*Original Research Article*

Allergy & Rhinology
Volume 12: 1–8

DOI: 10.1177/2152656721989288
journals.sagepub.com/home/aar
SAGE

# IFT46 Expression in the Nasal Mucosa of Primary Ciliary Dyskinesia Patients: Preliminary Study

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## Abstract

**Background:** Primary ciliary dyskinesia (PCD) is characterised by an imbalance in mucociliary clearance leading to chronic respiratory infections. Cilia length is considered to be a contributing factor in cilia movement. Recently, *IFT46* protein has been related to cilia length. Therefore, this work aims to study *IFT46* expression in a PCD patients cohort and analyse its relationship with cilia length and function, as it was not previously described.

**Materials and methods:** The expression of one intraflagellar transport (*IFT46*) and two regulating ciliary architecture (*FOXJ1* and *DNAI2*) genes, as well as cilia length of 27 PCD patients, were measured. PCD patients were diagnosed based on clinical data, and cilia function and ultrastructure. Gene expression was estimated by real-time RT-PCR and cilia length by electron microscopy in nasal epithelium biopsies.

Results and conclusions: While *IFT46* expression was only diminished in patients with short cilia, *FOXJ1,* and *DNAI2* expression were reduced in all PCD patient groups compared to controls levels. Among the PCD patients, cilia were short in 44% ($5.9 \pm 0.70\ \mu m$); nine of these (33% from the total) patients' cilia also had an abnormal ultrastructure. Cilia length was normal in 33% of patients ($6.4 \pm 0.39\ \mu m$), and only three patients' biopsies indicated decreased expression of dynein.

## Keywords

cilia length, ciliogenesis, ciliopathies, IFT46, primary ciliary dyskinesia

## Introduction

Mucociliary clearance is one of the most important mechanisms involved in airway epithelium's defence against inhaled pathogens and particulates. This process results from the coordinated action of the respiratory cilia, moving surface fluid, and mucus anteriorly to continuously cleanse the airway surface.[1] Therefore, mucus clearance is affected by three factors: the composition of the airway surface fluid, mucus viscosity and density, and ciliary activity.[2,3]

Cilia are microtubule-based organelles that protrude from most human cells' apical surface, excluding the epithelium lining the gastrointestinal tract, non-ciliated Clara cells, and T lymphocytes.[4,5] They are composed of more than 200 polypeptides and can be classified into two categories: primary and epithelial cilia.[6] Epithelial cilia are hair-like appendages present in the respiratory tract, female oviduct, male testicular efferent tubes, and ependyma (lining of the brain's ventricles). These cilia are motile and possess an intrinsic ciliary beat pattern. The correct

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operation of these complex structures is determined mainly by the cilia movement pattern, cilia beat frequency, and cilia length.[7,8] The length of epithelial cilia must fall within narrow limits to ensure effective mucociliary function.

Intraflagellar transport (IFT) is a conserved process in eukaryotes, which assembles, maintains, disassembles, and transduces cilium-generated signalling. IFT trafficking from the base to the tip of the cilium depends on the microtubules and is associated with two IFT protein complexes: IFT-A and IFT-B; IFT-B is essential for anterograde trafficking, and IFT-A is required for retrograde trafficking.[9]

IFT46 is a core component of the intraflagellar transport machinery and is required for the formation of all cilia. It is one of the highly conserved IFT complex B proteins. It has an essential role in vertebrate cilia development.[10] Knockdown of IFT46 caused shortening of the body axis as well as the formation of fewer and shorter cilia.[11] In *Paramecium*, a GFP-labelled IFT46 protein was found in basal bodies of some cilia, mostly those undergoing biogenesis. Additionally, RNA interference against IFT46 in *Paramecium* triggered severe defects in ciliary growth and architecture, including decreased cilia number and shortened cilia length.[12]

Alteration of cilia's normal structure or activity leads to a series of diseases known as ciliopathies, among which Primary Ciliary Dyskinesia (PCD, OMIM 242,650 ORPHA244) is one of the best known. PCD is a congenital respiratory disease in which the respiratory cilia are immotile, dysmotile, or both.[4,13–15] The PCD phenotype is characterised by impaired mucociliary clearance, which is believed to be responsible for chronic lung, sinus, and middle ear disease.[13,16] Approximately 80-85% of PCD cases are related to cilia's structural defects, while around 15-20% occur in patients with an apparently normal ultrastructure.[13]

It is demonstrated that IFT46 plays a crucial role in cilia development.[10,17] Additionally, the ciliary length is one little-studied factor affecting mucociliary clearance, while short cilia have been observed on the airway epithelium of patients with pulmonary diseases.[18] The aim of this study is to analyse *IFT46* expression in a PCD patients cohort in order to analyse its relationship with cilia length and function, as it was not previously described in this disease. With this purpose, we analysed *IFT46* gene expression by RT-PCR, TEM images to characterise cilia length in PCD patients and correlated these data with cilia function analysis.

## Materials and Methods

### Study Subjects

This study included 27 PCD patients with a clear diagnosis and 30 healthy volunteers. Samples of nasal epithelial cells and clinical data from patients were collected for the study after obtaining informed consent. The study protocol complied with the ethical guidelines of the 1975 Declaration of Helsinki[19] and was approved by the Ethical Comitee of the University General Hospital of Valencia (code 7/2016). Diagnostics were established with the study of ciliary motility using HSMV and ciliary structure by TEM, according to the criteria established by the European Respiratory Society guidelines.[20]

Clinical features are summarised in Table 1, including a deficit of sinus pneumatisation (hypoplasia or aplasia of the frontal sinus; in older than 15), pansinusitis (in patients older than 15), bronchiectasis, secretory otitis media, polyposis, family history of chronic respiratory disease, and respiratory allergies.[15] Male infertility was determined, only in adults, by a spermiogram after obtaining patients' informed consent. By contrast, females were considered infertile after three years of failure in their attempts to become pregnant.[21] Otherwise, they were classified as non-applicable.

### Samples Obtainment

Samples of ciliary airway epithelial cells were obtained from the middle nasal concha using curettage without local anaesthesia while patients were free of acute infection.[22] Three tissue samples were collected. The sample for HSVM analysis was immersed in 1-mL Dulbecco's modified Eagle's medium (DMEM, Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL). The sample for ultra-structural studies was immersed in 0.03 M phosphate buffer containing 2.5% glutaraldehyde, embedded, and sectioned. The samples used for gene expression analysis were preserved in liquid nitrogen.

### Ciliary Motility and Ultrastructure Analyses

Ciliary beat frequency (CBF) and pattern (CBP) were measured within 180 min after biopsy at room temperature by HSVM, as outlined previously.[15] To corroborate the results, each nasal biopsy was plated on tissue culture plates coated with human collagen (type IV, Vitrogen-100; Cohesion Technologies, Palo Alto, CA) and incubated in DMEM at 37 °C in a humidified atmosphere of 5% $CO_2$ in the air for 24 h. The CBF and the CBP were then measured a second time.

Ultrastructure analysis of cilia was performed by TEM, as reported previously.[23] Ciliary ultrastructure was classified according to the categories of dynein and microtubular abnormalities described by Afzelius.[24]

### Analysis IFT46 and Other PCD-Related Genes Expression

Total RNA was isolated from nasal turbinate biopsy specimens using TriPure Isolation Reagent (Roche,

**Table 1.** Clinical Demographics, Ciliary Ultrastructure, Cilia Length and Ciliary Beat Frequency of PCD Diagnosed Patients.

| Patient | KS | Age (years) | CBF (Hz) | Ciliary Ultrastructure | Cilia length (μM) | CBP | SP | PS | B | SOM | P | FHCRD | Fertility | RA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | NO | 40 | <1.0 | Non-Ciliated | NA | Uncoordinated | APSF | YES | YES | NO | NO | YES | NA | NO |
| 2 | NO | 46 | <1.0 | Non-Ciliated | NA | Uncoordinated | HSF | YES | YES | NO | YES | NO | NA | NO |
| 3 | NO | 6 | <1.0 | Non-Ciliated | NA | Vibratile | NA | NA | NO | NO | NO | NO | NA | YES |
| 4 | NO | 8 | 5,5 | Partial dynein deficiency | NA | Normal | NA | NA | YES | YES | YES | NO | NA | NO |
| 5 | YES | 72 | 1 | No dynein | NA | Uncoordinated | HSF | NO | YES | NO | YES | YES | NO | NO |
| 6 | YES | 48 | 5 | No dynein | NA | Uncoordinated | HSF | YES | YES | YES | YES | YES | YES | NO |
| 7 | YES | 15 | 2.5 | Partial dynein deficiency | 3.62 | Uncoordinated | HSF | YES | YES | NO | YES | YES | YES | NO |
| 8 | NO | 15 | <1.0 | Non-Ciliated | 4.31 | Vibratile | NORMAL | YES | YES | NO | NO | YES | NA | NO |
| 9 | YES | 14 | 0 | No dynein | 4.64 | Motionless | NA | NA | YES | YES | NO | YES | NA | NO |
| 10 | NO | 40 | <1.0 | Partial dynein deficiency | 4.88 | Vibratile | HSF | NO | YES | YES | YES | YES | YES | NO |
| 11 | NO | 38 | 9 | Partial dynein deficiency | 4.95 | Normal | ASF | YES | NO | YES | YES | YES | NO | NO |
| 12 | NO | 7 | <1.0 | Normal | 5.01 | Vibratile | NA | NA | YES | YES | YES | YES | YES | YES |
| 13 | YES | 47 | 1 | No dynein | 5.02 | Vibratile | APSF | YES | YES | NO | NO | NO | YES | NO |
| 14 | NO | 7 | <1.0 | Normal | 5.44 | Vibratile | NA | NA | YES | NO | NO | NO | NA | NO |
| 15 | YES | 12 | 7.0 | Normal | 5.68 | Normal | NA | NA | NO | YES | YES | NO | NA | NO |
| 16 | NO | 16 | <1.0 | Partial dynein deficiency | 5.75 | Vibratile | HSF | NO | YES | YES | NO | NO | YES | NO |
| 17 | NO | 3 | <1.0 | Partial dynein deficiency | 5.90 | Vibratile | NA | NA | YES | YES | YES | YES | NO | YES |
| 18 | NO | 8 | <1.0 | Partial dynein deficiency | 5.94 | Vibratile | NA | NA | YES | YES | YES | YES | NA | NO |
| 19 | NO | 41 | <1.0 | Normal | 5.99 | Uncoordinated | APSF | YES | YES | YES | YES | YES | YES | YES |
| 20 | NO | 17 | 5.0 | Normal | 6.11 | Normal | APSF | YES | YES | YES | NO | NO | YES | NO |
| 21 | NO | 13 | 0 | Normal | 6.12 | Motionless | NA | YES | NO | YES | NO | NO | NA | NO |
| 22 | NO | 8 | 0 | Normal | 6.28 | Motionless | HSF | NO | YES | YES | YES | YES | NA | YES |
| 23 | NO | 9 | <1.0 | Normal | 6.34 | Vibratile | NA | NA | YES | YES | YES | NO | NA | NO |
| 24 | YES | 53 | 2.5 | Partial dynein deficiency | 6.45 | Uncoordinated | HSF | YES | YES | YES | NO | NO | YES | YES |
| 25 | NO | 46 | <1.0 | Partial dynein deficiency | 6.51 | Vibratile | NA | NO | YES | YES | NO | NO | YES | NO |
| 26 | NO | 38 | 7.0 | Normal | 6.57 | Normal | NA | YES | YES | YES | YES | YES | NO | NO |
| 27 | YES | 12 | 0 | Partial dynein deficiency | 7.31 | Motionless | NA | NA | NO | YES | NO | YES | NA | NO |

KS: Kartagener's syndrome, CBF: ciliary beat frequency, CBP: ciliary beat pattern, SP: sinus pneumatisation (HSF: Frontal sinus hyperplasia; APSF: Frontal sinus aplasia), PS: pansinusitis, B: bronchiectasis, SOM: secretory otitis media, P: polyposis, FHCRD: family history of chronic respiratory disease, RA: respiratory allergy, NA: non-applicable. Ciliary beat frequency CBF and CBP were measured within 180 min after biopsy at room temperature by HSVM, as outlined previously.[16]

Indianapolis, IN, USA). cDNA was synthesised using 100-ng total RNA and the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was carried out in a 7900 HT thermocycler (Applied Biosystems) using 2× Gene Expression Master Mix (Applied Biosystems). Expression of *IFT46*, *DNAI2,* and *FOXJ1* was measured using gene-specific TaqMan® Gene Expression Assays (Applied Biosystems, CA, USA). Gene expression was normalised to that of *GAPDH* and compared according to the ΔΔCt method, as described previously.[3]

## *Cilia Length Analysis*

Cilia length was evaluated using the biopsy samples subjected to ultrastructural studies. The nasal epithelium was processed as above and sectioned into semi-thin slices, which were stained with toluidine blue. Ciliated areas were imaged at 40× magnification using a Leica DM108 microscope. Images were imported into the Image-Pro Plus 7.0 software (Media Cibernetics Inc., Rockville, MD, USA), which was first calibrated to match the photograph size and then used to measure

the length, in μm, of the cilia following methodologies described previously.[25] Figure 1 shows an example of cilia measure (1A), normal length cilia (1B), normal at the lower limit (1C), and short ones (1D). For each ciliated cell, six distinct cilia were measured, and for each studied case, up to 10 cells were measured (usually three measurable cells per image, with 3–4 images per case).

### *Data Analysis*

Data are presented as means ± SEM of n determinations. Statistical analysis was performed by analysis of variance (ANOVA) followed by Bonferroni's test, using the GraphPad Prism software (GraphPad, San Diego, CA, USA).

## Results

### *Cilia Structure and Function in PCD Patients*

Electron microscopy examination of ciliary sections from nasal biopsies revealed a partial deficiency in dynein in 10 patients, while four patients' cilia lacked dynein. Cilia were structurally normal in 9 patients. The ciliary ultrastructure of 4 patients was not assessed because too few cilia were present for analysis.

Ciliary activity was absent in 4 patients and significantly reduced in the other 23. Ciliary motility was uncoordinated in 7 patients, vibrating in 11 patients, and normal but slow in 5 patients.

### *Expression Analysis of* IFT46 *and Other PCD-Related Genes*

The expression of *IFT46* and other PCD-related genes (*DNAI2* and *FOXJ1)* was measured in PCD patients. Expression levels were compared with those of 30 healthy volunteers. As expected, global *FOXJ1* and *DNAI2* expression were significantly different in PCD patients compared to healthy volunteers (Figure 2(A)). In contrast, *IFT46* expression levels did not differ significantly from controls.

In order to analyse the relationship between *IFT46* and cilia length, patients were grouped according to

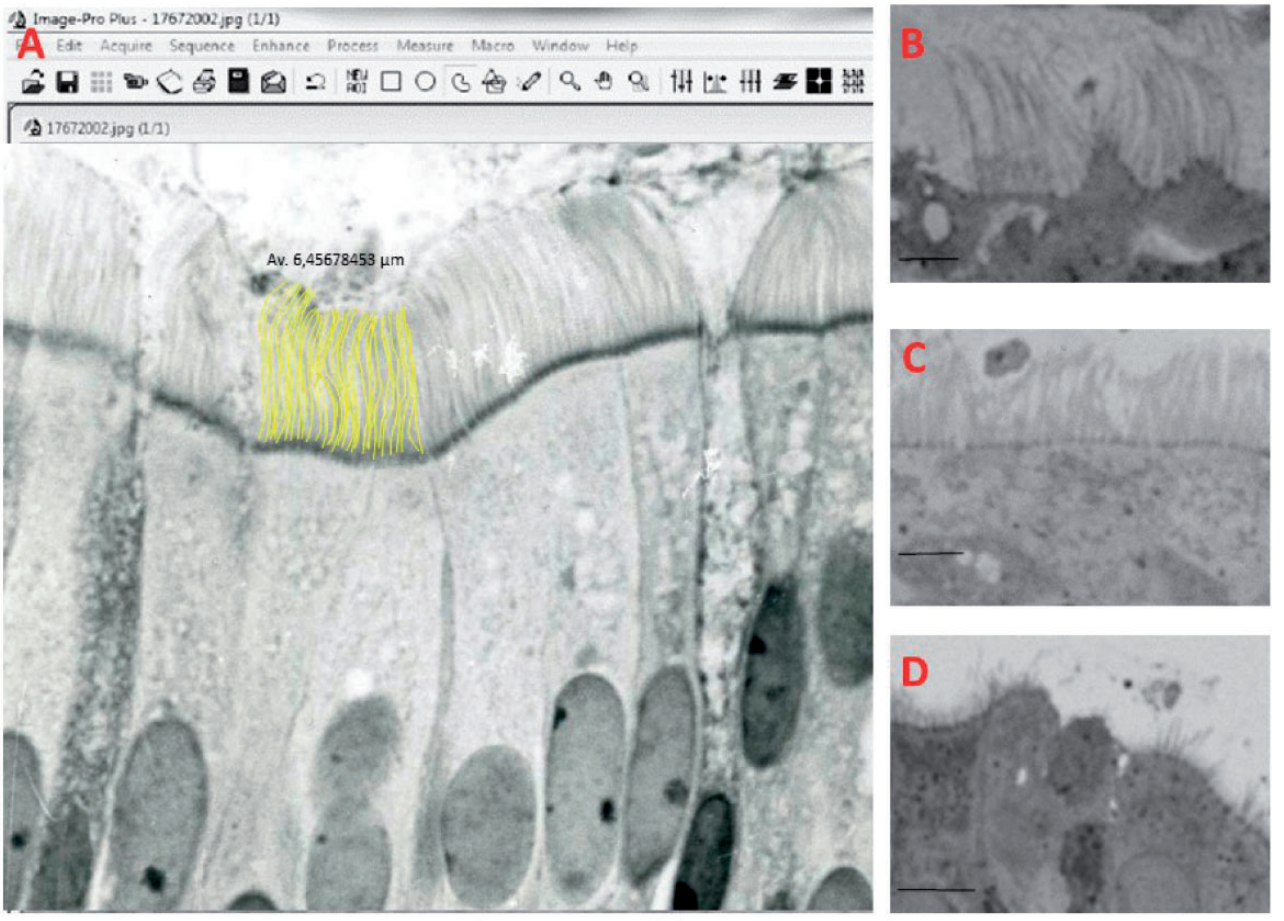


**Figure 1.** Example of cilia measure (1A), normal length cilia (1B), normal at the lower limit (1C), and short ones (1D). Images were taken at 40× magnification with a Leica DM108 microscope. Scale bar equals to 5 μm.

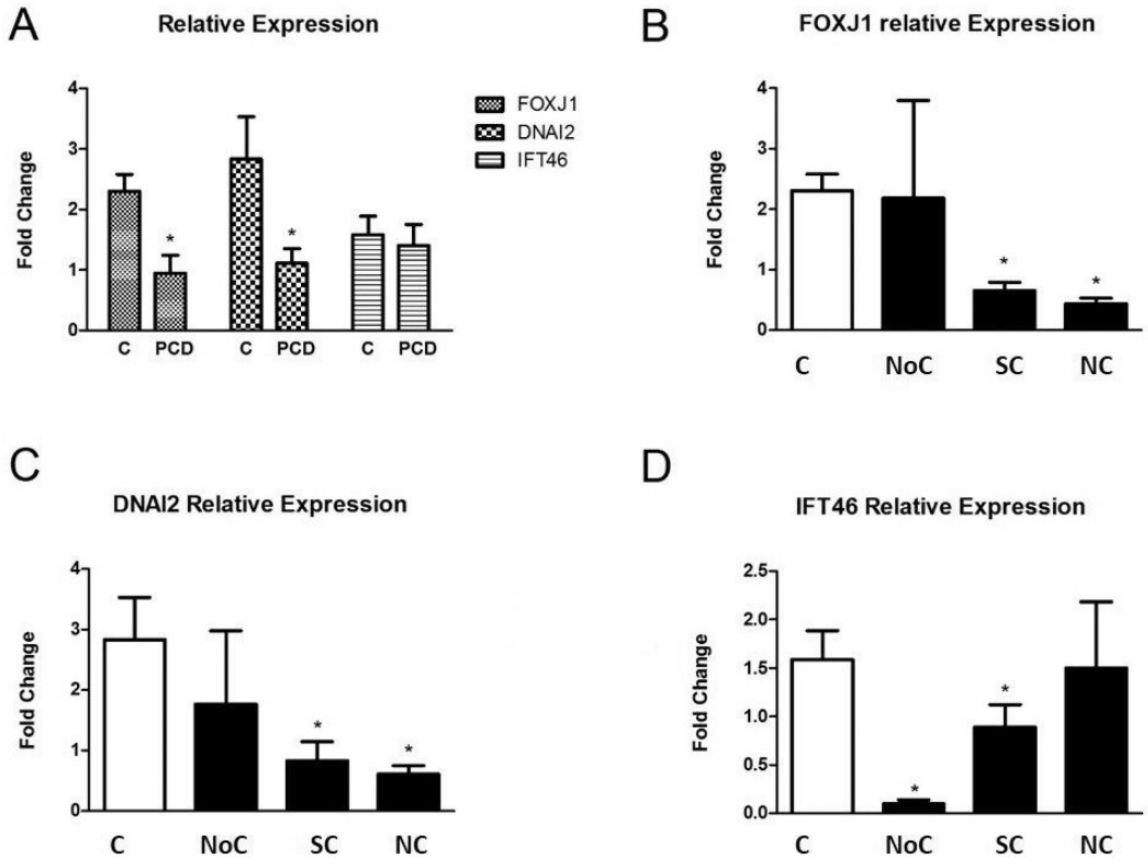


**Figure 2.** IFT46, DNAI2, and FOXJ1 gene expression analysis. Relative expression was calculated using $2^{-\Delta\Delta Ct}$ method. Fold change calculated with respect to control group is represented in Y-axis. Total RNA was extracted from nasal biopsies and analysed by real-time RT-PCR. 30 healthy volunteers (group C) and 27 primary ciliary dyskinesia well characterised (PCD group) patients were included. PCD patients were analysed together (panel A) or divided into three groups: NoC (non-ciliated; n = 3), SC (short cilia; patients showing a cilia length bellow 5.9 μM; n = 12) and NC (normal cilia; patients showing a cilia length above 5.9 μM; n = 9) for FOXJ1 (panel B), DNAI2 (panel C) and IFT46 (panel D) analysis. Results are represented as mean ± SEM. *$p < 0.05$ compared to healthy volunteers.

cilia length, and gene expression between normal and short cilia was compared. Results are summarised in Figure 2. All patients whose nasal epithelium lacked cilia expressed lower levels of all three markers compared to controls (Figure 2(B) to (D)). The relative expression levels of *FOXJ1* and *DNAI2* were similar in patients with both normal and short length cilia and significantly lower than the control group (Figure 2(B) and (C)). In contrast, expression of *IFT46* was significantly decreased only in patients with short cilia, while it was normal in patients with a cilia length >5.9 μm (Figure 2(D)).

## *Cilia Length in PCD Patients*

Of the 27 patients included in this study, cilia length was measurable in only 21. Patients 1-3 had too few cilia to yield reliable data, while the orientation of biopsies taken from patients 4-6 was not appropriate for length analysis. The remaining patients' cilia had lengths of 3.6–7.3 μm. The average cilia length was 5.7 ± 0.87 μm; median 5.9 μm. PCD patients were classified into the following three groups: normal cilia length (>5.9 μm), short cilia length (3.6–5.9 μm), and non-ciliated (patients 1, 2, and 3).

12 patients (44%) had short cilia (mean, 5.1 ± 0.70 μm). Only 3 patients had a normal ultrastructure, while the other 9 had dynein abnormalities (3 patients had no dynein while the other 6 had a partial deficiency of dynein affecting the internal dynein arms). In contrast, the cilia beat pattern was vibrating in 8, uncoordinated in 2, and normal but slow in the other 2. Cilia length was greater than 5.9 μm in 9 patients (33%); the mean in this group was 6.4 ± 0.39 μm. Within this group, the ciliary ultrastructure was normal in 6 patients, while 2 patients' biopsies included deviant cilia and 1 patient's cilia lacked ODA.

## Discussion

PCD is characterised by a malfunction (or absence) of respiratory ciliary activity, preventing proper mucociliary clearance, and thus leading to chronic respiratory infection.[13,15] Three principal factors regulate mucociliary clearance: ciliary activity, the composition of mucus and periciliary fluid, and cilia length.[3,18,23] The ciliary activity has been investigated extensively in PCD patients, and two major populations of PCD patients may be distinguished on this basis; the patients showing ultrastructural defects and those that exhibit normal ciliary ultrastructure but slow (or ineffective) beat activity due to dysfunction of metabolic enzymes involved in ATP or GTP synthesis, mutations in dynein genes such as *DNAH11*, or other unexplored factors.[13,26–28]

The periciliary fluid is composed of $H_2O$, ions, and mucins.[3] Lack of ciliary activity causes mucus accumulation in the upper and lower airways, which leads to chronic infections and consequent mucin hypersecretion. This mucus accumulation increases periciliary liquid's viscosity and further hampers mucus clearance, leading to pulmonary, sinonasal, and otic complications.[13] Another factor that could affect mucus clearance is the volume of periciliary liquid controlled by the activity of purinergic receptors.[2] Whether the activity of these receptors is altered in PCD patients has not been examined. The role of the final factor, cilia length, in PCD is likewise unexplored; however, short cilia have been reported in other pulmonary diseases.[29–31]

In this study, the cilia length of nasal epithelium biopsies from well-characterised PCD patients was measured. The main limitation of our study is the number of included PCD patients (n = 27). However, these are valuable data considering that PCD is a rare disease affecting 1/20,000–60,000 individuals.[13] The median cilia length was 5.7 µm, which is less than the normal length of upper respiratory cilia described by Afzelius et al.[32] According to this value, patients were divided into three subsets: non-ciliated (those without enough cilia to be measured), short cilia (<5.7 µm), and normal cilia (>5.7 µm). The majority of patients with short cilia were totally or partially deficient in dynein, which could be a result of abnormal IFT.[33]

To explore IFT, the expression of three genes whose products are involved in IFT was analysed: *FOXJ1*, which controls the expression of several genes whose products are involved in ciliary structure,[34,35] *DNAI2*, involved in ODA assembly[36] and *IFT46*, which plays an important role in cilia development.[10,37] Real-time RT-PCR analysis revealed that nasoepithelial expression of *FOXJ1* and *DNAI2* was decreased significantly in PCD patients relative to healthy volunteers, but it was statistically similar among the three PCD groups. That expression of these genes was downregulated in all patients suggesting that the loss of their function may be related to the development of PCD but not necessarily to decreased cilia length.

In contrast, *IFT46* expression was similar in PCD patients and control subjects but significantly reduced in the short cilia group. This suggests that loss of *IFT46*-mediated dynein transport across cilia may affect the overall cilia length. Our results are consistent with Shi et al. 2018, who demonstrated that RNA interference against IFT46 in *Paramecium* caused severe defects in ciliary growth and architecture, including a decreased number of cilia and shortened cilia length.[12]

PCD is a multifactorial disease in which many of the mechanisms involved are still unknown. This work, despite being preliminary, provides information regarding a new protein related to the disease pathology. However, more studies are necessary to demonstrate the involvement of IFT46 in PCD.

As previously described, there are a great number of PCD cases without a definitive diagnostic, as they have no clear ultrastructural or genetic defect. Despite the fact that more studies are needed, with these preliminary results, our work suggests that studying ciliary length and *IFT46* gene expression could be a new potential defect present in PCD patients that could help to patients' identification in the future.

### Acknowledgments

Authors are grateful for the professional English language editing of Mr Arash Javadinejad, English Instructor and Publication Editor at the IIS La Fe, Valencia, Spain.

### Author Contributions

Conceptualization: M. A.; Funding acquisition: M. M. and C. C.; Investigation: M. M. and A. R.; Methodology: J. Z., L. M., M. A. and A. R.; Project administration: M. M. and C. C.; Writing—original draft: A. R.; Writing—review & editing: M. M., J. Z., L. M., A. R., M. A., A. R. and C. C.

### Ethical Approval

This study was approved by our institutional review board.

### Statement of Human and Animal Rights

This study is with human samples and Informed consent from patients was obtained.

### Statement of Informed Consent

This study is with human samples and Informed consent from patients was obtained.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by grant PI16-01315 (MM) and PID2019-106099RB-C42 (MM) from the Ministry of Economy and Competitiveness of the Spanish Government and the Instituto de Salud Carlos III. CIBER-BBN and CIBERER are funded by the VI National R&D&I Plan 2008-2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions and the Instituto de Salud Carlos III, with assistance from the European Regional Development Fund.

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