Article

A revised protocol for culture of airway epithelial cells as a diagnostic tool for primary ciliary dyskinesia

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**Abstract:** Air-liquid interface (ALI) culture of nasal epithelial cells is a valuable tool in the diagnosis and research of primary ciliary dyskinesia (PCD). *Ex vivo* samples often display secondary dyskinesia from cell damage during sampling, infection or inflammation confounding PCD diagnostic results. ALI-culture enables regeneration of healthy cilia facilitating differentiation of primary from secondary ciliary dyskinesia. We describe a revised ALI-culture method adopted from April 2018 across three collaborating PCD diagnostic sites, including current University Hospital Southampton COVID-19 risk mitigation measures, and present results. Two hundred and forty nasal epithelial cell samples were seeded for ALI-culture and 199 (82.9%) ciliated. Fifty-four of 83 (63.9%) *ex vivo* samples which were originally equivocal or insufficient provided diagnostic information following *in vitro* culture. Surplus basal epithelial cells from 181 nasal brushing samples were frozen in liquid nitrogen; 39 samples were ALI-cultured after cryostorage and all ciliated. The ciliary beat patterns of *ex vivo* samples (by high-speed video microscopy) were recapitulated, scanning electron microscopy demonstrated excellent ciliation, and cilia could be immuno-fluorescently labelled (anti-alpha-tubulin and anti-RSPH4a) in representative cases that were ALI-cultured after cryostorage. In summary, our ALI-culture protocol provides high ciliation rates across three centres, minimising patient recall for repeat brushing biopsies and improving diagnostic certainty. Cryostorage of surplus diagnostic samples was successful, facilitating PCD research.

**Keywords:**  PCD; ALI-culture; bio-resource; primary nasal epithelium; diagnostics

Introduction

Primary ciliary dyskinesia (PCD) is a rare disease usually inherited as an autosomal recessive condition although autosomal dominant and X-linked cases exist [1]. The incidence of PCD is approximately 1:10,000, higher in consanguineous populations [2], and it is associated with impaired function of motile cilia in the airways, embryonic node, and reproductive system [3]. This causes a spectrum of symptoms including unexplained neonatal respiratory distress, persistent wet cough from infancy, repeated respiratory infections, rhino-sinus disease, organ laterality abnormality and subfertility [4]*.* Early diagnosis is essential to initiate treatment, with the aim of slowing disease progression and improving quality of life [5].

There is no “gold standard” diagnostic test for PCD [6]. ERS and ATS guidelines both recommend a multidisciplinary approach using a combination of tests to make a diagnosis [7-9]. *Ex vivo* nasal or bronchial samples obtained by brushing or curette biopsy are imaged by high-speed video microscopy analysis (HSVA) [10, 11] and ciliary motility analysed as a frontline functional test [12]. Transmission electron microscopy (TEM) is used to assess and quantify ultrastructural abnormalities of motile cilia [13, 14]. Immunofluorescence labelling can demonstrate the absence or mis-localisation of ciliary proteins [15, 16], particularly helpful in cases where no TEM abnormalities are detected such as with DNAH11 [17], DNAH9 [18] and *HYDIN* gene mutations [19]. Genotyping can detect pathogenic bi-allelic or X-linked hemizygous mutations in 50 PCD-related genes to confirm the diagnosis in approximately 70% of well characterized cases [1, 3, 20]. However, there are still many individuals without a genetic diagnosis. Some genetic defects result in subtle ciliary beat pattern abnormalities, which are difficult to differentiate from secondary defects (e.g. *GAS8* [21], *DNAH9* [18], *CCDC103* [22, 23] mutations) by HSVA and appear normal by TEM. *MCIDAS* [24], *CCNO* [25] and *FOXJ1* [26] mutations cause a lack of cilia rather than dyskinesia, and this could be mistaken for severe secondary epithelial damage.

*How can cell culture be used in PCD diagnostics and research?*

Secondary damage of cilia caused by infection, inflammation or sampling trauma is a common feature in *ex vivo* airway samples [27-29]. Also sample yield may not be sufficient to support the growing array of tests required to diagnose difficult cases. Therefore, to address poor quality, low yield or expanded diagnostic testing, cell culture can be used.

Airway epithelial cell monolayer mini-culture methods have been used to reduce secondary ciliary dyskinesia in the nasal brushings (from chronic sinusitis patients) to enable better ciliary function measurements by HSVA following 3 days in culture with 83% culture success and improved ciliary beat pattern visualization [30]. However, following recent infections, the airway cilia may be shed and this culture method does not allow for cilia re-growth.

Likewise, monolayer-suspension methods, initially developed for whole resected tissue [31], have been adapted for nasal brush biopsy [32] and are useful for reducing secondary abnormalities in culture for PCD diagnostics. Briefly, nasal brushing cell suspensions are seeded onto a 1% collagen gel substrate and non-adherent cell aggregates are harvested at 24 hours for suspension culture with continuous movement. After 24-48 hours, cell spheroids form, which are cultured for at least 21 days before analysis of newly formed cilia on the apical surface of the spheroid. This method confirmed a PCD diagnosis or resolved secondary defects in 46 of 59 cultures (78%) [32]. Pifferi et al., also described a later study in which of 151 subjects a PCD diagnosis could be confirmed in 36 patients using the suspension model optimally following a 5-day culture process [33].

Marthin et al., (2017) reported a more rapid nasal epithelial cell culture method, where spheroids spontaneously formed from terminally differentiated nasal epithelium, retaining their original cilia [34]. Spheroids formed in 82% of 18 samples, with the median number of days to harvest being 4 (1-5) in 7 healthy volunteers and 2 (1-5) in 8 PCD patients’ samples. Whilst retaining their original ciliary beat pattern and frequency, spheroids survived up to 16 days (albeit n=1) and provided ciliated spheroids for HSVA and immunofluorescence labelling of cilia [34]. However, using this method, spheroid numbers are not expanded and are limited by the original sampling yield, which may not support a multitude of tests. Also unhealthy or unciliated samples cannot be re-grown to resolve secondary damage since new cilia are not formed.

Alternatively, basal epithelial cells can be expanded in submerged culture before cells are differentiated on Transwell® inserts at an air-liquid interface (ALI) to stimulate cell polarisation and widespread ciliogenesis, which takes 4-6 weeks *in vitro*. Re-analysis of cilia function after ALI-culture gives a further opportunity to carry out HSVA, TEM and IF without secondary health issues [27, 35].

Cilia regeneration by spheroid suspension or ALI-culture can negate the need for patients, with insufficient or inconclusive test results, to undergo repeat nasal brushing biopsy, reduce the time to a diagnosis of PCD and increase the accuracy of HSVA [7, 12]. We have also previously shown how ALI-cultures may be used as an airway model to investigate nasal epithelial cell interactions with drugs [36-38], bacterial[37-41] and viral infections [40-43]

*A revised protocol with high diagnostic efficiency is being used within the UK PCD service*

Here we present our ALI-culture protocol using commercially available expansion and differentiation media, PneumaCult Ex Plus and PneumaCult ALI (STEMCELL Technologies). We report its efficacy at three UK diagnostic centres. We also report on creating a bio-resource for PCD research and the performance of cell cultures after cryostorage at UHS. Due to the current COVID-19 pandemic we have additionally described the protocol modifications at UHS used to mitigate risk of SARS CoV2 infection during patient interaction and sample handling since July 2020.

Methods

We collected culture data from 70 consecutive patient samples attending the PCD Centre at University Hospital Southampton (UHS) between 1st April 2018 and 1st April 2019 (Table 1). Local and national R&D and ethical approvals were complied with (Southampton and South West Hampshire Research Ethics Committee A CHI395, 07/Q1702/109). We further report data from 128 samples processed at Leicester and 45 samples processed at the Royal Brompton Hospital using the same culture protocol (with some minor variations depending on equipment and consumables).

**Table 1.** The characteristics of subjects referred for diagnostic testing to University Hospital Southampton PCD centre 1st April 2018 to 1st April 2019, grouped by final MDT diagnostic outcomes.



Patients were diagnosed as ‘PCD highly-unlikely’ if they had a non-suggestive clinical history and/ or PICADAR score [4], normal nNO, and ‘PCD unlikely’ HSVA on *ex vivo* nasal brushing. Patients with a suggestive clinical history and/ or PICADAR score with inconclusive/ insufficient or ‘PCD likely’ HSVA on *ex vivo* nasal brushings underwent further testing. This included TEM of cilia ultrastructure and IF labelling of ciliary proteins, and could include repeat HSVA following ALI-culture and/ or genetic testing. See HSVA outcome definitions below. Hallmark TEM or genetics diagnosed PCD and ‘PCD highly-likely’ cases were diagnosed according to the ERS guidelines [3, 7, 44]. All available diagnostic data were discussed at a multidisciplinary team (MDT) meeting to decide the patients’ outcome and follow up.

Nasal epithelial cell culture protocol (with additional UHS COVID-19 modifications):

Nasal brushing biopsies were taken from patients’ inferior turbinates, using a 3 mm bronchoscopy cytology brush (Conmed, NY, USA, #149R). Individually sterile wrapped brushes were opened in clinic and the wire handles were cut to approximately 15 cm to hold (with wire cutters). To maximise chances of a good yield of healthy ciliated tissue, brushings were performed when patients had been free from respiratory exacerbations for at least 6 weeks. Patients were seated (children on parent’s laps) and asked to remove spectacles, and to clear mucus secretions from their nose with a tissue. Clinicians placed one brush sequentially into each nostril (without anesthetic), ensuring that the patients nostrils were patent. Whilst applying gentle pressure to the inferior turbinate the brush was passed back and forth for approximately 5 seconds, whilst turning, to ensure coverage. The sample was then placed into a capped 5 ml round bottomed cytology tube (Fisher Scientific, #10186400) containing 1.5 ml Medium 199 (Fisher Scientific, #22350029) supplemented with 1% penicillin (5,000 U/ml)/ streptomycin (5,000 µg/ml) (Fisher Scientific, #15070063).

[Since the COVID-19 pandemic we have adopted several clinical and laboratory modifications to mitigate the risk of acquiring SARS CoV2 infection at UHS, which were approved by UHS and University of Southampton Health, Safety and Risk directorates. We recommend that all wanting to undertake nasal epithelial cell culture during the COVID-19 pandemic consult their local institute and government policy to ensure health and safety measures are in place. Patients are seen within 48 hours of a negative SARS CoV2 polymerase chain reaction (PCR) test. Patients are contacted directly before attending clinic to ensure that they had none of the main COVID-19 symptoms (as cited by http://[www.nhs.uk/conditions/coronavirus-covid-19/symptoms/#symptoms](http://www.nhs.uk/conditions/coronavirus-covid-19/symptoms/#symptoms) including ‘a high temperature, a new continuous cough and a loss or change to sense of smell or taste’). The clinicians wear full PPE including clinical scrubs with disposable aprons, gloves, FFP3 (personally fitted) face masks and plastic face shields. The clinical area is ventilated and the number of personnel and patients are restricted to ensure 2 m distancing before and after brushing sampling. To reduce risk of aerosol generation, patients wear disposable surgical face masks whilst in the hospital. Patients enter UHS via a patient only entrance and are temperature tested on arrival, and escorted to the clinical area directly before their appointment (to minimize interactions with other staff and patients whilst on site). Patients samples are contained and transported at room temperature (within an hour of sampling) to the onsite laboratory within a plastic sample bag within an anti-crush container. Couriered samples from other hospitals are not refrigerated and can take up to 3 hours to arrive. Samples received at our containment level 2 laboratory are handled within a class 2 microbiological safety cabinet (MSC). Specific to COVID-19 modified processing: 100 µl of the sample is sent for a SARS CoV2 PCR test via the Public Health England laboratory at UHS and 900 µl of the sample is kept at 4oC until the test result is returned (24-48 hours). Only those samples proven negative are cultured. We have so far not received a nasal brushing sample that has tested positive for SARS CoV2 by PCR. To remove mucus, the remaining 500 µl of sample is washed in 2 ml HBSS without calcium and magnesium, (Gibco, #10532003) and epithelial cells pelleted by centrifugation at 400 x g for 7 minutes. Cell pellets are resuspended in 500µl Medium 199 (including 1% pen/strep) for HSVA (100 µl), IF diagnostic testing (20 µl/ slide, n=10) and the remaining 200 µl is fixed in 4% glutaraldehyde for TEM processing.]

To remove mucus, cell suspensions (directly from the brush) intended for culture were washed in 2 ml HBSS without calcium and magnesium, (Gibco, #10532003) and epithelial cells pelleted by centrifugation at 400 x g for 7 minutes. Cell pellets were resuspended in 1 ml PneumaCult Ex plus medium (STEMCELL Technologies, Ex plus kit #05040) supplemented with hydrocortisone (0.1%) (STEMCELL Technologies, #07925) to directly seed cell clusters in 1-2 collagen (0.3 mg/ml, PureCol 5005.B CellSystems, Germany) coated wells of a 12-well culture plate (Corning, #3548). We did not digest or quantify cell numbers directly from the brush biopsies, and in our experience cell health is more important than yield. Under-seeding is to be avoided particularly if cell health is considered compromised after microscopic assessment. Cells were cultured in 37oC incubators with 5% CO2 and ~100% relative humidity and all culture medium contains additional 1% penicillin (5,000 U/ml)/ streptomycin (5,000 µg/ml) (Fisher Scientific, #15070063) and 0.002% nystatin suspension (10,000 U/ml) (Fisher Scientific, #15340029). Medium was replaced 3 times weekly and cells passaged with 0.25% trypsin EDTA (Gibco, 11560626) when at 50-70% confluence for both initial seeding passage 0 and at passage 1 in collagen coated T25 cm2 flasks, to give a final basal epithelial cell yield of 1-2 million. All centrifugations were at 400 x g for 5 minutes and cells washed twice in 7 ml HBSS to remove residual trypsin (without use of a trypsin inhibitor). At passage 2 100,000 cells were seeded per collagen coated 12 mm Transwell® with 0.4 µm pore polyester membrane insert (Corning Life Sciences, #3460) in a 12-well culture plate. Cells on Transwell® inserts were initially cultured submerged in 250 µl PneumaCult Ex plus medium on the apical side and 650 µl of the same medium on the basolateral side. When a confluent monolayer of basal cells was observed (usually between 1-2 days) cells were taken to air-liquid interface (ALI) by apical medium removal and replacement of the basolateral medium with 650 µl PneumaCult ALI medium (STEMCELL Technologies, ALI kit #05001) supplemented with hydrocortisone (0.5%) and heparin (0.2%) (STEMCELL Technologies, # 07925 and #07980 respectively), replaced 3 times weekly and with apical cell washing (briefly with 100 µl HBSS) aspirated to prevent a build-up of mucus. Cultures were harvested between 3 and 6 weeks to allow for optimal ciliation.

Bio-resource:

Between March 2018 and August 2020 UHS has cryopreserved surplus diagnostic cells from 181 PCD clinic patient samples and 30 healthy donor samples. Surplus cells from passage 1 were frozen 1 million per cryovial in 1 ml CryoStor® cell cryopreservation medium (Sigma, #C2874). Cells were initially frozen at -80oC (graduated freezing -1oC/ minute in a Mr. FrostyTM container ThermoScientific #5100-0001), then transferred to liquid nitrogen for longer term storage. After thawing, washed cells were seeded for research in a smaller Transwell® insert format in 24-well plates. Briefly, 50,000 cells per collagen coated 6.5 mm Transwell® with 0.4 µm pore polyester membrane insert (Corning Life Sciences, #3470) in 100 µl PneumaCult Ex plus medium supplemented (apical side) and 350 µl of the same medium on the basolateral side. Cultures were taken to ALI after 1-2 days replacing only the basolateral medium with 350 µl PneumaCult ALI medium supplemented and maintained as detailed above.

Post-ALI-culture high-speed video microscopy analysis:

Motile cilia were usually first observed by day 7 post-ALI by low power light microscopy with normal ciliary function and widespread coverage confirmed by HSVA [12] from day 20. Cultures were analysed between 3 and 6 weeks post-ALI when considered optimally ciliated. Cultures with no discernible cilia were analysed 6 weeks post-ALI, to examine for static cilia or ciliary aplasia. For HSVA, cells were scraped gently from the membrane with a pipette tip, washed and centrifuged to reduce mucus, transferred in 100 µl PneumaCult ALI medium into a 0.5 mm depth Coverwell imaging chamber (Sigma, #635051) and mounted on a glass slide.

Ciliary beat pattern (CBP) and frequency (CBF) were analysed during HSVA using a x100 objective lens, with samples equilibrated to 37°C. Videos were recorded at 500 frames per second and analysed at 30-60 frames per second from a minimum of 6 strips of ciliated epithelium as previously described [27]. Observers with extensive experience [45] in ciliary function analysis then denoted the sample as either ‘PCD likely’, where a widespread ‘hallmark’ beat pattern was observed that was unlikely to be caused by secondary factors alone; ‘PCD unlikely’ where normal ciliary function was observed in at least six areas and any minor abnormalities present could be attributed to obvious secondary factors; ‘inconclusive’ where abnormal ciliary beating was observed which was likely to be due to secondary factors but PCD could not be excluded; or ‘insufficient’ where the quality or quantity of ciliated epithelium was not sufficient for an accurate decision to be made.

Fast Fourier Transform analysis of cilia coverage:

We pseudo-quantified the percentage area of cilia coverage on ALI-cultures in-situ on a representative subset of 10 consecutive UHS ciliated samples where cilia were motile (cilia coverage on static cultures requires alternative imaging approaches e.g. by immunofluorescence labelling or SEM not discussed here). HSVA was carried out at 37oC with a x20 objective lens (to acquire larger area mean CBF measurements), imaging every 3rd field of view across the midline of each 12 mm Transwell® insert to collect non-overlapping representative data across the whole membrane. Fields of view with significant moving particulates or mucus debris were avoided. Fast Fourier transform analysis of HSVA .cih Photron video files was performed using an in-house written plugin for https://imagej.net/ (by Dr Peter Lackie) to determine the proportion of movement to non-movement (CBF in Hz) detected in the video, using a minimum box size of 4x4 pixels (Figure 1). The percentage area of movement detected within 16 fields of view was averaged to give a surrogate for cilia coverage. Of 10 samples the mean cilia coverage was 38.9%, which was within the expected range of 15-50% reported in the respiratory tract *in vivo* [46].



**Figure 1.** Fast Fourier Transform analysis of ciliary movement using HSVA data. Fast Fourier Transform analysis (left) ‘colour map’ corresponding to ciliary movement detected by HSVA (using a 20x objective lens) on the surface of an ALI-culture (right). The colour scale (left to right) depicts increasing CBF from 0 Hz (black) to 25 Hz (white). Black pixels also represent a CBF measurement outside of the detection threshold (below 2 Hz or above 50 Hz). Normal mean CBF at 37oC is 11-20 Hz. Scale bar represents 100 µm.

Trans-epithelial electrical resistance measurements:

One hour before trans-epithelial electrical resistance (TEER) measurements were taken, PneumaCult ALI medium was replaced on the test ALI cultures (ciliated at passage 2) (using 100 µl PneumaCult Ex plus medium on the apical side and 350 µl on the basolateral side in the Transwell® insert format in 24-well plates) and incubated at 37oC. A control well with an empty Transwell® insert containing only medium (no cells) was also prepared. A World Precision Instrument EVOM2 epithelial Volt/Ohm meter with STX2 electrode (chopstick probe) (Fisher Scientific, #15169112) was used. The chopstick probe was sterilized in 70% industrial methylated spirit for 5 mins and rinse in medium before and after use and between test and control wells. The mean of three measurements was taken and background control measurements subtracted before calculating mean Ω.cm2 (±SD).

Immunofluorescence labelling of ciliated ALI-cultures:

The membranes of ciliated ALI-cultures were excised at 4 weeks using a surgical scalpel blade (15) and placed into the well of a 24-well plate submerged in 100 µl PneumaCult Ex plus medium. The ciliated epithelial cells were scraped from the membrane surface using a pipette tip into the medium. Then 20 µl of cell suspension was dropped onto each coated Shandon™ Cytoslides™ (Fisher Scientific , #12026689) and allowed to air dry in the class 2 MSC. Once dry, slides were sealed in slide mailer boxes (Fisher Scientific, #11719885) and transferred to a -20 ˚C freezer for storage for up to 4 months. For immunofluorescence labelling, slides were thawed and fixed with 4% PFA for 15 minutes, washed in PBS with 0.1% triton X-100 (Fisher Scientific, #T/3751/08) then blocked with 5% milk powder in PBS-triton X-100 for 1 hour. After washing, primary antibodies (anti-RSPH4a 1:200, Atlas Antibodies, Sigma, #HPA031197; anti-alpha tubulin 1:500, Sigma #T9026) in PBS-triton X-100 were incubated for 2 hours at room temperature, followed by washing and secondary antibody (Alexafluor 488, Life Technologies, #A21121; Alexafluor 594, Life Technologies, #A11012) incubation at a dilution of 1:2500 in PBS-triton x-100 for 30 minutes at room temperature. DAPI (300 nM) (Molecular Probes, ThermoFisher, #D1306) was added to the final wash before mounting onto coverslips with Mowiol aqueous mounting media. The slides were kept in the fridge (at least over night) until imaging using a Leica SP8 laser scanning confocal microscope with Leica Application Suite X software.

Statistics:

Descriptive statistics are presented in Table 1. Normality was checked using the Shapiro Wilk test. Two sample comparisons were undertaken using the student t-test when normality test passed or Mann-Whitney test when normality test failed. Matched samples were analysed using the parametric paired student’s t test or non-parametric Wilcoxon test. For multiple comparisons One-Way ANOVA was used for parametric samples or Kruskal-Wallis test for non-parametric samples. Statistical analysis was performed in GraphPad Prism 8 (GraphPad, San Diego, USA). A P-value less than 0.05 was considered significant.

Results

*How did the nasal epithelial cell culture protocol improve diagnostic accuracy at UHS?*

Sixty-seven of 70 consecutive UHS patients’ (Table 1) samples were cultured and 64 of those 67 ALI-cultures successfully ciliated (95.5%). Of 3 samples that were not cultured, 2 were not suspected of PCD (due to a weak clinical history, normal nNO and ‘PCD unlikely’ HSVA on the *ex vivo* nasal brushing) and 1 was diagnosed as ‘PCD positive’ with hallmark TEM (absent outer dynein arms) with static cilia (by HSVA on the *ex vivo* sample) as determined at MDT and surplus epithelial cells were cryopreserved.

Of 67 *in vitro* ALI-cultures only 3 (4.5%) failed (1 insufficient sample, 2 due to infection) and HSVA was performed on the 64 ciliated cultures. The original *ex vivo* CBP was confirmed in 7 normal and 6 abnormal (PCD likely) ALI-cultured samples (19.4%). Fifty-one original *ex vivo* samples had an equivocal CBP and ALI-culture resolved a normal CBP in 30 (44.8%), an abnormal CBP (PCD likely) in 2 (3%) and remained equivocal in 19 samples (28.4%) (Figure 2). As expected, TEM analysis of ALI-cultures demonstrated that normal and PCD hallmark defects were replicated *in vitro* from representative samples (Figure 3). Example HSVA videos showing the quality of ALI-culture after equivocal or abnormal ‘PCD likely’ *ex vivo* samples are shown in Supplementary videos 1a-d.



**Figure 2.** Flow diagram of diagnostic sample processing for ALI-culture by repeat HSVA and MDT outcomes. Seventy patients’ *ex vivo* samples evaluated by HSVA (as part of the whole diagnostic process) and their ALI-culture outcomes were followed. HSVA on ALI-culture either confirmed the original HSVA finding, resolved an originally equivocal HSVA or remained equivocal despite culture. In bold, the ALI-culture HSVA outcomes are shown by MDT outcome (‘PCD’/ ‘PCD highly-likely’; ‘PCD highly-unlikely’ or ‘equivocal’ pending follow up, repeat tests or further tests (genetics or additional IF for example).



**Figure 3.** TEM of cilia in transverse section and CBF (by HSVA) in nasal samples and after ALI-culture.Representative TEM images a) and b) of *in vitro* ALI-cultured cilia in transverse section, showing a “9+2” microtubular arrangement. Cilia have normal ciliary ultrastructure in a) a ‘PCD highly-unlikely’ subject (with 3% microtubular defects, 18% inner and 4% outer dynein arm defects quantified from 102 cilia), and b) a ‘PCD positive’ subject (with 11% microtubular defects, 47% inner and 99% outer dynein arm absence quantified from 302 cilia). Scale bar represents 100 nm. Dot plot c) demonstrates the mean CBFs (Hz) of 57 *ex vivo* nasal brushing samples compared to their matched *in vitro* ALI-cultures (Wilcoxon paired test P=0.03). Data from *ex vivo* samples without a matched ALI sample were excluded (n=13), which was due to 7 *ex vivo* samples with variable CBP, 3 failed ALI-cultures and 3 not cultured. Normal CBF range of *ex vivo* samples at UHS is 11-20 Hz.

The mean CBF was determined in 57 patients’ *in vitro* ALI-cultures with unambiguous CBP (completely static cilia were recorded with a mean CBF as 0 Hz). Of the 57, 3 cases (2 ‘PCD highly-likely’ and 1 equivocal at MDT) with mostly static cilia and some residually moving dyskinetic cilia in the original *ex vivo* sample became completely static in culture, which we have reported before [27]. The Shapiro-Wilk test showed that *ex vivo* (W=0.99, P=0.81) and *in vitro* (W=0.96, P=0.06) sample CBFs were normally distributed only when static samples (3 in each group) were excluded. Compared to their matched *ex vivo* samples (n=57) the mean CBF of patients samples significantly varied after *in vitro* ALI-culture (median: 14.7 Hz ranging from 0-18.5 Hz *ex vivo*; 13.9 Hz ranging from 0-17.5 Hz *in vitro*) (Wilcoxon matched pairs P=0.03) (Figure 3). A mean CBF was not considered meaningful in 7 patients’ *ex vivo* samples caused by a mixed motile ciliary beat pattern, 3 samples were not cultured and 3 failed accounting for the remaining without a CBF.

The *in vitro* ALI-cultures’ results were compared by patients’ MDT outcome. Of 55 ‘PCD highly-unlikely’ patients a normal culture CBP was seen in 37 (67.3%) but remained equivocal in 16 (29.1%) and 2 were not cultured. Of 7 ‘PCD’/ ‘PCD highly-likely’ patients, an abnormal ‘PCD likely’ HSVA result was seen in 5 (71.4%), 1 was progressing but deliberately ‘paused’ and frozen due to ‘hallmark TEM defects’ [14] and 1 failed due to infection. (Figure 2).

There were 8 patients with an equivocal MDT outcome due to inconclusive tests. After all tests 5 patients with an unconvincing clinical history were considered to have equivocal diagnostic results at MDT, with patients advised to seek re-referral if symptoms persisted. Of this group 1 had a normal CBP, 3 were equivocal after ALI-culture and 2 failed (insufficiency/ infection). Two other patients with normal TEM and ‘PCD likely’ HSVA on *in vitro* ALI culture are being followed up with genetic testing.

*Was the nasal epithelial cell culture protocol reproducible across the UK PCD service?*

This culture method was simultaneously adopted by the Leicester and Royal Brompton PCD Centres. One hundred and five of 128 (82%) cultures from the Leicester PCD Centre ciliated and of these 21 of 29 (72.4%) insufficient biopsy samples were successfully differentiated at ALI-culture for diagnostic use, providing HSVA results where patients would otherwise have been recalled for repeat biopsy. At the Royal Brompton PCD Centre, 30 of 45 (66.7%) ALI-cultures ciliated. Therefore, 199 of 239 consecutive ALI-cultures ciliated across 3 UK PCD diagnostic sites, giving a combined ciliation success rate of 83.3%.

Since the COVID-19 pandemic UHS has cultured 44 patients’ samples with protocol modifications to mitigate the risk of acquiring SARS CoV2 infection from nasal brushing samples. This includes storing the nasal cell suspensions in Medium 199 (with 1% pen/ strep) at 4oC for up to 48 hours, whilst awaiting the outcome of a SARS CoV2 PCR test. Of the 44 samples cultured during this period 8 (18%) are still in progress, 28 (64%) ciliated successfully and 8 (18%) failed (3 due to bacteria, 3 due to fungus and 2 insufficient cell yield).

*Can a bio-resource extend diagnostic testing and research?*

Studies have been challenged by the limited number of PCD patients’ samples available for research, therefore since March 2018 UHS has cryopreserved surplus diagnostic cells from PCD clinic patient samples following consent (n=181 to August 2020 including 25 (13.8%) confirmed ‘PCD positive’/ ‘PCD highly-likely’) and 30 healthy donor samples (20 (66.7%) female; median age 37.4, range 21.4-58.3). We have to date recovered 6 confirmed ‘PCD positive’/ ‘PCD highly-likely’ samples, 25 confirmed ‘PCD highly-unlikely’ disease control samples and 8 healthy donors from liquid nitrogen storage, with a ciliation success rate of 100% at ALI-culture. Here we report representative data from a subset of the thawed samples (Figure 4). The ciliary beat patterns seen on original *ex vivo* samples (by HSVA) were recapitulated, scanning electron microscopy demonstrated excellent ciliation, and cilia could be immuno-fluorescently labelled in representative cases after cryostorage and ALI-culture differentiation (Figure 4).



**Figure 4.** Characteristics of *in vitro* ALI-cultures (ciliated and differentiated at passage 2) derived from frozen liquid nitrogen storage. a) There was no difference in the mean (±SD) TEER (Ω.cm2) of n=10 ‘PCD highly-unlikely’ and n=4 ‘PCD highly-likely’ ALI-cultures, recovered from liquid nitrogen cryostorage (post-LN2) (t test), or compared to n=4 healthy donor samples (non-frozen) controls (Mann Whitney test); measured in triplicate per transwell at 4 weeks ALI-culture, when cells were widely ciliated. b) The mean CBF (Hz) of n=4 matched PCD clinic samples differed before (*ex vivo*) and after liquid nitrogen storage (*in vitro* ALI-culture) P=0.01 (paired t test). c) A representative SEM image from a ‘PCD highly-unlikely’ ALI-culture showing typical ciliation at week 4 post-LN2. d) Representative PCD diagnostic immunofluorescence [15] images from an SP8 laser scanning confocal microscope, showing a PCD clinic ALI-culture after cryostorage with 4% paraformaldehyde fixation and immunofluorescence labelling with anti-alpha-tubulin (cilia marker-Alexa488 secondary antibody, green), anti-RSPH4a (radial spoke head protein-Alexa549 secondary antibody, red) and DAPI (nuclei DNA stain, blue). Scale 20 µm.

The physical barrier properties, measured by TEER at 4 weeks post-ALI, of n=10 thawed ‘PCD highly-unlikely’ 383 Ω.cm2 (SD± 86.5) and n=4 defrosted ‘PCD highly-likely’ 314 Ω.cm2 (SD± 153.9) samples were not significantly different to each other (t test) or to n=4 representative fresh (non-frozen) healthy donor *in vitro* ALI-cultures 299 Ω.cm2 (SD± 97.6) (Mann Whitney test) (Figure 4). HSVA demonstrated that n=4 representative ‘PCD highly-unlikely’ samples with mean nasal brushing CBF of 17.3Hz, SD±2.9 (*ex vivo*) resolved a normal CBP with mucociliary clearance with a significantly reduced mean CBF of 12.7 Hz, SD±2.5 in matched samples that were ALI-cultured (4 weeks) after liquid nitrogen storage (paired t test, P=0.01); while 3 of these cultures retained a normal CBP, 1 culture’s CBF fell just below the UHS normal range (11-20 Hz) [27] (Figure 4). Six ‘PCD positive’/ ‘PCD highly-likely’ samples that had predominantly static cilia (n=4) or uncoordinated and stiff dyskinetic cilia (n=2) on the *ex vivo* nasal brushing samples retained their abnormal CBP at week 4 of ALI-culture *in vitro* after freezing (CBF not shown). The quality of ALI-cultures prepared for HSVA after samples were defrosted from cryostorage compared to their original *ex vivo* samples are shown in Supplementary videos 2a-d.

Discussion

After revisions to our 2014 [27] culture protocol, employing the commercially available STEMCELL Technologies media system, we demonstrate an excellent 95.5% success rate of ALI-cultures differentiating cilia with 38.9% cilia coverage for analysis at the UHS PCD centre. Three UK PCD diagnostic centres share protocols[47, 48]. Therefore, this revised protocol was simultaneously adopted, enabling us to confirm a combined success of 83.3% ALI-cultures ciliated across our national service. Differences in success rates between our centres may have been due to several factors such as different patient demographics, sampling variability and physical management of samples due to our logistical set ups. For example, the UHS benefits from having laboratories on the same site as the patient clinics and we were able to repeat insufficient samples during clinic, whilst the patient was still onsite as needed. Since we have introduced COVID-19 mitigation measures, to limit our risk of acquiring the illness from our patients and samples, we have cultured 44 nasal brushing samples and 28 (64%) are so far ciliated, 8 (18%) ongoing and 8 (18%) failed since July 2020. This is encouraging that a high success rate can be maintained during the constraints of the current COVID-19 pandemic. Analyses following ALI-culture have negated the need to recall patients for repeat nasal brushing biopsy after inconclusive HSVA results due to secondary factors, and/or insufficient sample for analyses [27, 35]. The excellent yield of cilia coupled with the speed at which normally functioning cilia generate allow for repeat HSVA analysis and if needed repeat TEM to be reported within diagnostic time constraints, with sample also available for IF labelling of cilia and RNA extraction for further genetic splice variant screening (as well as genomic DNA genetic screening) if required. Results from ALI-cultures gave added confidence for patient discharge as ‘PCD highly-unlikely’ when abnormalities resolved in culture, or a diagnosis of ‘PCD positive’ or ‘PCD highly-likely’ to be given when abnormalities persist after culture.

Cell culture is an important tool in the diagnostic pathway but is a lengthy and technically demanding process. A reliable system for obtaining a good yield of healthy ciliated epithelial cells is vital. In our experience most failures occur early in the culture process due to either a lack of viable epithelial cells from nasal brush biopsy or viral/ bacteria/ fungal infections. To minimise failure rates, we recommend nasal brush biopsies are taken only from patients free from symptoms of infection for the previous six weeks. Basal epithelial cell survival, proliferation and differentiation *in vitro* rely on high cell densities [49]. With the patients still in clinic, typically cell yields from biopsies can quickly be checked by low power light microscopy to ensure enough material is present for all tests needed. In the event of a poor yield of healthy cells the patient may be approached for another brushing if tolerated. However, in light of the current COVID-19 pandemic, we are not practicing this to limit our exposure to nasal brushing samples in the laboratory. Although this could potentially be circumvented if microscope equipment can be housed within a customized class 2 MSC. As with all long-term cell culture, infection risks are high, maintaining the cultures in a designated culture facility with limited numbers of experienced users is important to maximise success rates.

Airway cells stored in a bio-resource and cultured at ALI retain their pre-frozen characteristics and provide amodel for PCD diagnostic testing and investigating the pathogenesis and treatments of respiratory disease (particularly for rare samples) without needing to recall patients for repeat brushings[36-39, 50]]. Exceptionally, 100% of samples recovered from liquid nitrogen successfully ciliated (n=6 ‘PCD positive’/ ‘PCD highly-likely’, n=25 ‘PCD highly-unlikely’ and n=8 healthy donor samples). In n=4 ‘PCD highly-unlikely’ samples with normal CBP and normal CBF (11-20 Hz at UHS) *ex vivo*, the CBF of ALI-cultures significantly (P=0.01) reduced after freezing despite maintaining normal CBP, either due to low sample numbers or the freezing/ defrosting process (Figure 4). The CBF reduction was not pronounced after the ALI culture process in 56 patients’ samples that were not previously stored in liquid nitrogen (Figure 3). We believe this method provides great scope for studies of both healthy and diseased ciliated epithelia, yet we advise for CBF studies that a baseline CBF of ALI-cultures derived from cryostored cells be established in-house. However, limitations of the model are the reliance upon commercially obtained culture media, and the undisclosed nature of the culture media components. In summary, we have presented an updated protocol for culture of airway epithelial cells, which has been stable and reliable, with consistently high success rates over the course of one year, based on the experience at three PCD diagnostic centres within a national service. Long-term, our patients will benefit directly from reduced recall for repeat samples, and advances in our understanding of disease phenotype and new treatment efficacy. Bio-resourcing will enable us to participate in national and international networks (http://bestcilia.eu/ and www.beatpcd.org/) that are collaborating to better characterise and treat PCD.

**Acknowledgments:** The National PCD Service is commissioned and funded by NHS England. PCD research in Southampton is supported by NIHR Southampton Biomedical Research Centre, NIHR Wellcome Trust Clinical Research Facility, AAIR Charity and NIHR RfPB (200470). PCD research in Leicester is supported by the NIHR GOSH BRC. Authors are members of BEAT-PCD (supported by COST Action BM1407 and ERS Clinical Research Collaboration). UHS and RBH are members of the European Reference Network for Rare Respiratory Diseases (ERN-LUNG) - Project ID No 739546. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research, or other organisations.

**Supplementary videos 1:** Removing secondary dyskinesia in ‘PCD highly-unlikely’ and confirming ‘PCD highly-likely’ in *ex vivo* clinic samples before and after *in vitro* ALI-culture. Videos were recorded at 500 fps and are shown at 30 fps playback. (UHS)

1. Inconclusive *ex vivo* nasal brushing (NB2322)
2. PCD highly-unlikely *in vitro* ALI-culture (NB2322)
3. PCD highly-likely *ex vivo* nasal brushing (NB2319)
4. PCD highly-likely *in vitro* ALI-culture (N2319)

**Supplementary videos 2:** Removing secondary dyskinesia in ‘PCD highly-unlikely’ and confirming ‘PCD highly-likely’ in *ex vivo* clinic samples before and after *in vitro* ALI-culture post LN2. Videos were recorded at 500 fps and are shown at 30 fps playback. (UHS)

1. Inconclusive *ex vivo* nasal brushing (NB2218)
2. PCD highly-unlikely *in vitro* ALI-culture post-LN2 (NB2218)
3. PCD highly-likely *ex vivo* nasal brushing (NB2129)
4. PCD highly-likely *in vitro* ALI-culture post-LN2 (NB2129)

**Conflicts of interest:** All authors certify that they have no conflicts of interest to declare.

References

1. Wallmeier, J., et al., *Motile ciliopathies.* Nat Rev Dis Primers, 2020. **6**(1): p. 77.

2. O'Callaghan, C., P. Chetcuti, and E. Moya, *High prevalence of primary ciliary dyskinesia in a British Asian population.* Arch Dis Child, 2010. **95**(1): p. 51-2.

3. Lucas, J.S., et al., *Primary ciliary dyskinesia in the genomics age.* Lancet Respir Med, 2020. **8**(2): p. 202-216.

4. Behan, L., et al., *PICADAR: a diagnostic predictive tool for primary ciliary dyskinesia.* Eur Respir J, 2016. **47**(4): p. 1103-12.

5. Lucas, J.S., et al., *Clinical care of children with primary ciliary dyskinesia.* Expert Rev Respir Med, 2017. **11**(10): p. 779-790.

6. Lucas, J.S. and M.W. Leigh, *Diagnosis of primary ciliary dyskinesia: searching for a gold standard.* Eur Respir J, 2014. **44**(6): p. 1418-22.

7. Lucas, J.S., et al., *European Respiratory Society guidelines for the diagnosis of primary ciliary dyskinesia.* Eur Respir J, 2017. **49**(1).

8. Shapiro, A.J., et al., *Diagnosis of Primary Ciliary Dyskinesia. An Official American Thoracic Society Clinical Practice Guideline.* Am J Respir Crit Care Med, 2018. **197**(12): p. e24-e39.

9. Shoemark, A., et al., *ERS and ATS diagnostic guidelines for primary ciliary dyskinesia: similarities and differences in approach to diagnosis.* Eur Respir J, 2019. **54**(3).

10. Jackson, C.L., et al., *Accuracy of diagnostic testing in primary ciliary dyskinesia.* Eur Respir J, 2016. **47**(3): p. 837-48.

11. Kempeneers, C., C. Seaton, and M.A. Chilvers, *Variation of Ciliary Beat Pattern in Three Different Beating Planes in Healthy Subjects.* Chest, 2017. **151**(5): p. 993-1001.

12. Rubbo, B., et al., *Accuracy of High-Speed Video Analysis to Diagnose Primary Ciliary Dyskinesia.* Chest, 2019. **155**(5): p. 1008-1017.

13. Shoemark, A., et al., *Twenty-year review of quantitative transmission electron microscopy for the diagnosis of primary ciliary dyskinesia.* J Clin Pathol, 2012. **65**(3): p. 267-71.

14. Shoemark, A., et al., *International consensus guideline for reporting transmission electron microscopy results in the diagnosis of primary ciliary dyskinesia (BEAT PCD TEM Criteria).* Eur Respir J, 2020. **55**(4).

15. Shoemark, A., et al., *Accuracy of Immunofluorescence in the Diagnosis of Primary Ciliary Dyskinesia.* Am J Respir Crit Care Med, 2017. **196**(1): p. 94-101.

16. Liu, Z., et al., *A quantitative super-resolution imaging toolbox for diagnosis of motile ciliopathies.* Sci Transl Med, 2020. **12**(535).

17. Dougherty, G.W., et al., *DNAH11 Localization in the Proximal Region of Respiratory Cilia Defines Distinct Outer Dynein Arm Complexes.* Am J Respir Cell Mol Biol, 2016. **55**(2): p. 213-24.

18. Loges, N.T., et al., *Recessive DNAH9 Loss-of-Function Mutations Cause Laterality Defects and Subtle Respiratory Ciliary-Beating Defects.* Am J Hum Genet, 2018. **103**(6): p. 995-1008.

19. Cindric, S., et al., *SPEF2- and HYDIN-Mutant Cilia Lack the Central Pair-associated Protein SPEF2, Aiding Primary Ciliary Dyskinesia Diagnostics.* Am J Respir Cell Mol Biol, 2020. **62**(3): p. 382-396.

20. Marshall, C.R., et al., *Whole-Exome Sequencing and Targeted Copy Number Analysis in Primary Ciliary Dyskinesia.* G3 (Bethesda), 2015. **5**(8): p. 1775-81.

21. Olbrich, H., et al., *Loss-of-Function GAS8 Mutations Cause Primary Ciliary Dyskinesia and Disrupt the Nexin-Dynein Regulatory Complex.* Am J Hum Genet, 2015. **97**(4): p. 546-54.

22. Panizzi, J.R., et al., *CCDC103 mutations cause primary ciliary dyskinesia by disrupting assembly of ciliary dynein arms.* Nat Genet, 2012. **44**(6): p. 714-9.

23. Shoemark, A., et al., *High prevalence of CCDC103 p.His154Pro mutation causing primary ciliary dyskinesia disrupts protein oligomerisation and is associated with normal diagnostic investigations.* Thorax, 2018. **73**(2): p. 157-166.

24. Boon, M., et al., *MCIDAS mutations result in a mucociliary clearance disorder with reduced generation of multiple motile cilia.* Nat Commun, 2014. **5**: p. 4418.

25. Wallmeier, J., et al., *Mutations in CCNO result in congenital mucociliary clearance disorder with reduced generation of multiple motile cilia.* Nat Genet, 2014. **46**(6): p. 646-51.

26. Wallmeier, J., et al., *De Novo Mutations in FOXJ1 Result in a Motile Ciliopathy with Hydrocephalus and Randomization of Left/Right Body Asymmetry.* The American Journal of Human Genetics, 2019. **105**(5): p. 1030-1039.

27. Hirst, R.A., et al., *Culture of primary ciliary dyskinesia epithelial cells at air-liquid interface can alter ciliary phenotype but remains a robust and informative diagnostic aid.* PLoS One, 2014. **9**(2): p. e89675.

28. Dixon, M. and A. Shoemark, *Secondary defects detected by transmission electron microscopy in primary ciliary dyskinesia diagnostics.* Ultrastruct Pathol, 2017. **41**(6): p. 390-398.

29. O'Callaghan, C., et al., *Ciliated conical epithelial cell protrusions point towards a diagnosis of primary ciliary dyskinesia.* Respir Res, 2018. **19**(1): p. 125.

30. Toskala, E., et al., *Culture of cells harvested with nasal brushing: a method for evaluating ciliary function.* Rhinology, 2005. **43**(2): p. 121-4.

31. Willems, T. and M. Jorissen, *Sequential monolayer-suspension culture of human airway epithelial cells.* J Cyst Fibros, 2004. **3 Suppl 2**: p. 53-4.

32. Pifferi, M., et al., *Simplified cell culture method for the diagnosis of atypical primary ciliary dyskinesia.* Thorax, 2009. **64**(12): p. 1077-81.

33. Pifferi, M., et al., *Rapid diagnosis of primary ciliary dyskinesia: cell culture and soft computing analysis.* Eur Respir J, 2013. **41**(4): p. 960-5.

34. Marthin, J.K., et al., *Patient-specific three-dimensional explant spheroids derived from human nasal airway epithelium: a simple methodological approach for ex vivo studies of primary ciliary dyskinesia.* Cilia, 2017. **6**: p. 3.

35. Hirst, R.A., et al., *Ciliated air-liquid cultures as an aid to diagnostic testing of primary ciliary dyskinesia.* Chest, 2010. **138**(6): p. 1441-7.

36. Ong, H.X., et al., *Primary Air–Liquid Interface Culture of Nasal Epithelium for Nasal Drug Delivery.* Molecular Pharmaceutics, 2016. **13**(7): p. 2242-2252.

37. Collins, S.A., et al., *Cephalosporin-3'-Diazeniumdiolate NO Donor Prodrug PYRRO-C3D Enhances Azithromycin Susceptibility of Nontypeable Haemophilus influenzae Biofilms.* Antimicrobial agents and chemotherapy, 2017. **61**(2): p. e02086-16.

38. Walker, W.T., et al., *Primary ciliary dyskinesia ciliated airway cells show increased susceptibility to Haemophilus influenzae biofilm formation.* Eur Respir J, 2017. **50**(3).

39. Walker, W.T., et al., *Ciliated cultures from patients with primary ciliary dyskinesia produce nitric oxide in response to Haemophilus influenzae infection and proinflammatory cytokines.* Chest, 2014. **145**(3): p. 668-669.

40. Smith, C.M., et al., *Ciliary dyskinesia is an early feature of respiratory syncytial virus infection.* Eur Respir J, 2014. **43**(2): p. 485-96.

41. Smith, C.M., et al., *Respiratory syncytial virus increases the virulence of Streptococcus pneumoniae by binding to penicillin binding protein 1a. A new paradigm in respiratory infection.* Am J Respir Crit Care Med, 2014. **190**(2): p. 196-207.

42. Blume, C., et al., *Barrier responses of human bronchial epithelial cells to grass pollen exposure.* European Respiratory Journal, 2013. **42**(1): p. 87-97.

43. Blume, C., et al., *A novel isoform of <em>ACE2</em> is expressed in human nasal and bronchial respiratory epithelia and is upregulated in response to RNA respiratory virus infection.* bioRxiv, 2020: p. 2020.07.31.230870.

44. Kuehni, C.E. and J.S. Lucas, *Diagnosis of primary ciliary dyskinesia: summary of the ERS Task Force report.* Breathe (Sheff), 2017. **13**(3): p. 166-178.

45. Lucas, J.S., et al., *Exploring the Art of Ciliary Beating: The Benefits of High-Speed Video Analysis.* CHEST, 2017. **152**(6): p. 1348-1349.

46. Serafini, S.M. and E.D. Michaelson, *Length and distribution of cilia in human and canine airways.* Bulletin europeen de physiopathologie respiratoire, 1977. **13**(4): p. 551-559.

47. Lucas, J.S., et al., *Diagnosis and management of primary ciliary dyskinesia.* Arch Dis Child, 2014. **99**(9): p. 850-6.

48. Lucas, J.S., et al., *Overcoming challenges in the management of primary ciliary dyskinesia: the UK model.* Paediatr Respir Rev, 2014. **15**(2): p. 142-5.

49. Fulcher, M.L., et al., *Well-differentiated human airway epithelial cell cultures.* Methods Mol Med, 2005. **107**: p. 183-206.

50. Lai, M., et al., *Gene editing of DNAH11 restores normal cilia motility in primary ciliary dyskinesia.* J Med Genet, 2016. **53**(4): p. 242-9.

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