first day of hospitalization, before receiving treatment or airway clearance. *PA1922* and *PA0121* are marker genes for the planktonic lifestyle of *P. aeruginosa*, while *norC*, *algA*, *glpD*, and *mreB* are marker genes for the biofilm lifestyle of this pathogen based on results obtained in a previous in vitro study [12]

research into the potential of shear-introducing therapies for treatment of CF lung infections.

This study tested IPV therapy as this technique is FDA-approved, commercially available, and utilized with comfort from neonatal to adult age. To date, it is used in a minority of CF patients as the few reported clinical studies conducted with IPV did not show significant health benefits [16, 20, 41, 42]. Yet, IPV was not explored from a microbiological point of view. Specifically, we evaluated the potential of IPV treatment to improve lung function in CF patients chronically colonized with *P. aeruginosa* and to direct *P. aeruginosa* gene expression from a biofilm into a planktonic mode of growth by introducing increased shear stress in the CF lungs. IPV at a high frequency significantly increased both FEV1 and FVC compared to autogenic drainage and IPV at medium frequency. To further evaluate the underlying reason for the improved lung function following IPV treatment at high frequency, we aimed at assessing bacterial load and the disruption of *P. aeruginosa* biofilms following treatment. When evaluating the bacterial sputum load following the different treatments, conflicting results were obtained. While one patient (patient 02) showed a promising strong decrease in bacterial sputum load during high-frequency IPV as compared to the other treatments, a second patient only showed a strong decrease in bacterial sputum load when receiving AD. These conflicting results might have been elicited by the addition and/or removal of one of the antibiotics in the antibiotic repertoire received by these patients before and during the clinical study. For instance, the addition of azithromycin might have resulted in a strong decrease of the bacterial sputum load, since this antibiotic was combined with the IPV400, but not the other treatments of patient 02, while patient 04 only received this antibiotic during AD (Table S1).

In agreement with the increase in lung function, the planktonic marker genes of *P. aeruginosa* that are associated with high fluid shear conditions were upregulated in response to IPV at high frequency, which was less pronounced at medium frequency or with AD for those patients where data sets could be compared. In addition, the *PA0121* planktonic marker gene and *algA*, one of the key genes in alginate biosynthesis, were inversely regulated in most sputum samples. It was difficult to draw general conclusions for the other marker genes since their expression in response to the different treatments varied strongly between patients. Similarly, in their recent study, Barthe and colleagues could not associate the expression of well-known marker genes (*algD*, *algR*, *antB*, *lasA*, and *pqsA*) with the clinical status of CF patients [43]. On the other hand, Gifford and colleagues [33] showed that there was minimal variation within as well as between sputum samples collected from four CF patients that were chronically colonized with *P. aeruginosa* when analyzing the transcription pattern of 75 genes, despite the fact that the patients received different antibiotics and had varying respiratory symptoms. Although it is generally accepted that *P. aeruginosa* undergoes convergent evolution during adaptation to the CF lung environment [5, 44], the different *P. aeruginosa* strains that colonize CF patients have a different origin, and hence genetic make-up. The difference in only one or a few regulators in the extremely complex regulatory network of *P. aeruginosa* [36] can result in major differences in the response of this opportunistic pathogen to the multiple stimuli it receives in the CF lung. The effect of a treatment on the response of *P. aeruginosa* may therefore greatly depend on the time that the bacterium has had to adapt to the CF environment and the different mutational routes that have led to this adaptation.

Conclusions

In this pilot clinical study, we evaluated the potential use of IPV in altering the behavior of *P. aeruginosa*, driving it from a biofilm lifestyle into a planktonic mode of growth. We found differences between AD, IPV at medium frequency, and IPV at high frequency in enhancing lung function. Combining all data sets (lung function, bacterial counts, and gene expression by *P. aeruginosa*), we can conclude that IPV at a medium frequency does not benefit the tested parameters. In contrast, AD and IPV at a high frequency do improve lung function, and this appears to be more pronounced for IPV at high frequency in patients that are chronically infected by *P. aeruginosa*. Although these results are promising, a larger group of patients should be included in a study that compares the effects of IPV at high frequency on lung function and *P. aeruginosa* biofilm formation to those of AD while avoiding changes in the antibiotic treatment during the study.

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Authors' contributions JD and HE wrote the manuscript, while AC, PM, RVH, PC, and AM contributed to the manuscript. PM performed the statistical analysis. Clinical data were obtained by HE and JW. JD performed all microbiological and molecular biological experiments. AC and AM conceptualized the study. The manuscript was read and approved by all authors.

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Compliance with ethical standards

Ethics approval and consent to participate This clinical study was approved by the Ethics Committee of the UZ Brussel (Approval number B.U.N. B14320095387). An informed consent was read and signed by the four patients that participated in this clinical trial.

Competing interests The authors declare that they have no competing interests.

Abbreviations AD, autogenic drainage; Bpm, bursts per minute; CF, cystic fibrosis; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; IPV, intrapulmonary percussive ventilation; LB, lysogeny broth; LFT, lung function tests; PBS, phosphate-buffered saline; RT, room temperature

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