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"Pulmonary Radioaerosol Mucociliary Clearance assessment: searching for
genotype-specific differences and potential as an outcome measure in PCD"

*June K. Marthin¹, *Mathias G. Holgersen¹, #Kim G. Nielsen¹,³, #Jann Mortensen ²,³

1: Danish PCD Centre, Danish Pediatric Pulmonary Service, Department of Pediatrics and Adolescent Medicine, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark.

2: Department of Clinical Physiology and Nuclear Medicine, Copenhagen University Hospital, Rigshospitalet, Denmark

3: Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

*: shared first authorship
#: shared last authorship

June K Marthin, Mathias G Holgersen, Kim G Nielsen and Jann Mortensen all meet the four ICMJE criteria for authorship. This manuscript has been seen and approved by all listed authors.

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Corresponding author:

June K Marthin, MD, PhD.
Email: June.kehlet.marthin.01@regionh.dk
Danish PCD Centre, Paediatric Pulmonary Service, ERN Accredited, Department of Paediatric and Adolescent Medicine, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark
ABSTRACT

Background: The Pulmonary Radioaerosol Mucociliary Clearance (PRMC) is a reliable method for assessing in vivo whole lung mucociliary clearance (MC) and used at the Danish PCD Centre as a supplementary diagnostic test for primary ciliary dyskinesia (PCD) for more than two decades. This study aimed to investigate genotype-specific differences in PRMC measures and evaluate its potential as outcome parameter.

Material and Methods: The study was based on a retrospective analysis on PRMC tests performed over a 24-year period (1999-2022) in individuals referred for PCD work-up and included patients with genetically confirmed PCD and non-PCD controls. Nebulized 99mTc-albumin-colloid was inhaled, and static and dynamic imaging following obtained. Three parameters were evaluated: 1-hour lung retention (LR1), tracheobronchial velocity (TBV), and cough clearance.

Results: The study included 69 patients from the Danish PCD cohort, representing 26 different PCD genotypes. MC by PRMC was consistently absent in most PCD patients, regardless of genotype. However, a single patient with a CCDC103 mutation preserved ciliary function, and normal nasal Nitric Oxide (NO) exhibited normal LR1 and low TBV values. Voluntary cough significantly improved clearance, with a median improvement of 11% (IQR: 4-24%).

Conclusion: Absent MC by PRMC should be expected in PCD regardless of genotype but residual ciliary function could result in measurable PRMC. This indicates a potential for PRMC to detect improvement of ciliary function if this can be restored. Addressing involuntary cough and peripheral deposition of radioaerosol is important if PRMC is to be used as outcome measure in future clinical PCD trials.
INTRODUCTION

Primary ciliary dyskinesia (PCD; MIM244400) is a multisystem ciliopathy that includes dyskinetic respiratory cilia. The resulting impairment of mucociliary clearance (MC) leads to chronic destructive infectious and inflammatory disease of the upper and lower airways. To date, approximately 50 genes have been identified to harbor variants attributed to cause PCD (1-3). Most mutations associated with PCD are autosomal recessive. Less frequently, X- chromosomal recessive and de novo autosomal dominant inheritance has been described (4).

Pulmonary Radioaerosol Mucociliary Clearance (PRMC) has been used as a supplementary diagnostic tool in the investigation of PCD in the national Danish PCD center for over two decades. It effectively identifies abnormal clearance patterns in patients with PCD (pwPCD) due to impaired MC caused by abnormal ciliary motility. The test is non-invasive technique and involves minimal radiation exposure (<1 mSv). Whole-lung retention (LR) of the radioaerosol is visualized by use of a gamma-camera. Previous studies have shown PRMC’s high sensitivity in predicting PCD and its ability to rule out PCD in non-PCD cases, even in children as young as 5 years old (5-9).

Growing interest to develop ciliary protein correctors for treatment of PCD has highlighted the need to establish relevant outcome parameters for future clinical trials. Currently, PRMC is the only available in vivo indicator of ciliary function and MC of the lungs (10). PRMC has already been used as an outcome parameter in randomized controlled trials (RCTs) for cystic fibrosis (11-12) and is obvious to consider for future clinical trials in PCD.

Long term lung function decline in pwPCD is heterogeneous (13-14) and growing evidence indicates that specific PCD gene defects may be related to different lung disease outcomes (14-16). With patient specific therapies targeting specific PCD genes on the horizon, exploring genotype-phenotype relationships within subgroups of pwPCD is more important than ever. With this study we evaluated PCD PRMC phenotypes in relation to a wide range of PCD genotypes.
HYPOTHESIS AND AIDS
We hypothesized that different PCD genotypes could result in different PRMC measures. Our primary aim was to assess these differences in patients from the Danish PCD Cohort. Three parameters were evaluated: 1-hour lung retention (LR1), tracheobronchial velocity (TBV), and cough clearance.
By introducing data on TBV as a new, not previously investigated PRMC parameter, we aimed to add this to the PRMC armamentarium of possible outcome measures. Additionally, we also studied the impact of voluntary and involuntary cough on PRMC measures, as coughing is both unavoidable and encouraged as a treatment maneuver in PCD.
As a secondary aim, we evaluated PRMC’s usefulness for generating outcome parameters in future PCD clinical trials.

MATERIAL AND METHODS
Study design
This retrospective, cross-sectional single-center study utilized data from the Danish PCD Registry, including re-analyzed diagnostic PRMC tests performed from 1999 to 2022. Additional exploratory diagnostic test results, including High Speed Video Microscopy (HSVM) assessment of nasal ciliary motility and Transmission Electron Microscopy (TEM) assessment of nasal ciliary ultrastructure, as well as PCD genetics and nasal Nitric Oxide (nNO), were incorporated from outpatient visits during this time.

Inclusion criteria:
Eligibility criteria for patients with PCD above five years of age included:
Confirmation of bi-allelic mutations in a known PCD-causing gene.
Previous PRMC test and a complete PRMC record for cough, conducted as part of PCD evaluation, with reliable determination of at least one of the defined PRMC outcome parameters: Retention in the lung of the inhaled radioaerosol after 1 hour (LR1), Tracheobronchial velocity (TBV), or voluntary cough clearance assessment.
Presence of clinical symptoms consistent with PCD and positive diagnostic tests (nNO, HSVM, and TEM) that align with the current ERS criteria for PCD diagnosis (17).

Exclusion criteria:
Cough registered within the first 1 hour after radioaerosol inhalation and
peripheral aerosol deposition that resulted in lack of visible central radioaerosol in trachea and main bronchi within the first 1 hour.

The control group consisted of individuals who underwent PRMC testing in 2022 as part of a full PCD evaluation including HSVM, TEM and nNO in addition to PRMC, with unambiguous negative results and where PCD ultimately had been ruled out. These patients needed to have conclusive PRMC results, including normal LR1, TBV and 24-hour LR.

**PRMC method**

PRMC tests were performed using identical technique and procedure throughout the 24 years of data acquisition. The PRMC imaging technique has previously been described (5,7,10). See also supplementary material.

In the present study data were re-analyzed with a view to assess the following three defined parameters: PRMC LR1, PRMC Tracheobronchial velocity (TBV), and PRMC voluntary controlled and involuntary random cough clearance. Whereas PRMC LR1 and TBV measurements were confined to only 60 minutes, cough clearance investigation was prolonged to 120 minutes. Cough was strictly monitored within these two hours.

From doorstep to doorstep a test lasted two hours: this included welcoming the patient, placing reference (57Co) markers on neck and back of the patient and taking the “zero”-acquisition with the markers, training the patient to do correct slow inhalation, and forced exhalation technique with isotonic saline, inhale the 99mTc- albumin colloid radioaerosol, measure static and dynamic PRMC acquisitions, monitor for cough, removing the reference markers and sending the patient home.

Examples of tracer movement from static acquisitions at 0, 30, and 60 minutes after radioaerosol inhalation in a pwPCD and a non-PCD referral is displayed in supplementary material.
Regional ventilation distribution was visualized by a $^{81m}$Kr-gas scintigram. The initial $^{99m}$Tc aerosol distribution was compared to the $^{81m}$Kr ventilation distribution to indicate how far the radioaerosol had penetrated the airways and lungs and hence had to move before being cleared from the airways. This penetration index (PI) was used to calculate each subject’s predicted values of LR from previously published reference equations (10), see appendix 1.

**Determination of PRMC TBV**

To determine PRMC TBV we re-read the existing digital films from the dynamic acquisitions performed during a PRMC test. Read directly from the screen, we identified distinctive radiomucous boli formed in the tracheobronchial area that further ascended within the main bronchi and the trachea, as displayed in supplementary movie 1a from a pwPCD and in supplementary file movie 1b from a non-PCD referral, which allowed for determination of PRMC TBV, if present. Two dynamic acquisitions of 20 minutes duration obtained within the first hour of the PRMC investigation were reviewed and analyzed for all included patients. Two readers re-analyzed each acquisition in a blinded fashion to the original diagnostic report. The transport distance between each frame was measured for any upward boli transport in the main bronchi and trachea.

As 1 frame equals 2 minutes (10 frames in a 20-minute acquisition), the PRMC TBV was calculated in mm/minutes using the following equation:

$$\text{PRMC TBV} = \frac{\text{mm of bolus transportation}}{(Y \text{ frames} \times 2 \text{ minutes})}.$$  

**PRMC voluntary controlled and involuntary random cough clearance**

Voluntary controlled cough clearance was performed in a subset of patients as repeatable cough maneuvers at the end of the PRMC measurements.

To avoid false negative results caused by cough clearance, any cases of involuntary random cough during the first hour of a PRMC test were discarded.

We compared these previously discarded tests for LR values at 30, 60 and 120 minutes with PRMC measurements in patients who did not cough during the PRMC investigation. This allowed us to analyze the impact of involuntary cough on PRMC.

**Additional diagnostic testing**

**PCD Genetics**
A Next Generation Sequencing (NGS) gene panel was used for analysis of the PCD genes mentioned below. Gene names are given according to the latest updated gene nomenclature (HGNC (HUGO (human genome organization) gene nomenclature committee; https://www.genenames.org/). For the convenience of the reader previous gene names are given in parenthesis:

ODAD1 (CCDC114), ODAD2 (ARMC4), ODAD3 (CCDC151), DNAH5, DNAH9, DNAI1, DNAI2, DNAL1, CFAP298 (C21orf59), CCDC103, CCDC65, CCDC39, CCDC40, CCNO, DNAH11, DNAAF1 (LRRC50), DNAAF2 (KTU), DNAAF3 (C19orf151), DNAAF4 (DYX1C1), DNAAF6 (PIH1D3), DRC1 (CCDC164), GAS8, HYDIN, DNAAF11 (LRRC6), MCIDAS, OFD1, RSPh1, RSPh3, RSPh4A, RSPh9, SPAG1, ZMYND10, and FOXJ1.

**Nasal NO measurement**

Triple nNO measurements (ppb) was performed using the CLD88sp stationary NO chemiluminescence analyzer (ECO MEDICS AG, Duernten, Switzerland) according to recommended standards (18) and supplementary material.

**HSVM and TEM**

Ciliary beat pattern and frequency was determined from HSVM analysis of nasal brush biopsy material, and nasal ciliary ultrastructure by TEM as per ERS criteria for PCD diagnosis (17). TEM material was obtained from a nasal scraping biopsy. There are no current consensus guidelines for performance of HSVM for PCD diagnostics, however in the lack of such we have decided in our laboratory to adhere to standards given by Kempeneers and colleagues (19). Normal ciliary beat frequency reference range was 8-11 Hz in our laboratory. TEM analyses were performed as standard diagnostic testing in all participants and according to ERS guidelines (20).

**Statistical analysis**

Non-parametric statistics were used to analyse the data due to the small sample size. Fisher’s exact test was used to compare count data in tables, while the Kruskal-Wallis test was used to compare three or more independent groups. Wilcoxon rank-sum test was used to compare means of individual and independent groups.

All statistical analyses were performed using R version 4.2.0. P-values <0.05 was considered statistically significant.
**Ethics**
Data collection was based on existing PCD registry data, of which written informed consent from all patients was already obtained. Hence, supplemental ethical approval was not necessary for this study.

**RESULTS**
Out of the total of 82 patients in our Danish PCD cohort who had undergone a previous PRMC test, we included 69 with verified biallelic PCD mutations. Patient characteristics are given in Table 1 and flow-diagram for inclusion and exclusion given in Figure 1. Among these 69 patients, we were able to include 49/69 (71%) for PRMC LR1 assessments and 34/69 (49%) for dynamic PRMC TBV assessments. Only 17 patients had a record for controlled cough assessment and could be included for voluntary cough clearance investigation. Six non-PCD controls provided PRMC TBV values for comparison (Table 1, Figure 3). In our previous publication on PRMC in PCD from 2007 (5) LR1 data were also included. There was an overlap of 26/49 (53%) patients with reported LR1 data in our previous study (5) and the LR1 data reported in the current cohort. Hence nearly half of the LR1 data reported now are new. TBV data and cough clearance data are all new as these data have not been previously published.

**PRMC measures and PCD genotype**
We compared the five most common genotypes in our PCD cohort (CCDC39/CCDC40, DNAH11, DNAH5, DNAI1, and HYDIN) and found no significant differences in either the LR1 values or retention z-scores (as shown in Table 2). Subgroup analyzes of PRMC values across TEM and HSVM groups also revealed no significant differences. The complete list of in- and excluded patients according to genotypes, HSVM ciliary motility patterns and TEM ultrastructure is provided in supplementary material.

Of the 34 patients included for dynamic PRMC assessment, all but one patient (33/34) had abnormal LR1 (91-100% retention) and no measurable velocity i.e., 0 mm/min, whereas
only one patient exhibited normal LR1 (73% retention) and a measurable TBV of 1.5 mm/min. TBV was significantly lower compared to the non-PCD referrals in the control group with a median (range) TBV of 4.0 (2.0 to 6.0) mm/min, P<0.00001 (Figure 2 and 3). The patient had a homozygous p.His154Pro CCDC103 defect, normal nNO levels of 968 ppb (319 nL/min), residual (subtle abnormal) ciliary beating with a low-to-normal CBF of 3.5-8.9 Hz on HSVM, and a moderate ODA deficiency (TEM cross sections counted was N=119, deficient ODAs were found in 56/119 (47%), no IDA or MT deficiency was detected).

**PRMC Voluntary cough clearance**

Voluntary controlled cough clearance from the central parts of the lungs was found to be median (IQR) 11 (4;24) % in 17 pwPCD. We found a statistically significant moderate negative linear correlation between the voluntary cough clearance and the PI, indicating that peripherally deposited aerosol (high PI value) is less easily coughed up from the main bronchi and the trachea than a centrally deposited aerosol (low PI value), Figure 4.

**Impact on involuntary cough clearance and peripheral aerosol deposition on PRMC results**

The majority of excluded LR1 data (19/20) were caused by cough within the first 1 hour (Figures 1 and 5). Overall, involuntary cough resulted in significant difference in LR at all timepoints, indicating false positive MC that accumulated over time from 30 minutes to 120 minutes after inhalation of the radioaerosol as shown below (and in Figure 6):

LR 30-minute, no cough (N=44): 99.2% (98.1-100) versus cough (N=6): 97.6% (96.2-98.3), P = 0.02544,

LR 60-minute, no cough (N=49): 99.9% (98.6-100) versus cough (N=19): 96.8% (93.5-98.8), P < 0.0001, and

LR 120-minute, no cough (N=34): 98.2% (96-99.6) versus cough (N=34): 91.4% (83.2-96.5), P = 0.0006.

The differences in LR between groups (no cough vs. cough) could not be explained by differences in PI's, as they were similar between groups at all time points (at 30 min: P=0.7653, at 60 min: P=0.9778 and at 120 min: P=0.8952).
The excluded TBV measurements (N=35) were caused by peripheral deposition in N=2/35 cases due to no visible tracer in the central airways at the time for the dynamic TBV assessments and by involuntary cough in N=1/35 cases. In 32/35 cases, where PRMC measurements had been performed in the first period of the 24 year of data collection, no digital films were available to re-read, as only the paper files existed.
DISCUSSION

Overall, our study showed that PRMC was extremely low or unmeasurable in all parameters regardless of PCD genotype, TEM ultrastructure and ciliary motility patterns. These findings are consistent with recently published data on nasal MC, which similarly demonstrated complete absence of clearance across various PCD genotypes (21). This is interesting, since previous studies have shown that certain PCD genes can be associated with either more severe or less pronounced clinical symptoms. Patients with CCDC39/40 defects, which is associated with ciliary ultrastructural microtubular disorganisation combined with IDA deficiency, tend to have poorer lung function outcomes and more severe HRCT abnormalities (14-15,22). Conversely, patients with DNAH11 defects, which are associated with normal ciliary ultrastructure (23), may have milder respiratory symptoms (14), higher FEV1 z-score, and fewer neonatal respiratory symptoms (24). Even though TEM is normal in DNAH11, the ciliary motility pattern is markedly compromised in these patients, as HSVM is characterized by stiff hyperkinetic and minimal movement (4,16).

In this study, we did not find indications that PRMC differed between groups of genotypes where PCD expectedly would be related to either more severe or more mild disease. In line with this, previous investigations by Vali and colleagues (9) have demonstrated that PRMC results are not associated with lung function.

One case was an exception. This patient was the only one to show normal LR1 values and measurable, although low, TBV and had a homozygous p.His154Pro CCDC103 defect, subtle ciliary motility pattern abnormalities, a normal nNO and only a moderate ODA deficiency. This particular defect has previously been associated with reduced-to-normal ciliary beating as well as normal range nNO (25). Even though it was only a single case and interpretation of the result obviously should be taken with caution, this finding illustrates that the PRMC method may have the ability to reflect even smaller variations in ciliary beating within PCD, which potentially could extrapolate to detectable improvement in PRMC as an outcome if ciliary function could be restored in pwPCD.

**PRMC cough clearance**
This study provides novel insights into the impact of cough on pulmonary clearance in individuals with pwPCD.
Involuntary cough, a challenge to suppress in pwPCD, had a notable impact on PRMC as it resulted in significant MC at all measured time points. Similarly, voluntary cough resulted in increased PRMC with an improved (reduction) on LR1 of median 11%.

With that, this study underscores that cough clearance plays a crucial role in PRMC measurements, as coughing during the test can significantly increase PRMC values and lead to false near-normal results. Additionally, our findings suggest that regular controlled coughing may significantly optimize airway clearance in pwPCD.

Strengths and limitations
Our study offers new insights into the potential use of the PRMC test as a source of outcome parameters for clinical trials. It is known that PRMC test results can significantly differentiate between individuals with pwPCD and healthy individuals (5, 7-9), while still retaining its diagnostic capability even with a shortened 60-minute test (9). However, the relationship between PRMC test results and specific PCD genotypes has not been previously described. In our study, we demonstrated the absence of PRMC across various genotypes in PCD. Although the evidence is limited, as it is based on a single patient, our findings suggest that residual ciliary function in PCD could lead to measurable PRMC, providing support for the use of PRMC testing as a valuable outcome parameter for detecting improvements in medically enhanced ciliary function.

Furthermore, the PRMC test can be conducted with relative speed and efficiency, as a complete measurement can be completed within a two-hour timeframe. This convenience enables the inclusion of patients from a wide hospital service area. Moreover, PRMC testing holds promise for studies involving younger children, as even children as young as five years old can actively participate and cooperate in PRMC measurements (5).

Data were collected retrospectively from our local PCD registry spanning 24 years. Despite the lengthy period of data collection our study was limited by small groups. During the 24 years, many cases of PCD were diagnosed using HSVM, TEM, nNO measurements, along with more recent genetic work-up, without the inclusion of a supplementary PRMC test which limited the overall number of included patients. In our pursuit of identifying PRMC variations among different PCD genotypes, we implemented rigorous data cleaning, and in this process, we excluded numerous recordings to maintain analysis integrity and mitigate the impact of cough. However, given the
challenge of suppressing cough in these patients, we acknowledge that our data cleaning approach may have been overly stringent which also explains low success rates for including LR1 data (71%) and the TBV data (41%).

Concerning the use of PRMC in clinical trials there are several challenges to consider, including radiation exposure, age restrictions, involuntary cough, and the potential impact on peripheral deposition. The inhaled amount of radioaerosol is the same for all individuals while the retention and thus radiation exposure differs. The half time of 99mTc is 6 hours, rendering radioactivity after 24 hours less than 7% due to physical decay. Due to the additional biological clearance, which is slower in patients with abnormal MC, residual activity is even lower than 7% both in healthy and diseased people. All in all, the effective exposure is 0.2-0.5 mSv per PRMC test - slightly higher in diseased patients than in healthy subjects – and will therefore always be lower than 1 mSv as stated, irrespective of a normal or abnormal PRMC where there still may be visible retention after 24 hours.

Although the radiation exposure associated with a PRMC measurement is low, it is important to consider, especially if multiple measurements are planned, e.g., before, during, and at the end of a clinical trial. Yet, even with three consecutive studies performed in a patient, the radiation exposure is lower than the yearly background radiation in Denmark of 4 mSv.

The successful execution of PRMC testing relies on the close cooperation of patients to perform controlled radioaerosol inhalation and to lie still for a minimum of 20 minutes during the scintigraphy phase of the test. These requirements make PRMC unsuitable for children under five years (5). Furthermore, age has been found to influence PRMC LR in healthy individuals, with children exhibiting faster MC. Currently, there are no established normal reference values for PRMC LR or TBV specific to different age groups (7). However, in a clinical trial utilizing PRMC for outcome parameters, such as baseline measurements at inclusion, during a trial, and at the end of trial, the patients would serve as their own controls. This approach would minimize the impact of age-related differences on the interpretation of the results.

In this study we demonstrated a significant impact of cough and on peripheral radioaerosol deposition on PRMC outcomes. The latter in alignment with previous published data on PI
impact on LR values (10). Uncontrolled cough should be avoided to prevent possible false positive PRMC outcomes. Minimizing peripheral deposition should be attempted to avoid false negative PRMC measures as peripherally deposited aerosol is less easily cleared than centrally deposited aerosol and because too peripheral radioaerosol deposition makes TBV evaluation difficult, as the tracer never reaches the central airways within time for assessment.

If repeated PRMC measurements are planned during a clinical trial, it will be crucial to target consistent PI values in the patients throughout the trial period. The deposition of the inhaled radioaerosol is predominantly influenced by aerosol particle size, inhalation pattern, and the extent of airway obstruction. While particle size remains constant and inhalation pattern can be controlled, the final aerosol deposition and resulting PI are primarily determined by the degree of airway obstruction. Since pwPCD often exhibit heterogeneous and variable peripheral obstruction, this poses an important limitation that requires attention.

To address these limitations, it is worth considering strategies aimed at reducing bronchial obstruction and tendency to cough where PRMC would be used as outcome parameter.

**CONCLUSION**

In conclusion, most pwPCD demonstrated a complete absence of PRMC, and no significant differences in PRMC were observed among different PCD genotypes. However, our study could suggest that PRMC parameters have the potential to improve with partial restoration of ciliary function.

Controlled coughing was found to enhance MC and could be beneficial for airway clearance in pwPCD. The PRMC test, completed within a convenient two-hour timeframe, allows for the inclusion of patients from a wide range of hospital service areas.

Overall, our results suggest that, after careful adjustment for pitfalls, there might be a potential for PRMC measurements to provide valuable outcome parameters for future clinical trials aimed at restoring ciliary motility and increasing mucociliary clearance. The main methodological challenges to address would be involuntary coughing and peripheral radioaerosol deposition.
ACKNOWLEDGEMENTS

Many thanks to the staff at the Department of Clinical Physiology and Nuclear Medicine at Rigshospitalet for their skillful help in performing the pulmonary radioaerosol mucociliary studies throughout the many years.
Table 1. Characteristics and numbers of non-PCD controls and patients with PCD included in the overall study and each sub-study based on outcome parameter.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non-PCD controls</th>
<th>Overall study pwPCD</th>
<th>LR1 1h lung Retention pwPCD</th>
<th>TBV pwPCD</th>
<th>Voluntary controlled cough clearance pwPCD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=6</td>
<td>N = 69</td>
<td>N = 49</td>
<td>N = 34</td>
<td>N = 17</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>23 (18;27)</td>
<td>15 (11;28)</td>
<td>14 (11;20)</td>
<td>13 (9;19)</td>
<td>10 (9;20)</td>
</tr>
<tr>
<td>Male sex</td>
<td>2 (33%)</td>
<td>31 (45%)</td>
<td>24 (49%)</td>
<td>12 (35%)</td>
<td>4 (24%)</td>
</tr>
<tr>
<td>Age at diagnosis (yrs)</td>
<td>IRR</td>
<td>10 (5;18)</td>
<td>9 (4;17)</td>
<td>8 (5;17)</td>
<td>7 (5;13)</td>
</tr>
<tr>
<td>Situs inversus</td>
<td>0</td>
<td>25 (36%)</td>
<td>15 (31%)</td>
<td>12 (34%)</td>
<td>8 (47%)</td>
</tr>
<tr>
<td>nNO (ppb)</td>
<td>475 (453;497)</td>
<td>44 (26;80)</td>
<td>51 (28;86)</td>
<td>38 (26;82)</td>
<td>34 (26;54)</td>
</tr>
</tbody>
</table>

Data reported as: medians (IQR) and numbers (%)

pwPCD: patients with PCD
TBV: tracheo bronchial velocity
nNO: nasal Nitric Oxide
IRR: irrelevant
### Table 2A: Genotypes

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CCDC39/CCDC40 (N = 7)</th>
<th>DNAH11 (N = 6)</th>
<th>DNAH5 (N = 4)</th>
<th>DNAH1 (N = 7)</th>
<th>HYDIN (N = 4)</th>
<th>Other (N = 21)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11 (10, 23)</td>
<td>10 (8, 17)</td>
<td>15 (13, 19)</td>
<td>19 (15, 31)</td>
<td>16 (12, 19)</td>
<td>14 (11, 20)</td>
<td>0.5</td>
</tr>
<tr>
<td>Male sex</td>
<td>5 (71%)</td>
<td>3 (50%)</td>
<td>0 (0%)</td>
<td>5 (71%)</td>
<td>2 (50%)</td>
<td>9 (43%)</td>
<td>0.2</td>
</tr>
<tr>
<td>Penetration index</td>
<td>0.52 (0.40, 0.65)</td>
<td>0.56 (0.45, 0.67)</td>
<td>0.63 (0.58, 0.70)</td>
<td>0.55 (0.42, 0.73)</td>
<td>0.42 (0.36, 0.50)</td>
<td>0.42 (0.34, 0.57)</td>
<td>0.4</td>
</tr>
<tr>
<td>1-hour lung retention</td>
<td>0.995 (0.992, 1.000)</td>
<td>0.996 (0.979, 1.000)</td>
<td>1.000 (0.996, 1.000)</td>
<td>1.000 (1.000, 1.000)</td>
<td>0.995 (0.979, 1.000)</td>
<td>0.990 (0.970, 1.000)</td>
<td>0.2</td>
</tr>
<tr>
<td>Predicted 1-hour lung retention</td>
<td>0.84 (0.80, 0.86)</td>
<td>0.84 (0.81, 0.87)</td>
<td>0.85 (0.83, 0.86)</td>
<td>0.81 (0.80, 0.89)</td>
<td>0.80 (0.79, 0.81)</td>
<td>0.80 (0.78, 0.84)</td>
<td>0.5</td>
</tr>
<tr>
<td>Z-score</td>
<td>1.69 (1.46, 2.23)</td>
<td>1.66 (1.36, 2.01)</td>
<td>1.62 (1.53, 1.73)</td>
<td>2.11 (1.20, 2.26)</td>
<td>2.13 (1.84, 2.28)</td>
<td>1.93 (1.35, 2.23)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

### Table 2B: Ultrastructure

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>BB mislocated (N = 2)</th>
<th>CC defect (N = 6)</th>
<th>No abnormalities detected (N = 12)</th>
<th>ODA defect (N = 16)</th>
<th>ODA+IDA defect (N = 5)</th>
<th>Tubular disorganization + IDA defect (N = 8)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11 (10, 13)</td>
<td>13 (11, 20)</td>
<td>13 (10, 19)</td>
<td>18 (15, 29)</td>
<td>11 (9, 13)</td>
<td>14 (10, 33)</td>
<td>0.12</td>
</tr>
<tr>
<td>Male sex</td>
<td>1 (50%)</td>
<td>3 (50%)</td>
<td>7 (58%)</td>
<td>6 (38%)</td>
<td>2 (40%)</td>
<td>5 (62%)</td>
<td>0.9</td>
</tr>
<tr>
<td>Penetration index</td>
<td>0.27 (0.19, 0.34)</td>
<td>0.51 (0.45, 0.64)</td>
<td>0.52 (0.43, 0.68)</td>
<td>0.52 (0.39, 0.69)</td>
<td>0.35 (0.34, 0.41)</td>
<td>0.48 (0.42, 0.62)</td>
<td>0.4</td>
</tr>
<tr>
<td>1-hour lung retention</td>
<td>0.994 (0.927, 0.960)</td>
<td>0.996 (0.974, 1.000)</td>
<td>0.996 (0.974, 1.000)</td>
<td>0.989 (0.970, 0.999)</td>
<td>0.994 (0.988, 1.000)</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Predicted 1-hour lung retention</td>
<td>0.76 (0.74, 0.78)</td>
<td>0.83 (0.81, 0.85)</td>
<td>0.82 (0.81, 0.87)</td>
<td>0.81 (0.79, 0.87)</td>
<td>0.79 (0.78, 0.81)</td>
<td>0.83 (0.78, 0.86)</td>
<td>0.6</td>
</tr>
<tr>
<td>Z-score</td>
<td>2.01 (1.95, 2.07)</td>
<td>1.39 (1.51, 2.16)</td>
<td>1.66 (1.28, 2.08)</td>
<td>2.01 (1.28, 2.23)</td>
<td>2.11 (1.91, 2.22)</td>
<td>1.82 (1.49, 2.13)</td>
<td>&gt;0.9</td>
</tr>
</tbody>
</table>

### Table 2C: HSVM motility pattern

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hyperkinetic/reduced proximal bending (N = 6)</th>
<th>Non-motile (N = 6)</th>
<th>Non-motile/residual flickering (N = 14)</th>
<th>Reduced number of cilia (N = 2)</th>
<th>Severely reduced amplitude/rigid axonemes (N = 8)</th>
<th>Slow rotational (N = 5)</th>
<th>Subtle abnormalities (N = 8)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>10 (8, 17)</td>
<td>12 (9, 16)</td>
<td>18 (15, 28)</td>
<td>11 (10, 13)</td>
<td>14 (10, 33)</td>
<td>12 (10, 14)</td>
<td>15 (13, 19)</td>
<td>0.2</td>
</tr>
<tr>
<td>Male sex</td>
<td>3 (50%)</td>
<td>2 (33%)</td>
<td>6 (43%)</td>
<td>1 (50%)</td>
<td>5 (62%)</td>
<td>2 (40%)</td>
<td>5 (62%)</td>
<td>&gt;0.9</td>
</tr>
<tr>
<td>Penetration index</td>
<td>0.56 (0.45, 0.67)</td>
<td>0.35 (0.34, 0.41)</td>
<td>0.53 (0.41, 0.71)</td>
<td>0.27 (0.19, 0.34)</td>
<td>0.48 (0.42, 0.62)</td>
<td>0.50 (0.43, 0.68)</td>
<td>0.51 (0.36, 0.59)</td>
<td>0.5</td>
</tr>
<tr>
<td>1-hour retention</td>
<td>0.996 (0.979, 1.000)</td>
<td>0.990 (0.975, 0.997)</td>
<td>1.000 (0.999, 1.000)</td>
<td>0.944 (0.927, 0.960)</td>
<td>0.994 (0.988, 1.000)</td>
<td>0.995 (0.987, 1.000)</td>
<td>0.995 (0.965, 1.000)</td>
<td>0.088</td>
</tr>
<tr>
<td>Predicted retention</td>
<td>0.84 (0.81, 0.87)</td>
<td>0.79 (0.78, 0.81)</td>
<td>0.81 (0.80, 0.87)</td>
<td>0.76 (0.74, 0.78)</td>
<td>0.83 (0.78, 0.86)</td>
<td>0.83 (0.80, 0.86)</td>
<td>0.82 (0.79, 0.84)</td>
<td>0.7</td>
</tr>
<tr>
<td>Z-score</td>
<td>1.66 (1.36, 2.01)</td>
<td>2.11 (1.91, 2.22)</td>
<td>2.06 (1.39, 2.24)</td>
<td>2.01 (1.95, 2.07)</td>
<td>1.82 (1.49, 2.13)</td>
<td>1.87 (1.39, 2.25)</td>
<td>1.63 (0.92, 2.06)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

BB: Basal Body, CC: Central Complex, HSVM: High Speed Video Microscopy, IDA: Inner Dynein Arm, ODA: Outer dynein Arm, TEM: Transmission electron Microscopy. Median (IQR); n (%) are given. P-values calculated by Kruskal-Wallis rank sum test; Pearson's Chi-squared test.
REFERENCES


Figure 1. Flowchart of patient inclusion.
Figure 2: 1-hour lung retention and indirect genotype measures

- **HSVM motility pattern**
  - Subtle abnormalities (DRC1, GAS8, HYDIN, RSPH1, CCDC103)
  - Hyperkinetic/reduced proximal bending (DNAH11)
  - Non-motile/residual flickering (DNAI1, DNAH5, DNAI2, CCDC151, CCDC114)
  - Slow rotational (RSPH9, RSPH4A)
  - Non-motile (PIH1D3, LRRC6, DNAAF4, DNAAF1, ZMYND10, C21orf59)
  - Severely reduced amplitude/rigid axonemes (CCDC40, CCDC65)
  - Reduced number of cilia (OFD1, FOXJ1)

- **TEM ultrastructure**
  - No abnormalities detected (DRC1, DNAH11, GAS8, HYDIN)
  - ODA (DNA1, LRRC6, CCDC103, DNAH5, DNAI2, CCDC151, CCDC114)
  - CP defect (RSPH9, RSPH1, RSPH4A)
  - ODA+IDA (PIH1D3, DNAAF4, DNAAF1, ZMYND10, C21orf59)
  - Tubular disorganization (CCDC40, CCDC65)
  - BB mislocated (OFD1, FOXJ1)
Figure 3. Comparison of PRMC velocity between included patients with PCD and non-PCD controls
Figure 4. Correlation between voluntary cough-clearance and aerosol deposition
Figure 5. Boxplots of retention measurements for the included patients with PCD stratified by records of cough.
Appendix 1

Procedure for calculation of reference values for PRMC and Residual Standard Deviations (RSD) based on a multiple regression model [10]:

\[
LR = k_0 + (k_1 \times \text{age}) + (k_2 \times \text{sex}) + (k_3 \times \text{Penetration index}) \pm SD
\]

Predicted \(LR_1\) (%): 

\[
72.63 + (-0.06 \times \text{age}) + (-1.72 \times \text{sex}) + (25.94 \times \text{Penetration index}) \pm 9.01
\]

\(k_0 = 72.63; k_1 = -0.06; k_2 = -1.72\) (sex: 1 = male, 2 = female); \(k_3 = 25.94\); \(SD_{LR1} = 9.01\)

Example:

Predicted \(LR_1\) calculated for a 6.9-year-old female (sex: 2, Penetration index: 0.46):

\[
72.63 + (-0.06 \times 6.9) + (-1.72 \times 2) + (25.94 \times 0.46) \pm 9.01 = 80.7 \pm 9.01
\]

**Normal range** for \(LR_1\) of this female (comprising 95% of the population):

\(LR_1\) (%) = 80.7 ± (2.0\(^a\) x 9.01) i.e., between 62.7% and 98.7%,

or more correctly since abnormal mucociliary clearance can only be too slow:

\(LR_1\) (%) = 80.7 + (1.67\(^b\) x 9.01) = 95.8% i.e., below 95.8% is normal.

\(^a\) t (N-2, P < 0.025); \(^b\) t (N-2, P < 0.05)

In this patient with PCD the measured lung retention after 1 hour was 100 % (i.e., nothing cleared within 1 hour)

**Calculation of RSD for LR1 in this patient**:

\[
RSD_{LR1} = \frac{(LR_{1\text{measured}} - LR_{1\text{predicted}})}{LR_{1\text{SD}}}
\]

\[
RSD_{LR1} = \frac{(100 - 80.7)}{9.01} = +2.1
\]

Thus, the measured 1-hour retention lies 2.3 RSD above the predicted value, which is abnormal (i.e., all RSD above 1.67 are abnormal)
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\]

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\[k_0 = 72.63; k_1 = -0.06; k_2 = -1.72 \text{ (sex: 1 = male, 2 = female)}; k_3 = 25.94; \text{SD}_{LR1} = 9.01\]

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\[
\text{RSD}_{LR1} = \frac{(100 - 80.7)}{9.01} = +2.1
\]

Thus, the measured 1-hour retention lies 2.3 RSD above the predicted value, which is abnormal (i.e., all RSD above 1.67 are abnormal)
"Pulmonary Radioaerosol Mucociliary Clearance assessment: searching for genotype-specific differences and potential as an outcome measure in Primary Ciliary Dyskinesia"

*June K. Marthin¹, *Mathias G. Holgersen¹, #Kim G. Nielsen¹,³, #Jann Mortensen ²,³

1: Danish PCD Centre, Danish Pediatric Pulmonary Service, Department of Pediatrics and Adolescent Medicine, Copenhagen University Hospital, Rigshospitalet, Copenhagen, Denmark.

2: Department of Clinical Physiology and Nuclear Medicine, Copenhagen University Hospital, Rigshospitalet, Denmark

3: Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark.

*: shared first authorship
#: shared last authorship
**PRMC method**

In brief, patients inhaled ultrasonically (model 35 B; Devilbiss; Somerset, PA) nebulized 99mTc-albumin colloid (Venticoll; GI PHARMA; Saluggia, Italy) during tidal breathing by 20 slow inspirations and forced expirations. Immediately after, subjects were placed in supine position against a posteriorly positioned gamma camera for detecting lung radioactivity and static and dynamic imaging were acquired for 60 and 120 minutes, and static acquisitions at 24 hours.

Examples of tracer movement from static acquisitions at 0, 30, and 60 minutes after radioaerosol inhalation in a pwPCD and a non-PCD referral is shown below: A series of static acquisitions showing radioaerosol tracer movement due to mucociliary transport in a patient with PCD (top row) and in a non-PCD individual (bottom row).

*Top row:* A patient with PCD. The radioaerosol is seen initially centrally deposited (0 min), and still, there is almost no movement of the tracer after 30 minutes (middle) and 60 minutes (right), indicating that mucociliary clearance is minimal in the patient. The swallowed radioaerosol is seen to pass from the left-sided stomach further to the duodenum and jejunum. Images are taken posteriorly.

*Bottom row:* A non-PCD referral where PCD was later ruled out. Centrally deposited radioaerosol inhaled at 0 minutes is quickly removed from the central airways,
ascending upwards from the main bronchi to the trachea from where it is further cleared. The initial tracer foci after inhalation are markedly cleared already after 30 minutes (middle) and further cleared after 60 minutes (right) with only little radioaerosol is left in the central airways, although some foci are still visible in the main bronchi and the trachea.

*Top row and bottom row:* Two $^{57}$Co markers seen over the cervical and lumbar spine were placed initially to help positioning the subjects.

**Nasal NO measurement**

Sampling flow rate was 0.33 L·min$^{-1}$. Conversion from nNO concentration to nNO production rate (nL·min$^{-1}$) was determined by multiplying nNO (ppb) by flow rate (L·min$^{-1}$).
Distribution of PCD genes among the included patients with relation to their TEM and HSVM findings.

**Legend:** Distribution of genes in relation to A) motility pattern and B) ultrastructure for the included patients in the study. CCDC39 and CCDC40 defects also include IDA defect. Dark grey annotates subjects excluded in the analysis of 1 hour retention.