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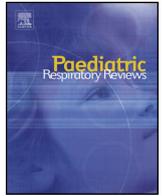
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# Paediatric Respiratory Reviews



## Mini-Symposium: Primary Ciliary Dyskinesia

### Diagnostic Methods in Primary Ciliary Dyskinesia

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#### EDUCATIONAL AIMS

The reader will be able to;

- Describe the basis of the various diagnostic tests for PCD
- Have an understanding of the strengths and limitations of each test
- Understand that there is no 'gold standard' test
- Understand why highly specialised centres should analyse samples and interpret results.

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#### SUMMARY

Diagnosing primary ciliary dyskinesia is difficult. With no reference standard, a combination of tests is needed; most tests require expensive equipment and specialist scientists. We review the advances in diagnostic testing over the past hundred years, with emphasis on recent advances. We particularly focus on use of high-speed video analysis, transmission electron microscopy, nasal nitric oxide and genetic testing. We discuss the international efforts that are in place to advance the evidence base for diagnostic tests.

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#### INTRODUCTION

Advances in diagnostic tests have accompanied technological developments and improved understanding of the pathophysiology of primary ciliary dyskinesia (PCD). Following the first case report of the syndrome, it took 70 years until transmission electron microscopy (TEM) was proposed as a diagnostic tool. Since then, a number of protocols and methods have been shown to aid diagnosis, but there remains no gold standard. In this review we discuss the historical developments that have led to the current state-of-the-art; we describe the most commonly used tests and consider the strengths and limitations for each (Table 1).

#### HISTORY OF PCD DIAGNOSTIC TESTING

In 1904, Siewart (or Zivat, Zivert, Sivert [1]) described a 21 year old with bronchiectasis associated with situs inversus. Kartagener later reported the triad of bronchiectasis, situs inversus and sinusitis in 1933, but it was not until the mid-1970s that Afzelius [2,3] and Pederson [4] recognized infertility as a feature and proposed the unifying role of cilia to explain the syndrome. Having noted absent dynein arms in the cilia of patients with the syndrome, Afzelius later demonstrated that the cilia were immotile, prompting the change of name from "Kartagener's Syndrome" to "Immotile Cilia Syndrome" [3]. These reports provided the evidence for TEM and assessment of motility as the basis of diagnostic testing. Recognition that outer dynein arm anomalies were not the only ultrastructural defect associated with the syndrome gave early insights to the underlying heterogeneity of the disorder [5,6]. Following recognition that a number of patients had motile but dysfunctional cilia [7,8] the name was further changed in the mid-1980s to "primary ciliary dyskinesia"

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**Table 1**  
The advantages and disadvantages of diagnostic tests in current use.

Diagnostic Test	Advantages	Disadvantages	Diagnostic accuracy
Nasal nitric oxide	<ol style="list-style-type: none"> <li>1. Guidelines exist for conduct of test [33]</li> <li>2. Meta-analysis demonstrates good sensitivity and specificity [32]</li> <li>3. Protocols can be standardized for multi-center use [29]</li> <li>4. Alternatives to 'gold standard' method (velum closure using chemiluminescence analyzer) have acceptable accuracy [35,37]</li> </ol>	<ol style="list-style-type: none"> <li>1. 'Gold standard' method impossible for young children and equipment expensive and non-portable.</li> <li>2. Standardised approach to use in PCD diagnostics and reporting of results needed</li> <li>3. Small % of patients have normal NO</li> <li>4. Normal reference values for younger age groups are lacking</li> </ol>	In consecutive patients for PCD diagnostic testing: <ol style="list-style-type: none"> <li>1. Cut-off 53 nl/min: sensitivity 0.92, specificity 0.96 [31]</li> <li>2. Cut-off 77 nl/min: sensitivity 0.98, specificity &gt;0.75 [29]</li> </ol>
HVMA	<ol style="list-style-type: none"> <li>1. Provides assessment of functional defect</li> <li>2. HSVMA is abnormal in all described cases of PCD.</li> <li>3. Correlates with TEM [47] and genetic findings [48]</li> </ol>	<ol style="list-style-type: none"> <li>1. Absence of standardized methods of reporting</li> <li>2. Abnormalities of CBP can be subtle</li> <li>3. Requires specialist equipment</li> <li>4. Requires rigorous adherence to quality control</li> <li>5. Secondary defects are common and experienced scientists are needed with expert knowledge of normal and abnormal findings</li> </ol>	Dyskinesia on >90% ciliated edges: <ul style="list-style-type: none"> <li>○ sensitivity 0.97</li> <li>○ specificity 0.95</li> </ul> to predict a TEM diagnosis [45]
TEM	<ol style="list-style-type: none"> <li>1. Provides assessment of the ultrastructural defects</li> <li>2. Correlates to genetics and HVMA</li> <li>3. Widely used</li> </ol>	<ol style="list-style-type: none"> <li>1. ≈30% of patients have no defect on TEM</li> <li>2. Potentially altered by secondary dyskinesia</li> <li>3. Requires specialist equipment and evaluation</li> </ol>	Sensitivity: 70–80% Specificity: 100% [46,61] (false positives occur, but can be avoided by evaluating sufficient cilia (>100) and adequate training of staff). Sensitivity: 65–80% (estimated) Specificity: 100% [19]
Genetic testing	<ol style="list-style-type: none"> <li>1. Indisputable and fast diagnosis of PCD in case of biallelic pathogenic mutations in known genes</li> <li>2. Has relevance to clinical phenotype</li> <li>3. Provides possibility for carrier testing in isolated populations with high frequency of PCD</li> </ol>	<ol style="list-style-type: none"> <li>1. Cannot rule out PCD (yet) as 20–35% is unknown</li> <li>2. Commercial testing does not offer complete gene/exon panel</li> <li>3. Can be difficult to prove pathogenicity/relationship to PCD in cases of mutations in novel (candidate) genes or novel mutations in known PCD genes</li> </ol>	Sensitivity: 65–80% (estimated) Specificity: 100% [19]
IF	<ol style="list-style-type: none"> <li>1. Much interest for IF to become more widely available as a diagnostic tool</li> <li>2. Useful research tool</li> <li>3. A number of antibodies are commercially available</li> <li>4. Relatively low cost</li> </ol>	<ol style="list-style-type: none"> <li>1. No evidence for use as a clinical tool yet published</li> <li>2. The antibodies currently available commercially will not detect all cases</li> <li>3. Absence of standardized methods or reporting</li> </ol>	No published data

[9,10]. Analysis of ciliary motility is recommended as a diagnostic test in European guidelines [11].

Since the first description of a gene causing PCD in 1999 [12], there has been an exponential increase in reports of PCD-associated genes. There are now over thirty known genes responsible for >60% of cases. Recent publication of mutations in genes causing reduced numbers of cilia with normal motility and ultrastructure [13,14] in patients with a PCD phenotype, brings to question whether yet another change of name is required. Gene panels are now used as part of the diagnostic pathway in a number of countries.

## DIAGNOSIS OF PCD: STATE OF THE ART

There is no single reference standard diagnostic test for PCD [15] and diagnosis usually requires a number of technically demanding, sophisticated investigations. The availability and combination of tests vary between countries [16], and there is no globally accepted consensus as to which diagnostic results constitute a definite positive diagnosis, possible diagnosis or excludes diagnosis. Moreover there is no global agreement regarding the standardisation of conduct or reporting of any of the methods used to diagnose PCD.

Diagnosis in Europe is generally based on the following criteria [11,15,17,18]: in a person with a clinical history consistent with PCD confirmation of the diagnosis by at least two of the following methods: (1) "hallmark" high speed videomicroscopy analysis (HVMA), (2) "hallmark" TEM, (3) biallelic disease-causing mutations identified by gene testing, (4) abnormally low nasal nitric oxide (nNO). If HVMA and nNO are the only abnormal tests, these tests should be repeated before making a diagnosis.

Diagnostic criteria differ in North America where diagnosis is currently based on a combination of nNO, TEM and genetic test results [19]. As the number of genes increases, the North American Genetic Disorders of Mucociliary Clearance Consortium propose using nNO testing in patients with a clinical phenotype to identify patients likely to have PCD, followed by genetic testing to confirm the diagnosis, reserving HVMA and TEM for cases that are not identified by PCD multigene panel testing [15]. The diagnostic pathway remains controversial, for example some groups believe that HVMA should not be used in clinical practice but reserved as a research tool [20,21].

## Who to refer for diagnostic testing

PCD diagnostic techniques are not widely available and require extensive expertise and experience. Therefore, it is recommended that diagnosis should only be made in specialized centers [11,19]. Though PCD is a rare condition, with a prevalence of approximately 1:10,000–20,000 people [16], its main presenting symptoms (recurrent respiratory tract infections) are common in the general and, especially, the pediatric population. The disease characteristics of PCD overlap with many more frequently occurring diseases like asthma, immune deficiencies and cystic fibrosis. In many children, respiratory tract symptoms are merely the result of frequent viral exposure without any underlying condition being present. It can be a challenge for a physician to decide which patients should be referred for further diagnostic testing.

The clinical suspicion of PCD should be raised in case of a combination of a clinical history of unexplained neonatal respiratory distress, early onset of nasal congestion, chronic wet



**Figure 1.** Example X-ray of a patient with PCD showing situs inversus totalis and partial atelectasis of the LEFT middle lobe.

sounding cough, recurrent serous otitis, and situs abnormalities (figure 1). However, some of these features can be mild or even absent in PCD patients [19]. In particular, 50% of PCD patients have normal cardiac and visceral organ laterality. Parents of PCD patients often report nasal discharge starting at the day of birth and it commonly persists throughout life. In addition, the majority of neonates with PCD show neonatal distress [22], suggesting that cilia have a role in the effective clearance of fetal lung fluid [23]. Serous otitis occurs frequently and, unlike in healthy children, persists throughout adulthood [24]. Early onset of wet coughing and lower respiratory tract infections occur in nearly all PCD patients. Although limited data on disease progression are available, bronchiectasis is present in about half the pediatric PCD patients and all adult patients [25]. In Table 2 clinical features are summarized that should raise the suspicion of PCD and prompt referral to a specialized diagnostic center [11]. In ethnic and consanguineous populations where PCD is known to occur more frequently, the threshold for diagnostic testing should be low.

#### Screening tests

Diagnosis of PCD requires access to a number of investigations which are technically demanding and only available in specialist centres. Screening of high risk individuals (i.e. individuals with an

**Table 2**

Who to refer for diagnostic testing. <br>Patients with early onset of recurrent respiratory tract symptoms and any of the following:

1. Situs inversus (SI) totalis or any heterotaxic syndrome (approximately 50% have normal situs)
2. Neonatal nasal congestion and/ or unexplained neonatal distress
3. Positive family history for PCD
4. Males with dysmotile sperm
5. Persistent productive cough/ bronchiectasis / severe upper airway after more common causes like allergies, asthma, immune deficiencies and CF have been excluded.
6. Early onset of the combination of both severe upper and lower respiratory tract infections
7. Persistent/ frequent intermittent serous otitis media (glue ear) associated with respiratory symptoms

affected sibling or with symptoms suggestive of PCD) helps identify patients for definitive testing.

Historically, estimation of nasal mucociliary clearance using the saccharine test was utilised as a simple screen. A small particle of saccharine was placed on the inferior turbinate and the patient asked to sit quietly for 1 hour; the test was considered positive if no taste of saccharine was noted after 1 hour. However, results were difficult to interpret, even in compliant adults, since patients with PCD find it difficult not to cough or sniff for the hour duration of the test. It is no longer recommended.

In 1994 it was reported that nNO concentrations were significantly lower in patients with PCD compared to healthy controls [26]. Whilst the reason for reduced nNO remains elusive [27], measurement became widely established as a screening test [11,17,19,28-31]. A recent meta-analysis of 11 studies comparing nNO during a velum closure breath hold reported a mean nNO output of 19 nl/min (SD 18.6) in PCD (n=478) and 265 nl/min (SD 118.9) in healthy controls (n=338) [32]. Although nNO is lower than normal in patients with CF [32] differentiation from PCD remains good. nNO readings in healthy infants and young children are lower than in older children, making false positive results more likely. This provides a major limitation of the test in the age range that should be targeted for diagnosis.

A multi-centre study from North America derived a cut-off of 77 nL/min to differentiate PCD patients from healthy controls [29]. They then validated the cut-off in consecutive patients referred for diagnostic testing. 77 nL/min differentiated PCD (based on genotype and/ or TEM) from patients with negative PCD test results with sensitivity of 0.98 and specificity of 0.75. Given that genetic testing and TEM fail to detect 20-30% of patients, the true specificity is likely to be higher and as new genes are added to panels, the reported specificity of nNO is likely to improve but is unlikely to approach 100%. Patients with nNO<77nL/min but normal TEM and genetics in the North American study had respiratory symptoms similar to patients with PCD, but only 9.5% had laterality defects suggesting that perhaps 19% had 'missed' PCD; if these 4 patients were reclassified as PCD positive, the specificity would moderately increase to 79%. In the authors' centre, 77nl/min distinguished PCD positive and negative outcomes based on HSVMA and TEM in consecutive referrals with sensitivity of 0.94 and specificity of 0.83 (unpublished). Therefore, although nNO is highly specific when differentiating PCD from healthy controls [29], it is less specific when differentiating PCD from patients with upper and lower respiratory tract symptoms [32]. It should be noted that the youngest child in the North American study was 5.1 years and the mean age much higher. Also reference data for young healthy children is sparse. Therefore strong evidence for use in the pre-school patient group, and reference norms is awaited. PCD associated with normal nNO has been reported in a number of studies [29,31,33], therefore nNO should not be used as a diagnostic test in isolation.

American Thoracic Society/ European Respiratory Society guidelines for the measurement of nNO [33] recommend aspiration of gas from one nostril with gas entrained via the other naris during a velum closure manoeuvre. The chemiluminescence analyser displays the nNO concentration in real-time allowing measurements to be taken from the peak-plateau. This method reliably differentiates patients with PCD from those with other respiratory diseases [29,31,32,34-36] as well as healthy controls. The limitations of this 'gold standard' method for measuring nNO are that velum closure breath hold is difficult particularly for younger children and that the chemiluminescence analysers are expensive and not easily transportable. To widen accessibility of nNO as a screening test a small number of studies have investigated the use of portable analysers [34,37,38]. These electromechanical analysers are more cost effective and have



**Figure 2.** Obtaining an epithelial biopsy using a curette. Only superficial biopsies are required, so minimal force is used. When adequately performed, patient discomfort is minimal.

185 excellent portability. The studies comparing portable and station-  
186 ary analysers suggest that portable analysers are a reliable means  
187 of measuring nNO, [32] but the machines have some drawbacks. In  
188 particular, the lack of real-time visual display of NO does not allow  
189 the technician to assess the acceptability of the measurement. In  
190 the author's experience, a number of patients referred to our centre  
191 with extremely low nNO measures using a portable device at the  
192 referring centre, had normal levels when tested at our centre using  
193 a stationary chemiluminescence analyser. Additionally the time  
194 required for the breath hold manoeuvre using portable devices is  
195 too long for many patients [37].

196 Although measurement is recommended during a velum  
197 closure breath-hold [33], tidal breathing produces a reasonable  
198 alternative if breath-hold cannot be achieved e.g. young children  
199 [32]. Tidal breathing measurements are lower in both PCD and  
200 controls [31,32,34,35,39,40] and are less discriminatory than  
201 measures during a velum-closure breath-hold.

202 In summary, nNO provides an excellent screening test for PCD,  
203 and we now need standardisation of methods of analysis and  
204 reporting.

#### 205 *Obtaining ciliated samples for analysis*

206 In order to evaluate ciliary motility and ultrastructure, a good  
207 quality epithelial sample is obtained from the upper or lower  
208 airways by a trained health care professional. Nasal samples are  
209 most easily obtained, but if the patient is having a bronchoscopy  
210 for other reasons, lower airway samples can be taken [41]. Epithelial  
211 cells can be collected by brush, curette or forceps (Figure 2). The  
212 advantages of brush biopsies are that they are less painful, bleeding  
213 is unlikely and samples can be taken without sedation [41]. The  
214 advantages of using a curette or forceps, are the larger amount of  
215 material obtained and the possibility to evaluate ciliary motility in  
216 the context of other epithelial cells [41]. Depending on local  
217 policies and age, sedation can be given during a nasal biopsy,  
218 although this is rarely required for brush biopsies. Good  
219 explanations to both child and parents about the procedure result  
220 in less stress and empower parents to provide support [42]. When  
221 sedation is required, it should be delivered by trained profes-  
222 sionals, using monitoring and with rescue facilities available  
223 [43]. It is useful to assess the quality of the sample using a light  
224 microscope while the patient is still in clinic so that a repeat  
225 sample can be taken immediately if necessary.

226 Samples for HVMA should be placed in buffered culture  
227 medium and analyzed as soon as possible [44] to provide optimal  
228 conditions for analysis. Biopsies taken for TEM are fixed in e.g. 3%  
229 buffered glutaraldehyde and can be stored.

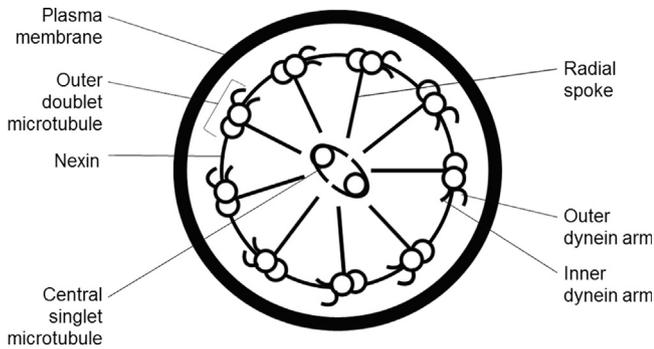
#### *High-Speed Video Microscopy*

230 Assessment of ciliary beat frequency (CBF) and ciliary beat  
231 pattern (CBP) using HVMA provide measures of ciliary function  
232 [45]. Ciliary frequency or pattern are abnormal in all patients with  
233 PCD, and HVMA has a pivotal role in the 30% of PCD patients in  
234 whom TEM is normal [46] and the 20–35% [19] of patients in whom  
235 the gene has not yet been identified.

236 Abnormalities of pattern associated with PCD include static,  
237 slow, rotating, stiff, hyperfrequent and vibrating cilia. These  
238 patterns correlate with abnormalities reported by TEM [47] (e.g.  
239 rotating patterns and central pair defects) and with some genetic  
240 findings (e.g. *DNAH5*-mutant cilia have a bent position with  
241 minimal movement) [48]. However, numbers of patients with  
242 individual genetic defects were extremely low in the only study  
243 [48], and larger multi-centre studies are called for to investigate  
244 genotype-ciliary phenotype relationships. Subtle functional  
245 defects are increasingly recognised to cause phenotypic disease  
246 [49,50] and analyses by scientists with substantial experience of  
247 normal and abnormal ciliary beating is essential. In expert hands,  
248 using HVMA to assess CBP, dyskinesia on >90% ciliated edges has  
249 97% sensitivity and 95% specificity to predict a TEM diagnosis of  
250 PCD [45].

251 Optical equipment should provide high magnification with  
252 excellent resolution to accurately analyse the pattern. The quality  
253 of the objective is critical as the main determinant of resolution  
254 and image quality. In the author's laboratory we observe cilia at  
255 x1000 magnification using an inverted microscope under bright  
256 field light conditions. Although lower magnification (e.g. x500) is  
257 acceptable for calculation of CBF, the CBP would be difficult to  
258 decipher. The camera should capture images at a fast rate so that  
259 they can subsequently be slowed down, allowing the microscopists  
260 to qualitatively analyse the beat pattern. We record at 500 frames  
261 per second; so for cilia beating at 15 Hz, each beat is represented on  
262 33 frames which can be played back at 30 frames per second for  
263 pattern analysis. Microscopic imaging is conducted under strictly  
264 controlled environmental conditions (e.g. temperature and pH)  
265 since minor variations effect ciliary function [51,52]. We analyse  
266 cilia within 4 hours of sampling, using buffered cell culture  
267 medium to control pH and the sample is maintained at 37 °C in an  
268 environmental chamber during analysis. Under these conditions  
269 our reference range for CBF is 11–20 Hz, but laboratories using  
270 different conditions will have different reference ranges.

271 Abnormalities of CBF and CBP can occur secondary to infection,  
272 damage during sampling or inflammation of the epithelia  
273 complicating the diagnostic picture. Following abnormal analysis  
274 it is therefore necessary to reanalyse CBF and CBP following culture  
275



**Figure 3.** Cartoon of transverse section of a respiratory cilium as seen by TEM. Motile cilia in the respiratory tract have a highly organized “9+2” arrangement running the length of the axoneme, with nine microtubule doublets surrounding a central pair of single microtubules. Nexin and radial spokes provide a scaffold for the structure. Attached to the peripheral microtubules are inner and outer dynein arms; dynein is a mechanochemical ATPase responsible for generating the force for ciliary beating, hence abnormalities of the dynein arms affect ciliary beating.

technicians with a high through-put of normal and abnormal samples. This restricts testing to a limited number of specialist centres.

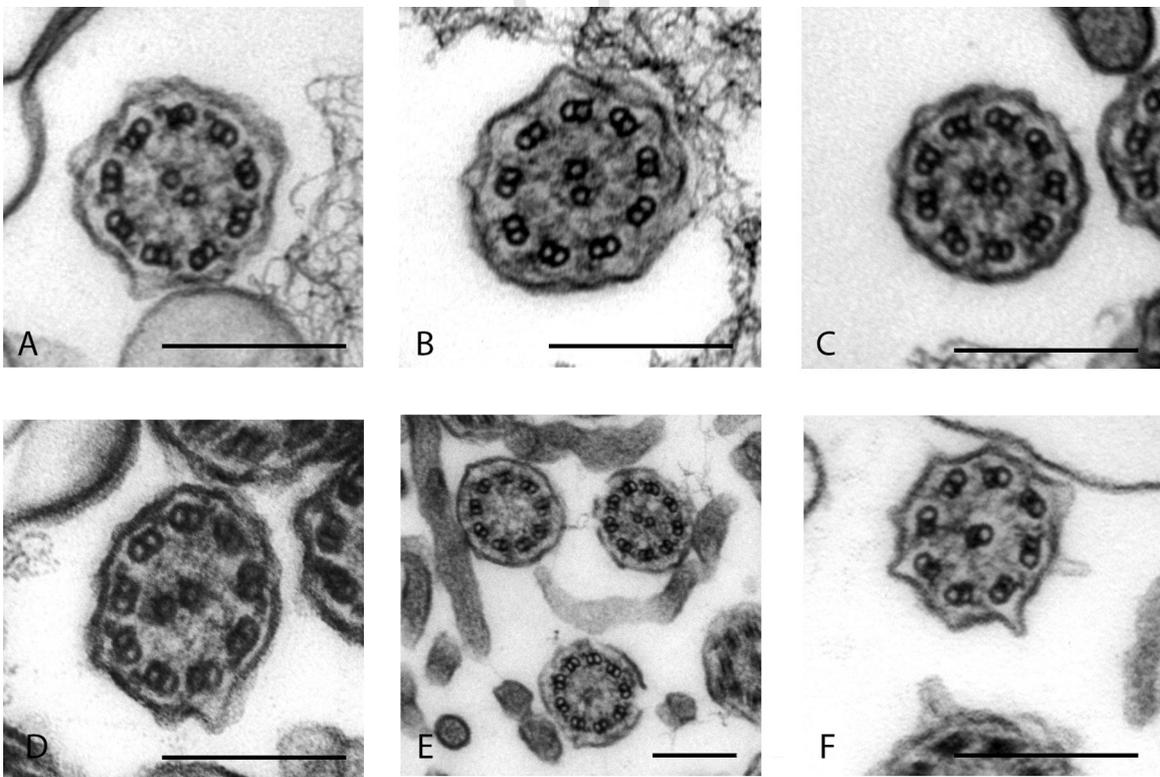
*Transmission Electron Microscopy*

(TEM) allows the ultrastructure (figure 3) of ciliary axonemes to be visualized. Ultrathin sections are prepared for TEM from fixed cells or tissue biopsies using standard methods [47]. Sections should be examined at magnifications sufficient to visualize the ultrastructural features (>x 60,000). Normal cilia have a structure of nine peripheral microtubular doublets and a central pair (9+2 arrangement) (Figure 3). The accessory axonemal components are the outer dynein arms (ODA), inner dynein arms (IDA), radial spokes and the nexin-dynein regulatory complex (N-DRC). Dynein arms contain adenosine triphosphatases and act as motors to achieve ciliary motion by sliding of adjacent microtubular doublets. Defects in any ciliary components can cause immotility or dyskinetic beating. Relationships between the ultrastructural defect and beating pattern have been described [47]. The most common ultrastructural defects are: ODA-defects (25-50%) and combined IDA- and ODA-defects (25-50%) [47,57-59] (figure 4). IDA defects associated with microtubular disorganisation occur in 15% of PCD, but isolated IDA defects as a cause of PCD are controversial particularly as no mutations have been identified in IDA proteins. IDA are difficult to identify due to the decreased repeats along the ciliary axoneme compared to the ODA [60] therefore false positive IDA defects are likely. Reporting of isolated IDA defects requires repeated testing or immunofluorescence staining of the IDA visualizing the entire axoneme [60]. Central pair defects occur less frequently (5-15%) and are associated with a mix of both normal and abnormal cilia [61,62], therefore adequate numbers of cilia need to be viewed in longitudinal and transverse section; in the author’s laboratory at least 100 and up to 300 cilia

of the epithelial cells or following a repeat brushing to confirm consistency of findings if genotype and TEM are not diagnostic.

Whilst direct measurement of CBP and CBF using HVMA is generally considered the most accurate and reproducible technique, it is time consuming and incurs risk of operator error due to selection bias. Several groups have attempted to overcome these problems by developing software to automate analysis from the digital images [53-55].

Although assessment of ciliary function by light microscopy is recognised as a critical diagnostic test used as ‘first-line’ in many centres [11,17,48,53,54,56] it should be noted that some clinicians question its value and recommend that HVMA should be reserved for research purposes [20]. A limitation of HVMA is the need for specialist equipment, rigorous quality control and experienced



**Figure 4.** TEM of representative nasal epithelium cilia from a healthy individual (A) and patients with PCD caused by (B) outer and inner dynein arm defects (C) outer dynein arm defect (D) microtubular disorganisation with inner dynein arm defect, (E) missing central pairs and (F) transposition defect: a peripheral microtubule doublet has crossed to take the position of a missing central pair. Scale bars = 200 nm. EM images obtained using FEI Tecnai 12 TEM (FEI UK Limited, Cambridge, UK) at 80 kV.

**Table 3**  
Overview of PCD genes and the encompassing ciliary ultrastructural and movement defects when mutated. Adapted from Paff [117] et al., 2014

Ultrastructural defect (by TEM)	Ciliary motion defect (by HVMA)	Clinical phenotype	Reference
<b>ODA</b>			
DNAH5	Immotile with occasional stiff moving cilia	Classic	Olbrich [71] et al., 2002
DNAI1	Unknown	Classic	Pennarun [12] et al., 1999
DNAI2	Unknown	Classic	Loges [73] et al., 2008
DNAL1	Decreased CBF	Classic	Mazor [74] et al., 2011
NME8 (TXNDC3)	Mixed populations: normal to immotile	Classic	Duriez [72] et al., 2007
CCDC103	Complete immotility or lack of coordination with reduced amplitude	Classic	Panizzi [108] et al., 2012
CCDC114	Largely immotile with some twitching cilia	Normal male fertility	Onoufriadis [70] et al., 2013
ARMC4	Complete immotility or reduced CBF and amplitude	Classic	Hjeij [81] et al., 2013
CCDC151	Complete immotility	Classic	Hjeij [80] et al., 2014
<b>ODA/IDA</b>			
DNAAF1 (LRRC50)	Complete immotility	Classic	Loges [109] et al., 2009
DNAAF2 (KTU)	Complete immotility	Classic	Omran [92] et al., 2008
DNAAF3	Complete immotility	Classic	Mitchison [90] et al., 2012
HEATR2	Near complete immotility	Classic	Horani [87] et al., 2012
LRRC6	Complete immotility	Classic	Kott [89] et al., 2012
ZMYND10	Complete immotility or reduced CBF and amplitude	Classic	Moore [91] et al., 2013**
SPAG1	Near complete immotility	Classic	Knowles [88] et al., 2013
C21orf59	Complete immotility	Classic	Austin-Tse [85] et al., 2013
DYX1C1	Largely complete immotility. Some cilia show reduced CBF	Classic	Tarkar [110] et al., 2013
<b>IDA/ microtubule disorganisation</b>			
CCDC39	Fast, flickery movement with reduced amplitude	Severe phenotype	Merveille [83] et al., 2011; Blanchon [84] et al., 2012; Antony [82] et al., 2012.
CCDC40	Fast, flickery movement with reduced amplitude	Severe phenotype	Becker-Heck [111] et al., 2011; Blanchon [84] et al., 2012; Antony [82] et al., 2012.
CCDC65	Stiff, dyskinetic moving cilia	No SI	Austin-Tse [85] et al., 2013
CCDC164	Increased CBF with reduced amplitude	No SI	Wirschell [112] et al., 2013
<b>CP defects</b>			
RSPH1	Mixed populations: low CBF to immotility and normal CBF with reduced amplitude	No SI. Mild phenotype	Kott [49] et al., 2013
RSPH4A	Mixed populations: low CBF to immotility and normal CBF and circular movement	No situs abnormalities	Castleman [50] et al., 2008
RSPH9	Mixed populations: low CBF to immotility and normal CBF with circular movement	No situs abnormalities	Castleman [50] et al., 2008
HYDIN	Mixed populations: immotility and reduced amplitude and lack of coordination.	No situs abnormalities	Olbrich [113] et al., 2012
<b>Aplasia/ basal body and rootlet mislocalisation</b>			
CCNO	Severe reduction in number of motile cilia. Cilia that are present function normally	No SI. Severe phenotype	Wallmeier [114] et al., 2014
MCIDAS	Severe reduction in number of motile cilia. Cilia that are present are immotile	No SI. Severe phenotype	Boon [14] et al., 2014
<b>Non specific defects</b>			
OFD1	Mixed populations: normal and chaotic beating pattern	Mental retardation	Budny [115] et al., 2006
RPGR	Mixed populations: motile and immotile cilia	Retinitis Pigmentosa	Moore [116] et al., 2006
<b>No defect</b>			
DNAH11	Mixed populations: increased CBF with reduced amplitude and low CBF to immotility	Classic	Schwabe [75] et al., 2008

selected from healthy cells are analysed in transverse section. It is also informative to examine the longitudinal sections as rare defects e.g. RSPH4A, can be identified by distinctive patterning [50].

Previously TEM was considered the “gold standard” but it is now recognized that 20–30% of patients have normal ultrastructure when analyzed by TEM [46,61]. For example, defects of nexin link components [63], central pair components [64], ciliary biogenesis defects [14] and defects caused by DNAH11 [65,66], usually cannot be visualized by classic TEM. Thus, normal ultrastructure cannot rule out PCD.

An additional limitation of TEM is that inflammation and infection, can alter the normal 9+2 arrangement. Therefore, it can be difficult to differentiate acquired defects from PCD [19]. Similar problems can occur if cells are poorly fixed. Even in samples obtained from non-inflamed tissue that have been properly fixed, correct identification of ultrastructural defects requires highly specialized personnel with substantial experience of the range of normality and abnormality.

Recently, electron tomography has evolved as a research tool providing 3D visualization of the ultrastructure of cilia. This technique has demonstrated ultrastructural defects in PCD patients with DNAH11 and HYDIN mutations, who did not appear to have defects on classic TEM [67] [64]. However, there are some limitations to this technique mainly concerning microscopic resolution, limited accessibility of the sample (penetration depth) and the speed of data processing. However, with the current progress in both hardware and software, this technique will be a valuable diagnostic tool in difficult PCD cases in the future.

#### Genetics

PCD is generally an autosomal recessive disease, though in rare cases other modes of inheritance have been described (X-linked or autosomal dominance). Currently 31 genes have been identified, explaining approximately 60% of cases (Table 3). There seems to be a clear association between genetic defects, ciliary ultrastructure

and motion defects (Table 3). However, the association between genetic defects and the clinical phenotype is largely unknown; international collaborations are developing large meta-cohorts to ensure sufficient numbers of patients with mutations in each PCD-associated gene. Some genotype-phenotype relations have been described, but caution needs to be kept as some descriptions are based on low numbers of patients: mutations causing reduced generation of multiple motile cilia (MCIDAS, CCNO) and those causing IDA with microtubular disorganization (CCDC39, CCDC40) have been reported to result in relatively severe lung disease [13,14,68]. In contrast, mutations in RSPH1 (a mutation in a gene coding for one of the radial spoke subunits) reportedly cause a mild phenotype [69]. Male patients with mutations in CCDC114 (coding for a docking protein for the ODA) are generally fertile [70]. Situs abnormalities are not observed in patients with mutations affecting the central pair [64] or radial spokes [49,50,69], nor in patients with reduced generation of multiple motile cilia [13,14]. However, it is likely that variable clinical pictures occur between patients with different mutations within a particular gene and even between patients with identical mutations.

To date mutations have been identified in 6 genes encoding for proteins that are part of the ODA (DNAH5, DNAI1, DNAI2, DNALI1, NME8 (TXNDC3) and DNAH11) [12,71–75]. Mutations in DNAH5 and DNAI1 are thought to account for the largest proportion of PCD patients: 30% and 9% respectively [19,76–79]. Mutations in these genes cause structural and functional ODA defects and consequently ciliary immotility [47,48]. However, in DNAH11, TEM is normal and thus not informative [65,66,75].

In addition, mutations in 3 genes have been described that, though not coding for proteins that are structurally part of the ODA, cause selective absence of ODA on TEM: CCDC114 [70], CCDC151[80] and ARMC4[81]. These genes code for ODA-associated docking complex proteins that enable attachment of the ODA to the axoneme.

Mutations in CCDC39 [82,83], CCDC40 [82,84], CCDC65 [85] and CCDC164 [63] cause variable inner dynein arm defects with microtubular disorganization. These genes encode for proteins of the N-DRC proteins that connect the peripheral microtubular doublets with each other. The cilia beat in a fast and stiff manner.

Patients with mutations in genes encoding for proteins that are part of the central apparatus and radial spokes (RSPH9, RSPH4A, RSPH1 and HYDIN) can pose diagnostic difficulties [49,50,64]. Patients have normal situs because healthy nodal cilia responsible for lateralization during embryogenesis differ from healthy respiratory cilia, lacking the central pair. Approximately half the cilia from patients with these mutations have normal ultrastructure on TEM and movement on HVMA evaluation. Additionally, RSPH1 mutations generally cause a relatively mild clinical picture and borderline to normal nasal NO concentrations [69].

Numerous proteins are involved in cytoplasmic biogenesis of cilia. The identification of genes involved in cytoplasmic assembly of cilia (HEATR2, DNAAF1, DNAAF2, DNAAF3, DYX1C1, CCDC103, LRRC6, ZMYND10, SPAG1, ARMC4 and C21orf59) has provided clear insights into this process [81,85–95]. Defects in the assembly line can result in ODA or ODA/IDA defects.

Recently, two genetic defects have been identified in patients previously classified as ciliary aplasia: mutations in CCNO and MCIDAS [13,14]. Mutations in CCNO and MCIDAS result in defective centriole generation and placement on the outer surface of the epithelial membrane. These basal bodies normally nucleate the motile ciliary axonemes. Consequently, there is a severe reduction in the number of motile cilia leading to the clinical picture of PCD. The few residual cilia in CCNO-mutants have

normal axonemal ultrastructure on TEM and do not show any obvious beating defects. Previously, these patients were often misclassified as secondary dyskinesia. In MCIDAS-mutants, the few residual cilia lack many of the axonemal motor components, and are immotile.

Thanks to high-throughput genetic testing, many disease-causing mutations have been identified, and genotyping can currently identify >60% of patients. However, for a significant proportion of patients the genetic defect has not yet been elucidated. Therefore genetic testing cannot be used to rule out the diagnosis of PCD yet.

#### Additional tests

Tests that are currently used by only a handful of centers, but with increased evidence may come into more common use include immunofluorescence (IF) labelling of ciliary proteins and radio-aerosol mucociliary clearance (MCC). IF was developed as a research tool to improve understanding of the impact of disease-causing genes on ciliary proteins [96]. Specific antibodies are used for subcellular localization of proteins in human respiratory epithelial cells using high-resolution IF imaging. A number of antibodies against ciliary proteins are now commercially available including DNAH5 (an outer dynein arm protein), DNALI1 (an inner dynein arm protein), RSPH4A (a radial spoke head protein- central pairs) and GAS8 (nexin links). Although the literature is confined to the research arena, several centers now use IF to aid diagnosis, and this is likely to increase as more antibodies become readily available. Antibodies against gene-related proteins associated with normal TEM would be a particularly useful (e.g. HYDIN and DNAH11). To promote IF as a diagnostic test, standardized protocols are required along with studies to establish the sensitivity and specificity.

Pulmonary radioaerosol MCC has been reported to differentiate PCD from healthy individuals [97,98]. The method is based on clearance patterns after the inhalation of a radioaerosol tracer, providing a whole-lung functional test for pulmonary radioaerosol MCC. Further data is now required to confirm whether the technique will be useful for differentiating PCD patients from people with secondary ciliary damage, or for diagnosing patients with inconclusive tests using other methods.

#### Cell culture

Culturing respiratory epithelial cells provides an opportunity to confirm that abnormalities seen on the fresh nasal brushing are due to PCD rather than a secondary dyskinesia. This removes the need to repeat nasal brushing to obtain a sample for reanalysis which is required if diagnosis depends on HVMA. The ciliary phenotype changes following cell culture helping to differentiate primary from secondary dyskinesia and this change is therefore advantageous [99]. Jorissen et al [57] first reported the use of cell culture to aid PCD diagnosis using a submerged method (monolayer-suspension cell culture). Culture followed by ciliation at air-liquid interface (ALI) [99–101] has the advantage of yielding more cells and cilia than the submerged method. Both submerged and ALI-culture techniques allow reanalysis by HVMA [57,99,101] and TEM [99,101,102] aiding the diagnosis of PCD. ALI-culture of respiratory cells from PCD patients additionally provides an excellent ex vivo model for research [103,104].

#### EQUIVOCAL OUTCOME FROM DIAGNOSTIC TESTING

There are a number of patients with a clinical phenotype suggestive of PCD, whose diagnostic tests are equivocal, or

abnormalities very subtle. In particular, in the experience of the authors, there are a small number of patients with subtle abnormality of ciliary pattern on PCD, with normal TEM and equivocal/normal nNO; we label this sub-group of patients 'possible PCD'. Having investigated them for any alternative diagnosis, we clinically manage them in the PCD service. We anticipate that further advances in PCD diagnostic testing, and in our understanding of the underlying pathophysiology of the condition will lead to a significant proportion of this group becoming positive over time.

## INTERNATIONAL PERSPECTIVES AND FUTURE NEEDS

Recent international networks and collaborations have led to advances in the understanding and conduct of diagnosing PCD, but there remains no 'gold standard'. A European Respiratory Society Taskforce (ERS TF 2007-9) provided a focus for paediatric pulmonologists to undertake epidemiological studies [16,105] and develop a consensus statement for diagnosis and management [11]. The taskforce highlighted disparity in diagnosis across Europe [16], with missed and delayed diagnosis particularly likely in countries with lower health expenditure. One of several projects being conducted by European Union's Seventh Framework Programme BESTCILIA (2012-15), aims to improve equity of care by establishing diagnostic centres in three areas of Europe currently unable to fulfil ERS consensus guidelines [11]: Cyprus, Poland and Greece. Recent advances in diagnostics have underpinned the development of a new ERS taskforce (ERS TF 2014-16), to develop evidence-based guidelines for the diagnosis of PCD. The taskforce is using rigorous methodology to develop practice guidelines for diagnostic testing including measurement of nNO, HVMA of ciliary function, TEM, genetics testing and IF labelling of ciliary proteins. In North America eight centres are members of the Genetic Disorders of Mucociliary Clearance Consortium [19]. The Consortium has developed standardized protocols for diagnostic testing, including nNO testing [29].

National collaborations are contributing to improved diagnostic management in a number of individual countries including France [58,106], and the United Kingdom [17,107].

## CONCLUDING DISCUSSION

Advances in the diagnosis of PCD have occurred over the decades since cilia were first implicated in the syndrome. National consortia and research from individual groups have moved us forward, but we still have no gold standard reference nor international standards for conduct of tests. A number of experts in the field are collaborating to develop evidence based guidelines for diagnostic testing through the European Respiratory Society PCD Task Force and COST Action BEAT-PCD. Initiatives are also underway to widen accessibility to diagnostic services (BEST-CILIA).

## CONFLICT OF INTEREST STATEMENT

JSL is a member of Aerocrine PCD Advisory Board and has received expenses and honoraria. TP, PG and EH have not declared any potential conflicts of interest.

## FUTURE DIRECTIONS

- Establish the evidence base to develop international standards for conduct and reporting of tests.
- Establish the evidence base for international development of a diagnostic algorithm for (i) definite PCD, (ii) probable PCD (iii) PCD excluded.

- Establish the accuracy (sensitivity, specificity, and predictive values of diagnostic tests in well designed 'blinded' studies.

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