

## Inner Dynein Arm Defects in Primary Ciliary Dyskinesia

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### Abstract

Primary ciliary dyskinesia (PCD) is a genetic condition in which there is a defect in cilia motility. This can be caused by the absence of the inner dynein arm motor (IDA). The IDA can be difficult to observe by electron microscopy and has been shown to transiently disappear, making accurate diagnosis difficult.

This study reviewed cases of PCD due to an IDA defect on the Royal Brompton Hospital database in order to confirm or retract the diagnosis. This was achieved using current methods of diagnosis (nasal nitric oxide, light and electron microscopy) and new techniques to improve visualisation of the IDA such as immunofluorescence analysis and 3D electron tomography.

Four patients received normal results and the diagnosis of PCD was reversed. Three patients had another structural defect in combination with absence of IDAs. The remaining five patients had an absence of IDA in mean (SD) 42(22) % of their cilia. In these group diagnostic findings were heterogeneous with a diverse range of cilia beat pattern and frequencies. Immunofluorescent antibodies revealed the presence of the IDA component protein DNALI1 in all patients and high resolution electron tomography confirmed the presence of some IDA structures. Nasal nitric oxide values ranged from 3 to 463 ppb.

In conclusion, PCD due to an IDA defect is difficult to diagnose as the IDA appears transiently absent on TEM. Diagnosis is reversed by repeat sampling in 38% patients. We suggest an isolated absence of IDA shown by TEM is not sufficient to confirm a diagnosis of PCD.

**Keywords:** Cilia; Diagnosis; Electron microscopy; Inner dynein arm; Primary ciliary dyskinesia

### Introduction

Primary ciliary dyskinesia (PCD) is a genetic condition affecting approximately 1 in 15,000 people [1]. In this condition abnormal or absent beating of cilia results in poor mucociliary clearance of the bronchial tree, which leads to recurrent lower respiratory tract infections and can cause bronchiectasis. Abnormal ciliary beating at other sites causes rhinosinusitis, middle ear disease and impaired fertility. Approximately 50% of patients also have situs inversus. Early diagnosis of PCD is important to prevent unnecessary ENT surgery and to allow physicians to limit disease progression in the lungs through introduction of physiotherapy and prompt antibiotic treatment of chest infections [1].

Diagnosis of PCD is made by patient history and examination, a nasal nitric oxide screening test, cilia beat frequency and pattern by light microscopy and assessment of cilia ultrastructure by transmission electron microscopy (TEM) [2].

The normal ultrastructural ciliary arrangement is a circle of nine peripheral microtubule doublets, each with an inner and outer dynein arm, plus a central pair of microtubules which are joined to the peripheral doublets by radial spokes (as shown in Figure 1). A number of abnormal ultrastructural phenotypes can cause PCD, but the majority of cases are due to a lack of dynein arms. The inner dynein arm (IDA) is frequently absent, sometimes in combination with either the outer dynein arms (ODA) or the radial spokes. Isolated absence of the IDA is reported to cause between 10 and 29% of PCD cases [3-5].

Ordinarily cilia beat in a co-ordinated fashion at 11-15 Hz clearing secretions from the respiratory tract [3]. The dynein arms act as motors which control the size and shape of the ciliary waveform. The IDA pattern is thought to repeat every 96 nm throughout the length of the

cilium. They are less dense than ODAs at TEM and therefore more difficult to visualize, resulting in reduced confidence in PCD diagnosis due to an isolated absence of IDAs compared to a diagnosis due to ODA or microtubular defects [6]. A recent report has shown that the IDA may transiently disappear at the TEM stage of diagnosis and be present

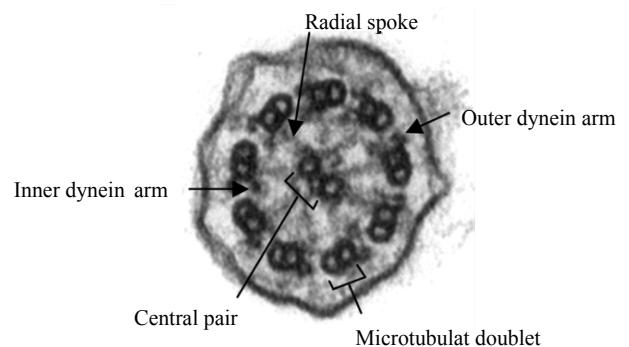


Figure 1: Cross section of a normal respiratory cilium.

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n	TEM result summary	% IDA absent at TEM	Mean nasal nitric oxide in ppb (StDev)	Mean ciliary beat frequency in Hz (StDev)	Cilia pattern*
4	Normal ultrastructure	2% (2.1)	461 (168)	11.0 (1.9)	Co-ordinated
5	Inner dynein arm defect	42% (19)	235 (211)	8.4 (4.4)	stiff [1], static [2], slow [2] and/or dyskinetic [2]
2	Radial spoke and inner dynein arm defect	90% (-)	57 (-)	0	Static
1	Inner and outer dynein arm defect	55%	31	0	Static
1	Insufficient material	NA	276	9.8	Dyskinetic
<b>Normal data from respiratory controls</b>		1 (2.3)% n=200	594 (262)ppb n=150	10.3 (2.3) n=840	

\*Some samples received more than one description. Numbers in brackets represent the number of samples per description

**Table 1:** Results from a diagnostic review of 13 patients listed on the Royal Brompton Hospital PCD database as PCD due to an IDA defect.

on a repeat sample obtained from a primary cell culture in the absence of infective and inflammatory stimuli [7]. These findings suggest an absence of IDA shown by TEM may not be sufficient to confirm a diagnosis of PCD. In light of these findings and case studies from our cohort of patients a clinical review of patients diagnosed with an isolated IDA defect was undertaken.

## Aim

The aim of this study was to review all cases of PCD due to an IDA defect on the Royal Brompton Hospital PCD database in order to confirm or retract the diagnosis.

## Methods

### Subjects

The Royal Brompton hospital holds one of the largest databases of PCD patients internationally. Of 252 patients 30 (12%) had been diagnosed by TEM as having PCD due to isolated absence of the IDA. Patients with a historical diagnosis of PCD due to an IDA defect were sent a written invitation to attend the Royal Brompton hospital for repeat diagnostic investigations and a review their condition. This did not include patients listed as a combined IDA and ODA defect or those with a combined microtubular disarrangement and IDA defect.

### Ethics

The study was approved by the London Harrow research ethics committee and all patients gave written informed consent.

### Investigations

**Nasal nitric oxide (NO):** Patients who were able to comply with the protocol had nasal NO measured on a chemiluminescence analyser (LR2500, Logan Research, Rochester, Kent) as previously described [8]. Two measurements were taken per nostril, at a flow rate of 250 ml/s. The mean of 4 readings were used in reporting of results.

**Nasal brush biopsy:** A nasal brush biopsy was taken from the inferior turbinate of each nostril using a shortened cytology brush. The first sample was split for light microscopy, TEM and tomography, and the second sample was used for immunofluorescence analysis.

**Light microscopy:** High speed video analysis of cilia was performed by light microscopy. The ciliated epithelia was visualised with a 100x oil immersion lens, and recording at 500 frames per second. Clips were replayed at 30 frames per second. The cilia beat pattern was analysed and the cilia beat frequency (CBF) calculated according to the method of Chilvers et al. [3].

**Transmission Electron microscopy (TEM):** Samples were fixed

in gluteraldehyde, post fixed in osmium tetroxide, spun into a pellet with agar, dehydrated and infiltrated with araldite. They were cut at 90 nm, stained with uranyl acetate and lead citrate and viewed under TEM (Hitachi H7000). Samples were methodically counted, quantifying primary and secondary ciliary abnormalities including microtubular arrangement, presence and absence of microtubular doublets and central pair microtubules, compound cilia, and presence/absence of dynein arms as previously described [9]. Briefly presence of dynein arms was assessed in high quality cross sections from healthy epithelial strips which were encountered. Typically 100 cross sections were assessed for arms per section. If one IDA per cross section was seen to be present or equivocal then that cross section was judged to have IDAs present.

**Immunofluorescence:** Samples were spread onto glass slides, air dried and stored at -80°C until use. Cells were treated with 4% paraformaldehyde, 0.2% Triton-X 100 and 1% skim milk prior to incubation with primary (at least 3 hours at room temperature or over night at 4°C) and secondary (30 minutes at room temperature) antibodies. Appropriate controls were performed omitting the primary antibodies. Polyclonal anti-DNALI1 antibody was reported previously [12]. Monoclonal mouse anti-acetylated- $\alpha$ -tubulin antibody was purchased from SIGMA (Germany). Highly cross adsorbed secondary antibodies (Alexa Fluor 488, Alexa Fluor 546) were obtained from Molecular Probes (Invitrogen). DNA was stained with Hoechst 33342 (Sigma). Immunofluorescence-images were taken with a Zeiss Apotome Axiovert 200 and processed with AxioVision 4.7.2.

**Electron tomography:** Thin sections were cut (150 nm) and labelled with 10nm gold particles before staining with uranyl acetate and lead citrate. Images of transversely and longitudinally orientated specimens were acquired over a tilt range of  $\pm 70^\circ$  at  $2^\circ$  intervals in a Philips CM200 TEM [10].

These images were processed using IMOD to generated tomographic volumes allowing three-dimensional visualisation of the cilia. IMOD was used to average each of the microtubule doublets [1-9] from nine individual axonemal cross sections. This helped to remove noise and enhance the quality of the IDA structures. A montage (100 nm thickness) was created in protomo (v1.1.4) for each averaged microtubule doublet [11]. The presence or absence of the IDA structure was observed through the slices of the montage. Subtomographic average projections were created to summarise this data.

## Results

13 patients responded to a written invitation to review their condition and attended the Royal Brompton Hospital for a clinical history and repeat diagnostic investigations (NO and nasal brush biopsy). The results of these investigations are shown in Table 1.

### Diagnosis reversed in four patients

Four patients previously recorded as having an IDA defect received normal test results. Table 2 shows diagnostic results for these four patients at initial diagnosis and at review. In the four patients who received a normal result nasal NO testing was not available at initial diagnosis (diagnosis was made before 1994 or the patient was too young to perform the NO test). Three of the four had situs inversus. One had minor bilateral hearing loss, there were no other recent ENT symptoms. Two patients had a dry cough but no other respiratory symptoms. None had bronchiectasis. At initial diagnosis sinopulmonary symptoms were present in all patients. One had respiratory distress at birth. Ciliary beat frequency was within the normal range at initial diagnosis and again at review. Ciliary beat pattern was disorganised and dyskinetic at initial diagnosis but was co-ordinated and effective on review. Ultrastructure by TEM was normal at review. Although IDAs differed there was no difference in the number of microtubular changes (<8% cross sections showed abnormal microtubules) between initial visit and review.

### Five patients had a depletion of inner dynein arms at TEM

Results from patients in whom reduction in IDA number remained are shown in Table 3. Results of nasal NO screening tests and ciliary beat frequency were diverse and overlap with normal values. The heterogeneous presentation and diverse results in this group make it unlikely that they are caused by a single genetic defect

### Immunofluorescence

Antibodies to DNALI1, a dynein light chain homologous to p28 in chlamydomonas IDA and predicted to be part of several single-headed IDA complexes in human respiratory cilia, revealed presence of this IDA component in all patients tested (Figure 2). This confirms that at least a subset of IDAs can be assembled in these cases and excludes a complete IDA absence.

Clinical details				
Gender	M	F	M	M
Situs inversus	Yes	Yes	No	yes
Respiratory distress at birth	No	No	Yes	No
Consanguinous parents	Yes	Yes	No	No
Results from initial diagnosis				
Nasal nitric oxide	N/A	N/A	N/A	N/A
CBF (Hz)	15.6	14.5	16.3	10.2
Cilia beat pattern	dyskinetic	dyskinetic	dyskinetic	dyskinetic
% IDA absent at TEM	79	53	55	25
Age	4 yrs	10 yrs	1 yr	3 yrs
ENT symptoms	Yes	Yes	Yes	Yes
Lower respiratory tract symptoms	Yes	Yes	Yes	Yes
Results at review				
Nasal nitric oxide (ppb)	700	605	276	503
CBF (Hz)	8.5	10.9	13.3	11.5
Cilia beat pattern	co-ordinated	co-ordinated	co-ordinated	co-ordinated
% IDA absent at TEM	0	3	0	5
Age	7 yrs	15 yrs	7 yrs	14 yrs
ENT symptoms	Yes	No	No	No
Lower respiratory tract symptoms	Yes	No	No	No
Presence of IDA component DNALI1	Yes	Yes	Yes	Yes

Table 2: Diagnostic test results for patients with normal ultrastructure at review.

Patient	1	2	3	4*	5*
Clinical details					
Gender	M	F	F	F	M
Age	23 yrs	18 yrs	39 yrs	5 yrs	9 yrs
Situs inversus	No	Yes	No	Yes	Yes
ENT symptoms	Yes	Yes	Yes	Yes	Yes
Respiratory distress at birth	No	No	No	No	No
Productive/wet cough	Yes	Yes	Yes	Yes	Yes
Bronchiectasis	Yes	Yes	Yes	No	No
Recurrent chest infections	Yes	Yes	Yes	Yes	Yes
Results					
Nasal nitric oxide (ppb)	25	293	3	463	394
CBF (Hz)	12.6	8.5	0	11.5	8.9
Cilia beat pattern	Static or stiff, rapid incomplete beat	slow	Mainly static, some slow dyskinetic movement	Dyskinetic and rapid incomplete beat	Static, dyskinetic and co-ordinated beat
% IDA absent at TEM	42%	36%	78%	21%	33%
Immunofluorescence to DNALI1	Yes	Yes	Yes	Yes	Yes

\*Patients's 4 and 5 were siblings

Table 3: Diagnostic test results for patients with consistent loss of IDA at review.

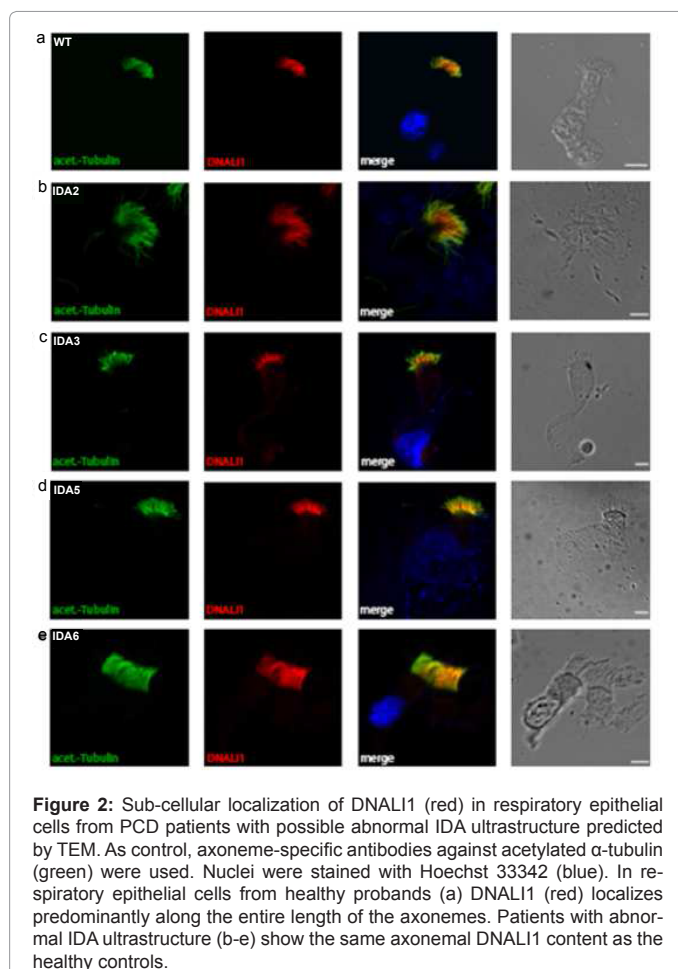
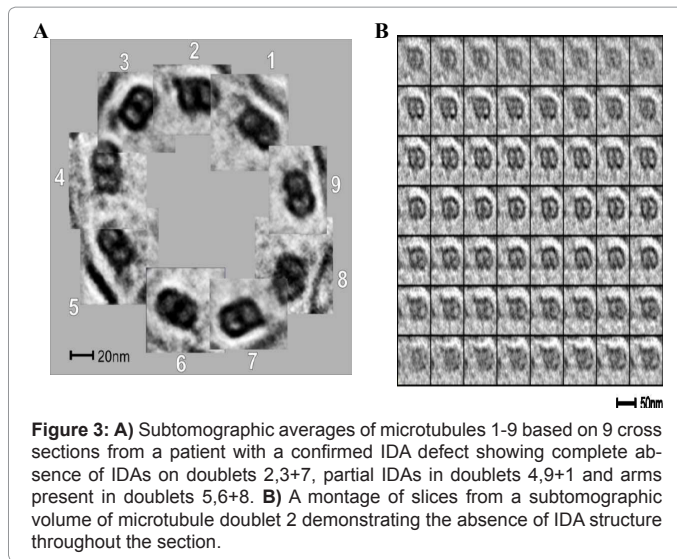


Figure 2: Sub-cellular localization of DNALI1 (red) in respiratory epithelial cells from PCD patients with possible abnormal IDA ultrastructure predicted by TEM. As control, axoneme-specific antibodies against acetylated  $\alpha$ -tubulin (green) were used. Nuclei were stained with Hoechst 33342 (blue). In respiratory epithelial cells from healthy probands (a) DNALI1 (red) localizes predominantly along the entire length of the axonemes. Patients with abnormal IDA ultrastructure (b-e) show the same axonemal DNALI1 content as the healthy controls.





### Electron tomography

Electron tomography on patient 1 from Table 3 revealed the presence of some IDAs. Some microtubules appeared to have IDAs present (doublets 5, 6 and 8) or partially present (doublets 4,9,1) whereas others did not (doublets 2, 3 and 7). Outer dynein arms also looked unusual in this patient. Data is shown in Figure 3.

Three patients had confirmed PCD with an IDA defect in combination with another defect, all 3 had static cilia and nasal NO <100 ppb.

### Discussion

The findings from this study suggest that a diagnosis of an IDA defect is less common than previously thought, and may have been over diagnosed in the past [3-5]. We suggest that ultrastructure results should be considered in conjunction with results of all the other diagnostic information, including nasal NO, before a diagnosis can be established. In addition, confirmation of a defect should be made on the basis of repeated samples either another primary sample when the patient is well or a culture of the original sample. Repeat samples should depict identical findings on TEM and video microscopy. Repeating the diagnostic tests in this study has led to normal ultrastructural findings in 30% of patients. It can take six weeks for repair of ciliated epithelium following a viral infection therefore symptoms should be treated and an appropriate timeframe passed before repeat testing.

The presence of the IDA and an apparently normal TEM ultrastructure in these patients does not necessarily rule out a diagnosis of PCD since some genetic defects causing PCD have been identified which do not show a structural defect using traditional TEM [13]. In a recent review up to 15% of cases believed to have PCD on clinical grounds have normal ciliary ultrastructure on TEM [14]. It is therefore possible that some of our patients suffer from PCD caused by mutations in genes that result in PCD with normal ultrastructure (DNAH11 mutations or subtle defects often not identified by TEM). Although upper and lower respiratory tract symptoms were no longer present at review in our patients. Three of the four had situs inversus. One had respiratory distress at birth.

In five patients there remained a depletion of IDAs by TEM. In this group diagnostic findings were heterogeneous with a diverse range of

NO screening test, cilia beat pattern and frequency results making it unlikely that this group represents a single genetic defect. Interestingly Immunofluorescent antibodies revealed the presence of the IDA component protein DNALI1 in all these patients. This is in contrast to combined ODA and IDA defects caused by KTU and LRRC50 mutations as well as in respiratory cilia characterized by combined dynein regulatory complex (DRC)/IDA defects caused by *CCDC39* and *CCDC40* mutations, where DNALI1 is absent respectively [15-18]. This immunofluorescence finding confirms that at least a subset of IDAs can be assembled in these cases and excludes a complete IDA absence explaining difficulties at TEM. In addition IDA structures are identified by electron tomography in patients with the IDA defect. In the patient studied they appeared to be present on certain microtubules, but absent on others. Despite their presence these structures are observed less frequently than IDAs in normal healthy ciliary axonemes. Standard TEM findings revealed on average 58% of cross sections contained at least one IDA.

This study has only looked at one follow up and it is possible, given the immunofluorescence and tomography results that a third sample might result in finding normal ultrastructure in further patients. In particular it would be of interest in those patients in the group with a near normal nasal NO result.

The presence of some IDAs in a defective sample may be a consequence of the number of different IDA isoforms which are present in the axoneme. In human respiratory cilia at least two distinct ODA types are present: type 1: DNAH9-negative and DNAH5-positive (proximal ciliary axoneme); type 2: DNAH9- and DNAH5-positive (distal ciliary axoneme) [19]. Human ODA complexes contain two axonemal heavy chains (e.g. DNAH5 and DNAH9). IDAs are more heterogeneous in their structure however, and repeat less frequently throughout the axoneme compared to the ODAs. The IDA in the human respiratory tract is thought to be well conserved and consistent with that of *Chlamydomonas reinhardtii* in which there are seven different dynein isoforms making up the IDA. These dyneins consist of 8 different heavy chains (dimers a-g have a single heavy chain whereas f (or I1) has a double heavy chain). This is therefore fewer electrons dense and more diverse than the ODA where there is one repeated isoform with 2 heavy chains [20]. Since there are a number of different IDA isoforms it is possible that in the IDA defects some isoforms are present and others are absent. In addition, like ODA complexes, IDA complexes probably vary in composition along the ciliary axoneme.

Studies with *Chlamydomonas* mutants show that the different isoforms of IDA have different functions and can effect ciliary motion and beat in unique ways. Although not all IDA isoforms are necessary for cilia movement, certain combinations are required [21]. The absence of different IDA isoforms when an IDA is confirmed may explain the heterogeneity of this condition and its ciliary beat patterns. In comparison to an ODA defect or transposition defect where the static or circling beat pattern is quite distinct, we found that the IDA defect when present on two samples had a variety of beat patterns, and frequency, between samples and within a single biopsy. The defects also had a wider range of nasal NO values compared to those observed in other PCD cases. The borderline NO values in these patients stress the importance of performing a nasal brush biopsy where the clinical history is strong, despite a seemingly normal nasal NO screening result.

O'Callaghan et al show that IDAs can be absent on TEM cross-sections in respiratory cilia but present when cells are grown in sterile culture conditions [7]. Consistent with this observation we show that in some nasal brush samples where they had previously appeared to

be reduced, they appeared normal by re-sampling. It is possible that the TEM sampling site is important and it is expected that the IDA composition visualised in cross sections vary along the length of the ciliary axoneme. Thus, possibly absence of IDAs on some cross-sections might be normal, although not recognized because the site of the cross-section cannot be determined accurately. It is also possible that this structure is occasionally not stable during processing for TEM. The effect of fixation and tissue processing technique is not known, but it has been shown that the average number of IDAs per cross section that can be visualised by different research groups where fixation and processing techniques are different can vary from 1-9 [22]. The quantitative method used overcomes this problem to an extent by judging 1 arm present as presence of arms on the entire cross section. However, if the structure appears to be absent as a consequence of *in vivo* absence or an *in vitro* processing problem remains to be established. In previous studies it has been demonstrated that patients with chronic respiratory infections exhibit a decreased number of dynein arms [23].

Despite testing multiple candidate genes (DNAH3, DNAH7, IC140, HP28, DPCD) no disease-causing mutations for isolated IDA defects in humans have been identified [24-26]. Therefore the isolated IDA remains a heterogeneous and poorly understood PCD ultrastructural defect. Enhancement of the IDA through tomography, computer modelling or immunofluorescence labelling with antibodies targeting different IDA components, as well as genetic studies should be explored further and may aid the diagnosis of this defect in the future.

In conclusion, PCD due to an IDA defect is difficult to diagnose as the IDA appears transiently absent on TEM. When confirmed on a second biopsy the diagnosis results in a heterogeneous poorly defined disorder where partial presence of the IDA structure remains. Consequently immunofluorescence labeling for DNAHLI does not aid diagnosis. Diagnosis is reversed by repeat sampling in 38% patients therefore a full diagnostic work up including nasal NO and analysis of a second sample should be performed before PCD is confirmed

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